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IMMUNOCHEMICAL AND BIOCHEMICAL ANALYSIS OF THE GUANINE NUCLEOTIDE - BINDING PROTEIN, $G_o\alpha$, IN THE NEUROBLASTOMA x GLIOMA HYBRID CELL, NG108 -15.

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" Glasgow is, indeed, a very fine city; the four principal streets are the fairest for breadth, and the finest built that I have ever seen in one city together; in a word, 'tis the cleanest and beautifullest, and best built city in Britain. "

Daniel Defoe [1726] in "A Tour Through The Whole Island Of Great Britain."

" Science herself is over - specialised her right hand knoweth not what her left hand doeth; scientists in bulk inhabit a city of water - tight compartments. Each of them is busily engaged in investigating the interior of his own compartment; but by the irony of the situation, the compartments are not quite water - tight, and each investigator finds that the results of some one else's investigations sooner or later percolate into his own place, and often transform the whole aspect of his interior. "

Julian Huxley [1926] in "Preface to Essays in Popular Science."

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Abbreviations.

A : amps

ADP : adenosine 5' - diphosphate

AppNHp : adenylyl 5' - imidodiphosphate

ATP : adenosine 5' - triphosphate

BME : 2 - mercaptoethanol

8 - bromo cAMP : 8 - bromoadenosine 3' : 5' - cyclic monophosphate

Bromophenol blue - 3', 3", 5', 5" - Tetrabromophenolsulfonphthalein

BSA : bovine serum albumin

cAMP : adenosine 3' : 5' - cyclic monophosphate

cDNA : complimentary DNA

CP : creatine phosphate

CPK : creatine phosphokinase

cpm : counts per minute

CRE : cAMP responsive element

C - terminus : carboxyl terminus

DADLE : enkephalin (2 - D - alanine - 5 - D - leucine)

DALAMID : enkephalinamide (2 - D - alanine - 5 - D - leucine)

Db cAMP : N^6, $O^{2'}$- dibutyryl adenosine 3' : 5' - cyclic monophoshate

DMEM : Dulbecco's Modified Eagle's Medium

DMSO : dimethylsulphoxide

DPM : disintegrations per minute

DTT : dithiothreitol

EDTA : ethylenediamine tetra-acetic acid

FBS : foetal bovine serum

Forskolin : 7b-acetoxy-8,13-epoxy-1a, 6b, 9a-trihydroxy-labd14-ene-11-one

G_i : inhibitory G - protein of adenylyl cyclase regulation

G_o : G - protein of unknown function

G_s : stimulatory G - protein of adenylyl cyclase regulation

GRE : glucocorticoid responsive element

GppNHp : guanylyl 5' - imidodiphosphate

G - protein : GTP - binding protein

GTP : guanosine 5' - triphosphate

GTPγS : guanosine 5' - O - (2 - thiodiphosphate)

HAT : hypoxanthine, aminopterin, thymidine

IBMX : isomethyl butyl xanthine

IEF : isoelectric focussing

Ig : immunoglobulin

kDa : kilodaltons

KLH : keyhole limpet haemocyanin

mA : milliamps

mRNA : messenger RNA

NAD^+: nicotinamide adenine dinucleotide

NEM : N - ethylmaleimide

N terminus : amino terminus

PAGE : polyacrylamide gel electrophoresis

PBS : phosphate - buffered saline

PGE_1 : prostaglandin E_1

pI : isoelectric point

RIA : radioimmunoassay

rpm : revolutions per minute

SDS : sodium dodecyl sulphate

TBS : tris - buffered saline

TCA : trichloroacetic acid

Td : transducin

TEMED : N,N,N',N' - tetramethylethylenediamine

Tris : tris(hydroxymethyl)aminomethane

Tween 20 : polyoxyethylenesorbitan monolaurate

V : volts

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SUMMARY.

The aim of the work presented in this thesis was to investigate the status of the guanine nucleotide - binding protein, G_o, in the neuroblastoma x glioma hybrid cell line, NG108 - 15 and to assess any functional changes brought about by morphological differentiation of these cells.

The guanine nucleotide - binding proteins (G - proteins) are a family of highly homologous proteins which function to couple a considerable range of cell surface receptors for hormones, neurotransmitters and growth factors, either to enzymes involved in the generation of intracellular second messengers or to specific ion channels. Each G - protein consists of three subunits termed α, β and γ, although it is the diversity in the sequence of the α subunit which defines the identity of each G - protein. Amongst this family is G_o, originally the G - protein of unknown function, which is implicated in the regulation of voltage - sensitive Ca^{2+} channels.

The first part of this thesis describes the production and characterization of specific antisera directed against unique peptide stretches of the α subunit of G_o obtained from molecular cloning sequence analysis. Three antisera, each raised against different regions and specific for $G_o\alpha$ were produced using this strategy. These were characterized using

1. Immunoblot analysis with purified preparations of $G_o\alpha$.
2. Comparison of immunoblot analyses with various tissues known to contain $G_o\alpha$.
3. Mixing experiments with existing well characterized $G_o\alpha$ antisera and other G - protein antisera.

Morphological differentiation of the neuroblastoma x glioma hybrid cell line,

NG108 - 15, can be achieved with any of a number of pharmacological agents which exert their function by raising intracellular cAMP. When subjected to ADP - ribosylation by pertussis toxin, a marked increase in incorporation of radiolabel was observed in membranes from these treated cells when compared to controls. Subsequent quantitative immunoblot analysis with the specific $G_o\alpha$ antipeptide antiserum, IM1, revealed there to be a marked increase in $G_o\alpha$ immunoreactivity over control in the membranes prepared from each of the treated cells. The other $G_o\alpha$ antisera produced similar results, confirming the original observations. However, the levels of the β subunit did not change appreciably with differentiation.

As well as increases in the levels of $G_o\alpha$, differentiation of these cells with db cAMP (1 mM, 6 days) resulted in a marked decrease in the levels of $G_i2\alpha$ as determined using two specific antisera. Stimulation of high - affinity GTPase activity in response to opioid peptides, which in this cell line interact with an opioid receptor of the δ subclass, was much decreased in the db cAMP - differentiated cell membranes in comparison with membranes of untreated cells. Also, inhibition of adenylyl cyclase by these same opioid peptides was almost entirely attenuated in differentiated cell membranes although it was noted that opioid receptor number was also decreased in these membranes in comparison with the control cells.

Bovine brain has recently been shown to contain two forms of $G_o\alpha$, namely $G_o\alpha$ and $G_o\alpha$*. Two - dimensional analysis consisting of an isoelectric focussing first dimension followed by SDS - PAGE and immunoblotting revealed two species of immunoreactive $G_o\alpha$, with isoelectric points of 5.5 and 5.8, in membranes from NG108 - 15 cells. Differentiation with all of the treatments resulted in an increase in the levels of only the more acidic isoform. The possibility that these isoforms differed by some form of covalent modification was considered. Both were shown to be substrates for ADP - ribosylation by pertussis toxin and incubation with alkaline phosphatase,

under conditions that dephosphorylate $G_i2\alpha$, had no effect on either of the $G_o\alpha$ isoforms.

Separation of the two isoforms was achieved in one - dimension using SDS - urea PAGE analysis. The more slowly migrating band was the more acidic form and corresponded exactly in mobility with the major form of $G_o\alpha$ from both rat and mouse brain. However, there was no equivalent in brain of the more rapidly migrating form of $G_o\alpha$ in the cells suggesting that this protein was not identical to $G_o\alpha$*.

Chapter 1.

INTRODUCTION.

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1. 1. Historical Perspective - The involvement of GTP in the hormonal control of adenylyl cyclase.

Many hormones, growth factors and neurotransmitters exert their actions on target cells through modulations in the levels of a number of intracellular second messengers. It is now apparent that many of these effects are elicited via a family of closely related guanine nucleotide - binding proteins [G - proteins] which act as transducing elements.

In a pivotal series of experiments performed in the late 1950's and early 1960's Earl Sutherland and his colleagues discovered that cAMP was able to mediate the actions of adrenaline upon canine liver [Rall *et al.*, 1957; Sutherland *et al.*, 1962]. Sutherland suggested that the enzyme responsible for regulating cellular cAMP levels, adenylyl cyclase [**E.C. 4. 6. 1. 1.**] , could be hormonally stimulated and that this activation was mediated through specific receptors on the cell surface. Once the receptor was filled by a hormone, a conformational change in the catalytic moeity of the enzyme resulted in a switching - on of the cAMP apparatus.

In the ensuing two decades much effort was expended into the investigation of hormonal regulation of cyclic nucleotide synthesis and in the identification of the various components involved in that regulation.

Rodbell and his co - workers first demonstrated that GTP was required for glucagon activation of adenylyl cyclase in plasma membrane fractions from rat

hepatocytes and that GTP enhanced the dissociation rate of radiolabelled glucagon in these membranes [Rodbell *et al.*, 1971a; Rodbell *et al.*, 1971b; Harwood *et al.*, 1973]. Subsequent studies using radiolabelled β - adrenergic antagonists and purified erythrocyte plasma membranes gave essentially the same results as for glucagon thus confirming a central role for GTP in receptor - mediated activation of adenylyl cyclase [Rodbell, 1980].

Using non - hydrolysable analogues of GTP [namely GTPγS and GppNHp] Schramm & Rodbell noted that it was possible to irreversibly activate adenylyl cyclase, even in the absence of hormone [Schramm & Rodbell, 1975]. The effect of hormone on this process was to increase the rate of formation of the irreversibly activated enzyme [Cautrecasas *et al.*, 1975] suggesting that the role of the hormone - receptor complex is to make it possible for activation of adenylyl cyclase by guanine nucleotides. A divalent metal ion was also needed for activation and indeed no irreversible activation was seen in its absence [Rodbell, 1980].

Hormonal stimulation of adenylyl cyclase results in the hydrolysis of GTP to GDP. The demonstration of this receptor - controlled event and its apparent involvement in termination of the hormonal response was first described by Cassel & Selinger. They showed that activation of β - adrenoreceptors in turkey erythrocytes simultaneously caused an increase in high affinity GTPase activity and resultant GTP - dependent stimulation of adenylyl cyclase [Cassel & Selinger, 1976]. Although GTP involvement was shown in hormone activation of adenylyl cyclase, its means of interaction with the components of the signalling pathway was still largely unknown.

Since these pioneering studies showing involvement of G - proteins in hormonal signalling, it has become increasingly apparent that a family of highly homologous G - proteins are the targets for guanine nucleotides in these processes and that these proteins act as the main transducing element between the

receptor and its effector [**Figure 1. 1.**].

Classical G - proteins involved in the control of hormone action are however a subset of a larger group of GTP - binding proteins. Members of this superfamily include the proteins that control protein synthesis of which Elongation Factor Tu (EF - Tu) is the most studied [for review see Allende, 1988] and a group of " small " G - proteins with molecular masses of 20 - 25 kDa. Included in this group are the *ras* proto - oncogene product [Barbacid, 1987], G_p, a protein isolated from placenta [Evans *et al.*, 1986] and the products of the yeast YPT1 and SEC4 genes [Salminea & Novick, 1987]. However, this introduction will concentrate mainly on those proteins involved in hormonal signal transduction.

1. 2. G - Proteins - Characterization, Structure and Function.

1. 2. 1. Characterization of the G - Protein α subunit.

All of the G - proteins so far characterized appear to exist as heterotrimers comprising 3 non - identical protein subunits termed α, β and γ in order of decreasing mass. They are classified by the identity of their distinct α subunit and it is believed, in the majority of instances, that it is the α subunit which defines the nature of the interactions between that G - protein and both the receptor and the effector moieties with which it interacts.

To date nine genes that encode for the α subunits have been identified [Kaziro, 1990]. 12 polypeptides are known to be the the products of these genes [**Table 1. 1.**]. The α subunits range in size from 39 - 46 kDa from primary sequence information as derived from corresponding cDNA clones. The twelve α subunits characterized in this way are :

Figure 1. 1.

Schematic diagram of a generic G - protein - coupled receptor and effector.

Activation of receptor (R) by the first messenger leads to interaction with and activation of the heterotrimeric G - protein (G) associated with the cytoplasmic surface of the membrane which in turn interacts with and regulates an effector (E) that generates an intracellular second messenger.

Figure 1. 1.

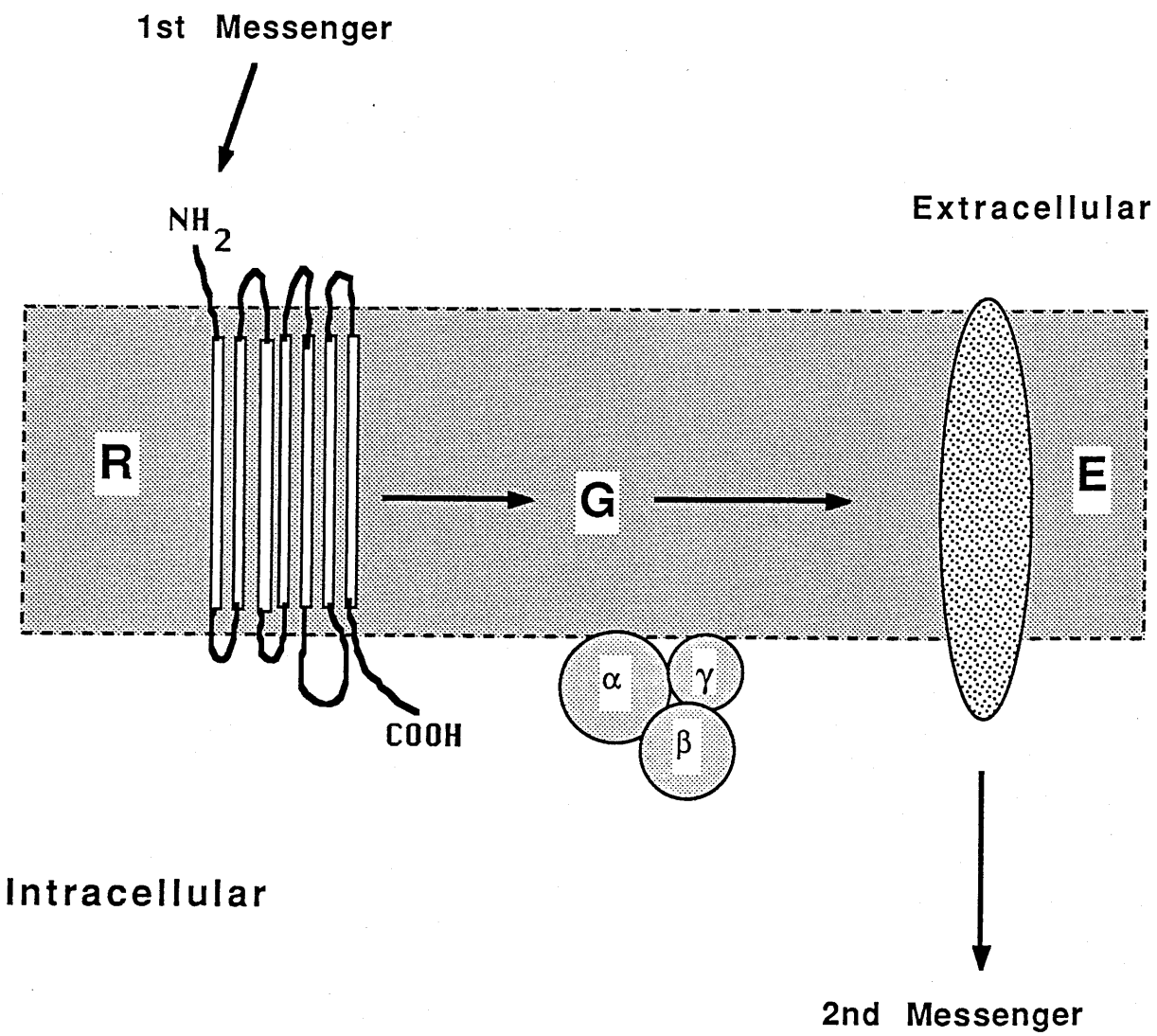

1st Messenger
NH2
Extracellular
R
G
E
α
γ
β
COOH
Intracellular
2nd Messenger

Table 1. 1.
The structure and function of the " classical " G - proteins.

G - protein	Sensitivity to ADP - ribosylation by bacterial toxins	Function	Distribution
G_s (x4)	cholera toxin	stimulation of adenylyl cyclase, activation of dihydropyridine - sensitive Ca^{2+} channels	universal
Td1	cholera toxin pertussis toxin	activation of cGMP phosphodiesterase	rod outer segments
Td2	cholera toxin pertussis toxin	activation of cGMP phosphodiesterase	cone outer segments
G_{olf}	cholera toxin	stimulation of adenylyl cyclase	olfactory sensory neurones
G_z (G_x)	none	undefined, stimulation of phospholipase C ?	undefined, but restricted
G_i1	pertussis toxin	undefined	limited; high levels in brain
G_i2	pertussis toxin	inhibition of adenylyl cyclase, stimulation of phospholipase C	universal
G_i3	pertussis toxin	regulation of K^+ channels	undefined; universal ?
G_o	pertussis toxin	regulation of Ca^{2+} channels	limited

$G_s\alpha$ .Two forms of $G_s\alpha$ corresponding to polypeptides of 46 and 44 kDa have been identified [Northup *et al.*, 1980]. However, molecular cloning has revealed the existence of four forms of $G_s\alpha$ cDNAs ($G_s\alpha$ 1 - 4) which have been isolated and characterized from human brain. $G_s\alpha 1$ and $G_s\alpha 3$ are identical except that $G_s\alpha 3$ lacks a single stretch of 45 nucleotides. $G_s\alpha 2$ and $G_s\alpha 4$ have three additional nucleotides to $G_s\alpha 1$ and $G_s\alpha 3$ at the 5' end of exon 4 [Bray *et al.*, 1986]. Bovine adrenal gland has been shown to contain two $G_s\alpha$ cDNAs that correspond to $G_s\alpha 1$ and $G_s\alpha 4$ which when expressed in COS - m6 cells generated the 52 and 45 kDa forms of the G_s protein [Robishaw *et al.*, 1986a; Robishaw *et al.*, 1986b]. Translation of the four forms indicated that the two short forms of $G_s\alpha$ consist of 380 and 381 amino acids and have Asp^{71} - Glu^{72} and Asp^{71} - Ser^{72} at the splice junction respectively. The two long forms of $G_s\alpha$ consist of 394 and 395 amino acids, having a 15 amino acid insert in place of the Asp^{71} residue.

The human $G_s\alpha$ gene isolated by Kozasa *et al.*, 1988 contained thirteen exons and twelve introns spanning approximately 20 kilobases of the genomic DNA. Comparison of the four human $G_s\alpha$ cDNAs with this human genomic sequence suggests that four types of $G_s\alpha$ mRNAs may be generated from the single $G_s\alpha$ gene. Analysis of the nucleotide sequence upstream of the initiation codon reveals a high G + C region suggesting the possible binding site for the transcriptional factor Sp1. No typical TATA box or CCAAT box was found in the promotor region of the $G_s\alpha$ gene [Kozasa *et al.*, 1988].

G_s has been unequivocally established as the stimulatory regulatory element of adenylyl cyclase as demonstrated by its ability to reconstitute hormonal, fluoride and guanine nucleotide regulation and cholera toxin sensitivity to the $G_s\alpha$ - negative adenylyl cyclase system of *cyc*$^-$ S49 lymphoma cells [Northup *et al.*, 1980; Codina *et al.*, 1983]. Purified preparations of G_s have also been shown to stimulate the fully

purified catalytic subunit of adenylyl cyclase [May *et al.*, 1985]. G_S has also been implicated to have a role in the stimulation of dihydropyridine - sensitive Ca^{2+} channels. Yatani and coworkers were able to demonstrate that both GTPγS and a purified preparation of G_S were able to prolong the survival of, and indeed stimulate, this class of calcium channels in membrane patches excised from guinea pig cardiac myocytes and bovine cardiac sarcolemmal vesicles incorporated into planar lipid bilayers [Yatani *et al.*, 1987]. These results were confirmed when analysis using skeletal muscle T - tubule calcium channels inserted into lipid bilayers were also regulated by G_S. The advantage of these channels over cardiac channels is their stability, thus allowing assessment of the effects of G_S under steady state conditions [Yatani *et al.*, 1988]. These findings seemed to challenge the hypothesis that although one G - protein can interact with several receptors, a given G - protein can only interact with one effector. However, since G_S may consist of up to four distinct polypeptides, it is conceivable that each variant may couple to a different effector species. To test this, Mattera and coworkers looked at the ability of the splice variants of $G_S\alpha$ to interact with both adenylyl cyclase and calcium channels. These workers utilised recent advances in recombinant DNA techniques to synthesize three of the four splice variants and were able to demonstrate that each of these polypeptides activated both adenylyl cyclase and the calcium channels to some extent [Mattera *et al.*, 1989].

Td1α, Td2α : The visual transduction cycle exists as a parallel to the hormonal transduction cycle. Both systems consist of receptor activation of a G-protein resulting in its interaction with effector and subsequent modulation in the production of second messengers. In the case of the rod photoreceptor cells, phototransduction occurs in the retinal rod outer segment. Activation of the photoreceptor, rhodopsin, by light results in its photoisomerization from the 11 - *cis* - retinal to the all - *trans* - retinal form enabling rhodopsin to interact with transducin (Td), the G - protein of the phototransduction cascade. Transducin then undergoes exchange of GDP for GTP and subsequent dissociation from its heterotrimeric

conformation. This enables the activated α subunit of transducin to stimulate a specific cGMP phosphodiesterase which rapidly hydrolyses cytosolic cGMP [for review see Stryer, 1986]. Activation of a single receptor molecule causes the hydrolysis of 10^5 cGMP molecules in a fraction of a second. The transient decrease in the levels of cGMP causes the closure of the cGMP - sensitive cation channels on the plasma membrane of the cell resulting in the hyperpolarization of the cell. Deactivation of the activated transducin occurs upon hydrolysis of the bound GTP by the intrinsic GTPase activity of transducin and subsequent reassociation of the holomeric protein. A similar transduction system exists in cone photoreceptor cells, however this system uses a cone specific form of transducin (termed Td2 as opposed to Td1 for rod transducin). Both forms of transducin have been cloned and the primary amino acid sequence homology between the two transducins is more than 85 % [Lochrie *et al.*, 1985; Lerea *et al.*, 1986].

G_{olf} : As well as visual transduction, G - proteins appear to be involved in other sensory systems. Evidence has been presented to show that, at least for some odorants, olfactory signal transduction is mediated by an adenylyl cyclase cascade coupled through a G - protein [for review see Bruch & Gold, 1990]. Previously, Jones & Reed identified five distinct cDNAs encoding the α subunits of G_s, G_o, and G_i1-3 from a rat olfactory cDNA library [Jones & Reed, 1987]. However, rescreening of this library using low stringency hybridization with a fully degenerate oligonucleotide probe directed towards a portion of the highly conserved GTP - binding domain and subsequent elimination of the known α subunit cDNAs left a single class of clones that hybridized weakly with $G_s\alpha$. These cDNAs encoded for a single olfactory neuroepithelium - specific $G_s\alpha$ - like protein which the authors called G_{olf} [Jones & Reed, 1989] and was exclusive to olfactory tissue, particularly the olfactory neurons. The predicted primary strucure of G_{olf} reveals a 44 kDa polypeptide consisting of some 381 amino acids sharing 88 % identity with $G_s\alpha$.

G_x (G_z) : This α subunit was cloned simultaneously from human brain [Matsuoka *et al.*, 1988] and human retina [Fong *et al.*, 1988] and hence the confusing nomenclature. It has 355 amino acids and is 41 - 67 % identical in amino acid composition with those of other known α subunits, being most similar to the $G_i\alpha$ family of proteins.

G_i1, G_i2, G_i3 : Originally designated as the G - protein which mediated the inhibition of adenylyl cyclase, molecular cloning has revealed, to date, the presence of three highly similar α subunits termed G_i1, $G_i2$and G_i3 based on the order in which they were cloned. The term G_i is ambiguous, while it first defined the G - protein mediating inhibition of adenylyl cyclase, it is now used widely to define those G - proteins, other than G_o, which have α subunits that are substrates for pertussis toxin catalysed ADP - ribosylation. The distribution of these proteins is markedly different. Whilst G_i2 and G_i3 appear to be present in all tissues so far examined, G_i1 is mainly expressed in brain tissue. Heterogeneity of these proteins has been confirmed both immunologically [Milligan,1988; Milligan,1990] and through their deduced primary amino acid sequences obtained by molecular cloning [Jones & Reed, 1987; Itoh *et al.*, 1988].

There is evidence to suggest that, in some cell types, G_i2 is the G - protein that mediates hormonal inhibition of adenylyl cyclase [McKenzie & Milligan, 1990] and that G_i3 is involved in the ability of muscarinic receptors to stimulate a certain class of K+ channels in atrial and pituitary membrane patches [Yatani *et al.*, 1987]. However, no such function has yet been ascribed to G_i1.

G_o : cDNA cloned from libraries made with mRNAs from a number of sources predict a polypeptide of 354 amino acids which has most homology to the $G_i\alpha$ class of polypeptides. This protein is more fully discussed in **section 1. 4.**

Cloning studies have revealed there to be a high degree of species homology between the members of the G - protein family. **Figure 1. 2.** shows the complete predicted amino acid sequences for several cloned α subunits. The amino acid sequence of $G_s\alpha$ is strongly conserved between human and rat, only 1 out of 394 amino acids is different and the sequence of bovine $G_i1\alpha$ is identical to that found in human. Even a form of $G_s\alpha$ found in *Drosophila* is 71 % identical with that from mammalian sources [Kaziro, 1990].

To date genomic clone analysis has been performed on a small number of the G - proteins. Screening of human genomic libraries with the corresponding rat cDNA's has led to the isolation of human genes coding for $G_s\alpha$, $G_i1\alpha$, $G_i2\alpha$, $G_i3\alpha$ and $G_o\alpha$ as well as the α subunit of G_z (G_x).

1. 2. 2. Properties of the G - protein α subunit.

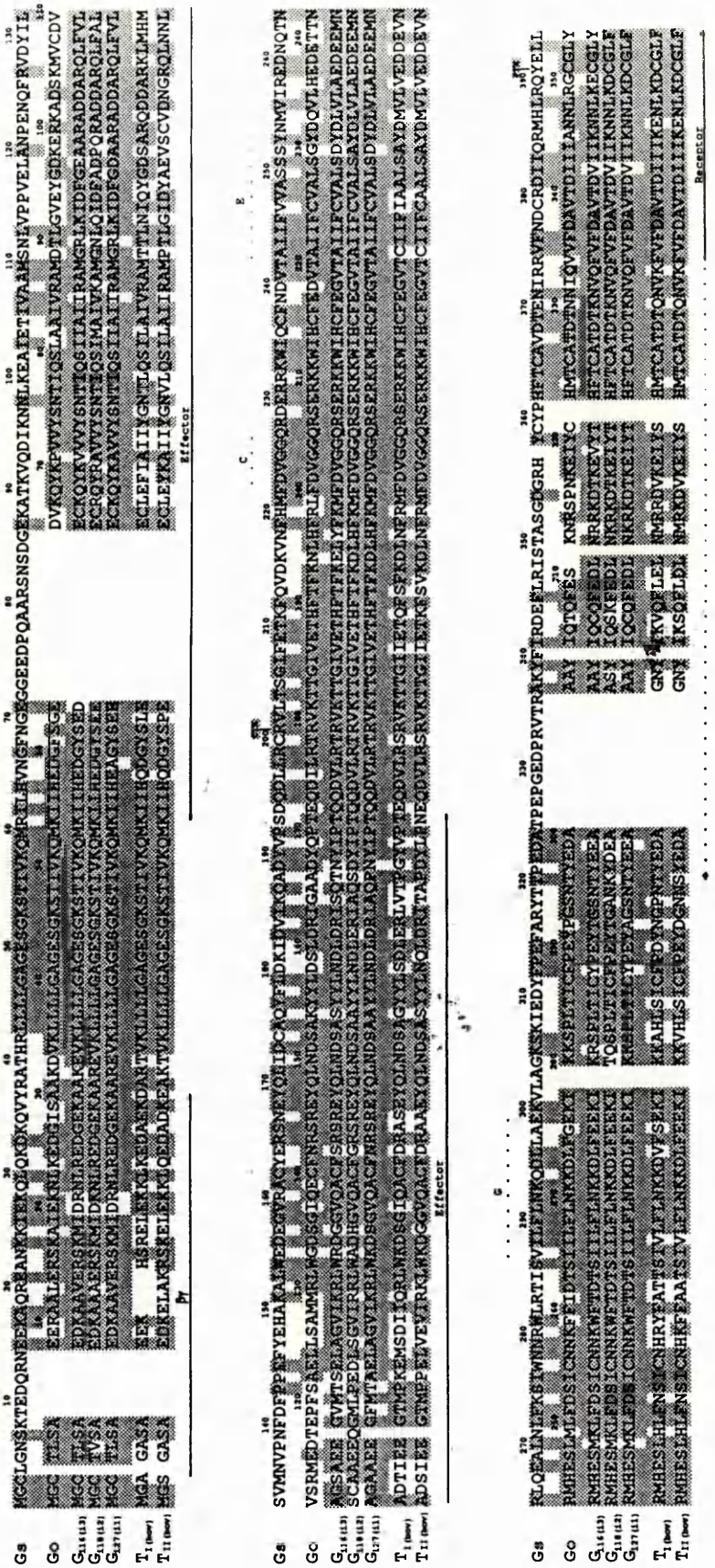
G - protein α subunits contain the guanine nucleotide - binding site and are able to bind GTP in the submicromolar range. The α subunit also contains the intrinsic GTPase activity which is necessary for the role of the G - protein in signal transduction. Since the function of these G - proteins is to couple agonist - activated receptors to various effector systems which are able to alter the levels of a number of discrete second messengers, this regulation must be under strict control. Thus the activation of effectors must be of limited duration and the G - proteins involved in that particular coupling should undergo subsequent deactivation. In the absence of a hormone - receptor complex, the rate of dissociation of GDP, the product of the GTPase reaction, limits the rate of the GTPase, since the catalytic rate is roughly 10 - fold faster than the GDP dissociation rate [Freissmuth *et al.*, 1989]. The α subunit also contains at least one high - affinity binding site for divalent metal ions; Mg^{2+} is the physiological ligand required for the formation of the activated GTP - bound species. In addition, Mg^{2+} appears to interact with a low - affinity binding site within the GDP -

Figure 1. 2.

Primary sequences of the α subunits of the currently identified pertussis toxin - sensitive G - proteins expressed in rat.

Areas of identity are shaded. In cases in which identity at particular residues does not extend across all the polypeptides, then the closest homolgy to the " G_i - like " subfamily is indicated. Where each of G_i1, G_i2 and G_i3 is represented by a different amino acid at one position then no further homology to G_o, Td1 or Td2 is noted. The sequences for G_o, G_i1, G_i2 and G_i3 are taken from Jones & Reed [1987], that for Td1 from Tanabe *et al.* [1985] and that for Td2 from Lochrie *et al.* [1985]. This figure is reproduced from Milligan [1988].

Figure 1. 2.



liganded α subunit and the affinity of this site may be regulated by the agonist - bound receptor [Gilman, 1987].

Figure 1. 3. shows the classical G - protein cycle [Gilman,1984; Gilman, 1987]. In the resting state the holomeric G - protein exists with GDP bound to the guanine nucleotide - binding site of the α subunit. Upon receptor activation the rate of release of GDP, which appears to be the rate limiting step in the G - protein activation / deactivation cycle, is enhanced and the the released GDP is replaced with GTP. The holomeric αβγ complex is now able, in the presence of Mg^{2+}, to dissociate into an activated α subunit with GTP bound and free βγ. The active α subunit is now able to interact with the catalytic moiety of its particular second messenger generating system e.g. adenylyl cyclase, phospholipase C, or a variety of ion channels to alter the rate of production of the second messenger. Hydrolysis of the terminal phosphate of the bound GTP by the intrinsic GTPase activity of the α subunit results in its deactivation. The α subunit, now with GDP bound, is able to reassociate with βγ to restore the G - protein to its resting state. Interruption of this cycle can occur when non - hydrolysable GTP analogues such as GTPγS or GppNHp are introduced. These have the effect of permanently activating the G - protein due to the complete blockade of the ' turn off ' GTPase activity [Gilman, 1987].

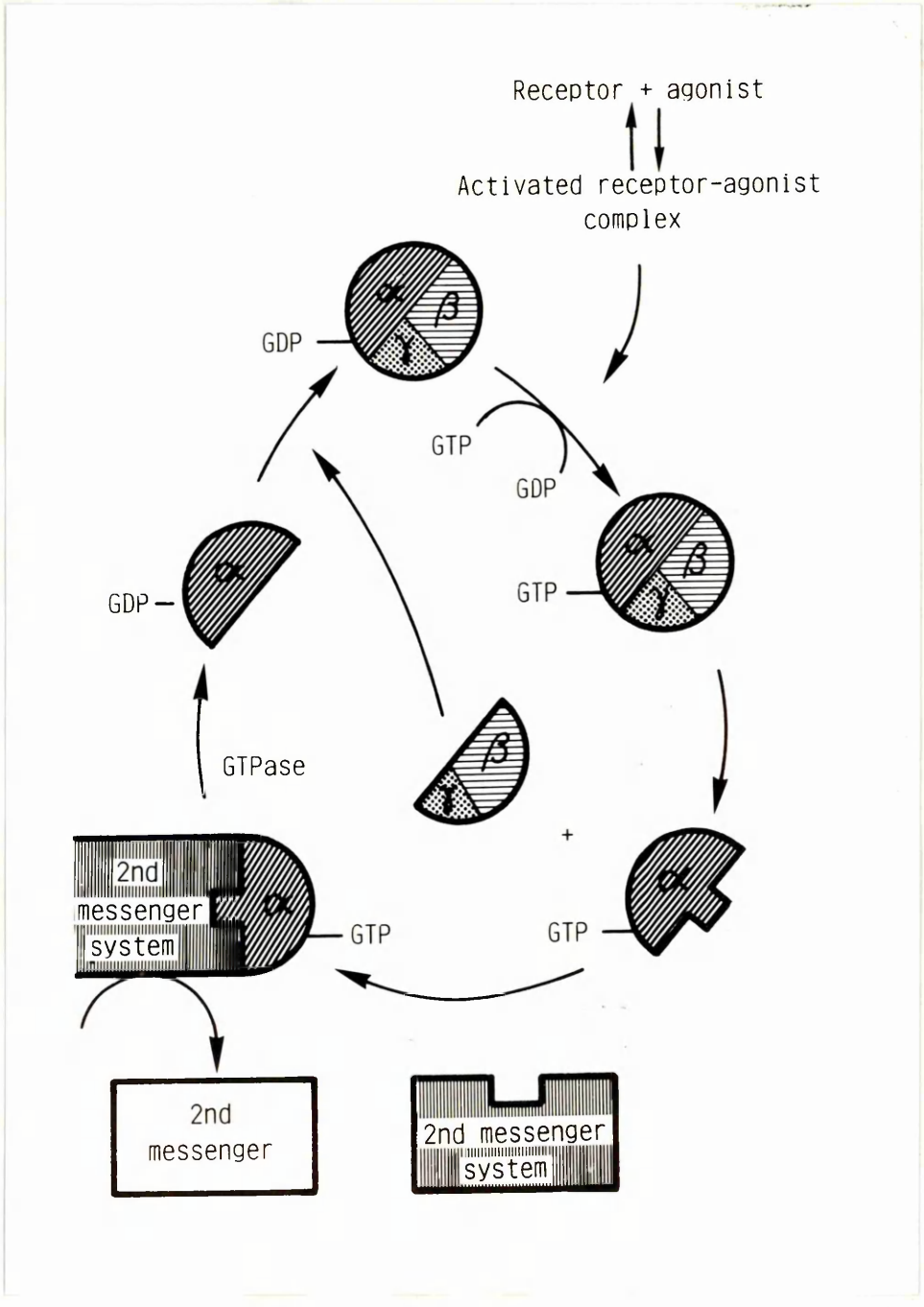
Another means of permanently activating the α subunit artificially has been to use certain fluorometallic compounds which simulate the terminal phosphate of GTP. Sternweis and Gilman were able to show that the activating effect of millimolar concentrations of F^- ions depended on the presence of micromolar amounts of aluminium and suggested that the real activating species could be the ion complex AlF_4^- [Sternweis & Gilman, 1982]. Bigay and coworkers then measured the stoichiometric binding of one aluminium per transducin and furthermore, the presence of GDP binding to transducin was essential to this activation. Noticing strong structural

Figure 1. 3.

The role of GTP binding and hydrolysis in the activation and deactivation of a schematic G - protein.

The function of the G - proteins is to couple agonist - activated receptors to their effector systems. This process appears to be cyclical and of limited duration. This figure is reproduced from Milligan [1988].

Figure 1. 3.


Receptor + agonist
Activated receptor-agonist complex
GDP
GTP
GDP
GTP
GDP
GTPase
2nd messenger system
GTP
GTP
2nd messenger
2nd messenger system

analogies between AlF_4^- and a phosphate group they proposed that the metallofluoride complex bound in the nucleotide site and mimicked the presence of the terminal phosphate of GTP, hence conferring on the G - protein its active conformation [Bigay *et al.*, 1985; Bigay *et al.*, 1987].

G - protein α subunits contain sites for covalent modification by various bacterial exotoxins which catalyse NAD^+ - dependent ADP - ribosyltransferase reactions. One such toxin, cholera toxin from *Vibrio Cholerae* has been shown to ADP - ribosylate both G_s and transducin. The toxin catalyses the ADP - ribosylation of an arginine residue at position 201 in $G_s\alpha$ [Van Dop *et al.*, 1984]. Covalent modification of these polypeptides by the toxin stabilises the GTP - form of the α subunit, inhibiting its intrinsic GTPase activity and resulting in the permanent activation of the α subunit in much the same way as the non - hydrolysable GTP analogues [Milligan, 1988; Spiegel, 1988]. In the case of G_s, adenylyl cyclase is now maximally stimulated and no longer under hormonal control.

The primary structure around this substrate arginine is highly conserved in all of the G - proteins which have been so far identified and the arginine itself is invarient in the equivalent position in the other G proteins. Using amphipathic and secondary structure analysis of the primary sequences of G_s, Td1, Td2, G_i and G_o, Masters and her coworkers were able to predict the secondary structure of a composite α subunit which they termed α_{avg}. This subunit was shown to contain four short regions of sequence homology to regions in the guanine nucleotide - binding domain of bacterial Ef - Tu. Similarities between the predicted secondary structure of α_{avg} and the known secondary structure of EF - Tu allowed for the construction of a 3 - D model of the guanine nucleotide - binding region of α_{avg} [Masters *et al.*, 1986]. Arg 201 was found to be close to the guanine nucleotide - binding domain in this model.

Pertussis toxin, an exotoxin from the organism *Bordetella Pertussis* has been extremely useful in the identification of a subset of G - proteins which are themselves substrates for mono ADP - ribosylation by the toxin. The toxin itself consists of six subunits one of which, the S1 subunit, displays an ADP - ribosyltranferase activity [for review see Gill, 1977]. Like cholera toxin, pertussis toxin was originally shown to produce sustained elevation in the receptor - mediated production of cAMP in pancreatic islets, hence the toxin originally being called Islet Activating Protein (IAP) [Katada & Ui, 1979; Katada & Ui, 1981].

Amongst the substrates for this toxin are Td1, Td2, G_i1, G_i2, G_i3 and G_o. Pertussis toxin appears to exert its effect on the G - protein cycle in a manner different from that of cholera toxin. Instead of inhibiting the intrinsic GTPase activity of the α subunit by converting GTP into a superactive nucleotide thus irreversibly activating the simulatory G - protein, pertussis toxin appears to prevent the receptor - mediated dissociation of the $\alpha\beta\gamma$ complex resulting in the inability of the G -protein to interact with its effector. The site of pertussis toxin - catalysed ADP - ribosylation in G - proteins that are substrates for this toxin is a conserved cysteine residue located four amino acids from the C - terminus [Masters *et al.*, 1986]. Interestingly, in G_x (G_z) the Cys residue is replaced by Ile indicating that this G - protein is refractory to modification by pertussis toxin [Matsuoka *et al.*, 1988; Fong *et al.*, 1988] and G_s, which is also resistant, contains a Tyr instead of Cys at this position [Itoh *et al.*, 1986].

1. 2. 3. Characterization of the G - Protein β subunit.

There is also diversity in the nature of the β subunit. Two forms have been isolated : a major form running at 36 kDa on SDS - PAGE (β1) and a minor one running at 35 kDa (β2) [Winslow *et al.*, 1986; Evans *et al.*, 1987; Woolkalis & Manning, 1987]. Both forms are widely distributed [Hinsch *et al.*, 1989] with the

exception of retinal tissue which appears to contain only β1 and placenta where β2 is the major form [Evans et al., 1987]. β1 and β2 are immunologically distinct [Roof *et al.*, 1985; Evans *et al.*, 1987] but have a 90 % homology in primary amino acid sequence [Fong *et al.*, 1987; Gao *et al.*, 1987].

The two β subunits are not the result of alternate splicing of a single gene but are the products from two separate genes [Amatruda *et al.*, 1988]. These genes have been located on separate chromosomes in human, β1 on chromosome 1 and β2 on chromosome 7 [Lochrie & Simon, 1988]. A third β subunit (termed β3)has recently been sequenced from a cDNA from brain. This protein, with a M_r of 37 kDa, has close homology to the other β subunits. The amino acid sequence of β3 has 83 % identity to β1 and 81 % identity to β2 although the 3' untranslated regions of the three cDNAs encoding for the proteins show no significant homology [Levine *et al.*, 1990].

1. 2. 4. Characterization of the G - Protein γ subunit.

The γ subunits exist as a group of small (8 - 10 kDa) distinct proteins which under all physiological conditions remain tightly bound to the β subunit in a complex generally refered to as the βγ complex.Although the total number of γ subunits is not yet known, immunoblotting studies suggest the existence of at least four different γ subunits with possible tissue - specific distribution. These are the γ_5 and γ_6 subunits associated with brain G_S, G_i andG_o [Robishaw *et al.*, 1989], γ_t which is associated with retinal transducin [Hildebrandt *et al.*, 1985] and a form found exclusively in placenta [Evans *et al.*, 1987].

To date only the retinal γ subunit [Hurley *et al.*, 1984; Ovchinnikov *et al.*, 1985] and the brain γ_6 subunit [Gautam *et al.*, 1989; Robishaw *et al.*, 1989] have been cloned and sequenced. The retinal γ clone produced a cDNA which encoded a

peptide consisting 74 amino acids with a calculated molecular weight of 8.4 kDa whereas the brain γ_6 clone encoded for a protein with three amino acids fewer. When aligned, comparison of these two predicted amino acid sequences revealed significant variability, with substitutions in no less than 41 of 71 of the aligned residues. The majority of these differences occured in the amino - terminal halves of the proteins and were mostly non - conservative substitutions, often resulting in the replacement of charged residues in the retinal γ subunit with uncharged residues in the brain γ_6 subunit [Robishaw *et al.*, 1989].

Fung and coworkers have recently demonstrated, in brain $\beta\gamma$, that the γ subunit is methyl esterified on the α - carboxyl group of a C - terminal cysteine residue and that this modification is likely to be associated with lipidation at the sulphydryl group of the same cysteine [Fung *et al.*, 1990], thus explaining the tight membrane binding property of the $\beta\gamma$ complex. This event has a precedent, in *ras* proteins, the C - terminal cysteine appears to be polyisoprenylated in the biosynthetic step prior to the attachment of a palmitoyl group on a nearby cysteine residue [Buss *et al.*, 1987].

Fukada and coworkers recently reported that it was possible to separate the transducin $\beta\gamma$ complex into two components, each of which appeared to differ only by the γ subunit. They found that one of the retinal $\beta\gamma$ complexes, which they termed T$\beta\gamma$-2, enhanced the binding of GTP to the α subunit of transducin in the presence of metarhodopsin II by about 30 - fold when compared to the other $\beta\gamma$ complex(T$\beta\gamma$-1) indicating that a specific γ subunit is essential for the physiological function of transducin [Fukada *et al.*, 1989]. In a more recent article the same authors reported that as well as being methyl esterified, a farnesyl moiety is attached to the C- terminal cysteine residue of the γ subunit in the T$\beta\gamma$-2 complex, which is the active form [Fukada *et al.*, 1990]. Such modifications were missing from the other $\beta\gamma$ complex suggesting that the attached farnesyl group is indispensable for the GTP - binding

activity of transducin. The possibility that such modifications occur in the γ subunits of the other heterotrimeric G - proteins is unknown at this time.

The diversities in the number of both β and γ subunits raises the possibility of the existence of numerous different holomeric isotypes of the holomeric G - proteins. For example three β subunits and four γ subunits produce twelve possible βγ complex combinations. The ability of each separate βγ to interact with each of the known fourteen α subunits would be able, in theory, to provide specifity for the signal transducing pathways known to interact with G - protein - linked receptors.

1. 2. 5. The role of the βγ complex.

The role of the βγ complex has still to be conclusively determined. Although detergents are required to solubilize the hormone receptor - linked G - proteins from the plasma membrane, once solubilized the α subunits behave as hydrophilic molecules suggesting that a possible function of the βγ complex could be to anchor the α subunit to the plasma membrane [Neer & Clapham, 1988]. Certainly the γ subunit contains a site for possible membrane attachment, a cysteine residue which may undergo lipidation upon membrane insertion [Fung *et al.*, 1990] and since the βγ complex does not dissociate under physiological conditions its potential role in anchorage of the α subunit must be considered. Furthermore, removal of the 2 kDa amino terminal region of $G_i1\alpha$ and $G_o\alpha$ with trypsin, although not affecting their GTPase activities, prevents the association of these polypeptides with βγ. Digestion of the carboxyl terminus of the α subunits with carboxypeptidase A did not interfere with the formation of the heterotrimer [Neer *et al.*, 1988]. However α subunit anchorage by βγ cannot be the whole story, since the GTP - induced dissociation of the αβγ heterotrimer does not result in the rapid release of G - protein α subunits from the membrane. It has been noted, however, that analogues of GTP but not of GDP or ATP will cause a slow, time

- dependent release of the α but not the βγ subunit of pertussis toxin - sensitive G - proteins from membranes of both NG108 - 15 and C6 BU1cells. This guanine nucleotide - dependent release does not require proteolytic cleavage of the G - proteins as the released forms are the same size in SDS - PAGE as the membrane attached forms. Further, antisera directed against both the extreme N - and C - terminal regions identified both the released and membrane bound forms [McArdle *et al.*, 1988]. Certain of the G - protein α subunits are able to undergo a number of different covalent modifications. Immunoprecipitation of $G_o\alpha$, $G_i\alpha$ and $G_s\alpha$ from astrocytoma cells prelabelled with [^{3}H] myristic acid have demonstrated the presence of amide - linked myristate on G_i and G_o but not on G_s [Buss *et al.*, 1987; Schultz *et al.*, 1987], thus the observed release of the α subunits may have more to do with the turnover of myristate in the membrane than with any function ascribed by their association with the βγ complex.

In solution, activation of the G - proteins by non- hydrolysable analogues of GTP results in the dissociation of the heterotrimer into the activated α - GTPγS and free βγ. By mass action, increasing the concentration of βγ should favour the reassociation of the subunits, thus deactivating the G - protein. Northup was able to show that increasing the βγ concentration reversed the the activation of adenylyl cyclase by GTPγS - liganded $G_s\alpha$ [Northup, 1985], leading to the proposal that elevation of free βγ concentration could be a general mechanism for hormone receptor - mediated inhibition of adenylyl cyclase. Bokoch was able to reinforce this idea when he demonstrated that addition of exogenous, purified βγ was able to inhibit forskolin - activated adenylyl cyclase in a dose - dependent manner. Further this inhibition was reversed upon addition of proteins which were able to bind βγ e.g. G - protein α subunits [Bokoch, 1987]. A similar scheme has been proposed by Moriarty and coworkers as model for the regulation of phospholipase C. The *xenopus* oocyte has a muscarinic receptor - activated Cl^- channel that is mediated by inositol [1,4,5] trisphosphate. Cells microinjected with human erythrocyte or bovine brain βγ complex

showed a dose - dependent reduction of up to 95 % in the evoked Cl^- current [Moriarty *et al.*, 1988].

However, if reversal of adenylyl cyclase activation of $G_s\alpha$ by the release of a $\beta\gamma$ complex from a variety of activated G - proteins was the only mechanism of hormonal inhibition of the enzyme, then inhibition ought never to be observed in the absence of stimulation by $G_s\alpha$. In fact, somatostatin is able to inhibit adenylyl cyclase in the mutant S49 lymphoma cell, *cyc*$^-$, which is entirely lacking in $G_s\alpha$ [Hildebrandt *et al.*, 1982] thus inhibition must be occuring via some other method, possibly by direct inhibition of the enzyme by $G_i\alpha$.

The dissociation of the major retinal rod outer segment G - protein, transducin (Td1), upon activation by light and the subsequent activation of cGMP phosphodiesterase has been the subject of much study [Stryer, 1986; Hingorani & Ho, 1990]. It has been demonstrated that light also activates phospholipase A_2 in dark - adapted rod outer segments of bovine retina via a transducin - dependent mechanism [Jelsema, 1987] and, more recently, that addition of purified $\beta\gamma$ – stimulated phospholipase A_2 activity in dark - adapted, transducin - poor rod outer segments. Addition of the α subunit of Td1, which itself slightly stimulated phospholipase A_2 activity in this system, inhibited the $\beta\gamma$ - induced increase in enzymic activity probably as a result of subunit reassociation since the GTPγS - liganded α subunit (which cannot associate with $\beta\gamma$) was not able to inhibit the activation of phospholipase A_2 induced by $\beta\gamma$ [Jelsema & Axelrod, 1987].

The initial observation that the muscarinic gated - K^+ channel ($I_{K.Ach}$) in cardiac myocytes was dependent upon a pertussis toxin - sensitive G - protein [Pfaffinger *et al.*, 1985; Breitweiser & Szabo, 1985] and that this channel can be studied in isolated patches of membranes has led to the development of sophisticated

reconstitution assays that involve application of purified G - protein subunits onto the patches themselves. Using such a protocol, Logothetis and coworkers reported that nanomolar concentrations of purified brain βγ subunits activated $I_{K.Ach}$ in chick embryonic patches [Logothetis *et al.*, 1987].

1. 3. Isolation of G_s and G_i.

Gill & Meren demonstrated that the A1 subunit of cholera toxin, an exotoxin from the organism *Vibrio Cholerae*, was able to activate adenylyl cyclase in pigeon erythrocytes. This activation was shown to require GTP, was irreversible and as such resembled that produced by the non - hydrolysable analogues of GTP. Activation in pigeon erythrocytes could be correlated with the appearance a [^{32} P] - labelled polypeptide of 42 kDa when the membranes were incubated with the toxin and [^{32}P] NAD^+ [Gill & Meren, 1978]. Cholera toxin contains an ADP - ribosyltransferase activity which allows for the transfer of the ADP - ribose moeity from the radiolabelled substrate onto a protein acceptor. From these studies it was first deduced that the GTP sensitivity in the hormone signalling pathway was transduced via a stimulatory GTP - binding protein or G - protein first named N_S [stimulatory nucleotide - binding protein] but now called G_S [Rodbell, 1980].

As well as GTP and the toxin itself there is a third requirement for toxin - catalysed ADP - ribosylation of G_S. Gilman and his colleagues noted that the presence of an intrinsic membrane protein was also necessary for the toxin to have its effects [Schleifer *et al.*, 1982]. This protein termed the ADP - Ribosylation Factor (ARF) has been found in in every preparation of plasma membranes tested including those from cultured mouse lymphoma cells, and human and turkey erythrocytes [Kahn & Gilman, 1984]. ARF has been purified from from rabbit liver membranes as a 21 kDa polypeptide [Kahn & Gilman, 1984]. More recent studies have shown ARF to contain

a high affinity guanine nucleotide - binding domain and can therefore be classed among the small molecular weight G - proteins. Binding of GTP or its analogues to ARF is necessary for the activity of the cofactor in cholera toxin action although no ARF GTPase activity could be detected [Kahn & Gilman, 1986].

Having identified a role for a G - protein in the hormonal stimulation of adenylyl cyclase resulting in an increase in intracellular cAMP, attempts were made to resolve the system further. Using affinity chromatography, Pfeuffer was able to dissociate G - protein from a pigeon erythrocyte adenylyl cyclase preparation with Lubrol PX solubilization. This resulted in a loss of guanine nucleotide and fluoride activation, both of which could be restored upon reconstitution reinforcing the proposed involvement of a GTP - sensitive element which was discrete from adenylyl cyclase [Pfeuffer, 1977]. Subsequently Pfeuffer & Cassel showed that the 42 kDa cholera toxin substrate was specifically bound and eluted from the GTP - binding column by the same proceedure and so contained a GTP - binding domain. They concluded that cholera toxin affected the adenylyl cyclase system by catalyzing an ADP - ribosylation of this 42 kDa polypeptide G - protein thus showing that this G - protein, G_s , was a separate molecular species from adenylyl cyclase [Cassel & Pfeuffer, 1978].

The resolution of G_s as a signal transducing element was unequivocally obtained using genetic varients of the S49 murine lymphoma cell line. In this cell line cAMP is cytotoxic to the cell enabling Bourne and his coworkers to select for a clone which was unable to synthesize cAMP [Bourne *et al.*, 1975]. This clone named *cyc*$^-$ because it was originally thought to be deficient in adenylyl cyclase has proved to be a valuable tool in the elucidation of the various elements of transmembrane signalling.

Using detergent extracts from the wild type S49 cells in which the adenylyl cyclase activity was inactivated, Ross and Gilman were able to restore cyclase activity to

membranes from *cyc*⁻ and that the reconstitution was sensitive to both fluoride and guanine nucleotides. They concluded that the *cyc*⁻ cells were actually deficient in G_s and not adenylyl cyclase, an observation which was confirmed when they were able to show the presence of the enzyme in these cells by direct stimulation of of its catalytic moeity [Ross & Gilman, 1977a; Ross & Gilman, 1977b; Ross *et al.*, 1978]. Subsequent cholera toxin ADP - ribosylation of these membranes revealed that whereas the wild type S49 contained two cholera toxin substrates at 52 kDa and 45 kDa, the *cyc*⁻ membranes lacked any endogenous acceptors for the ADP - ribose transferred by the toxin [Northup, 1985]. The use of *cyc*⁻ and the ability to restore adenylyl cyclase activation in reconstitution provided an ideal assay to check further G_s purification protocols. Northup and his colleagues used such a reconstitution as an assay for G_s when it was eventually purified to homogeneity from rabbit liver [Northup *et al.*, 1980].

Purification of G_s brought with it a number of surprises. Analysis of the purified protein by denaturing SDS - PAGE revealed an oligomeric structure with three distinct polypeptides. These comprised the α subunit which consisted of two subtypes with molecular weights of 52 and 45 kDa and two smaller subunits, β and γ, which had molecular sizes of 35 and 10 kDa respectively.

The organization of the G - protein oligomer in the hormonal cycle and the means by which it was able to interact with it were unknown at this time. It could be shown that the G - protein appeared to exist in two distinct states, non - activated and activated. In its heterotrimeric formation G_s could be shown to be in the non - active basal state whilst its activated form was shown to be the GTP - bound free α subunit [Northup, 1985]. Activation of the G - protein either directly by hormone or indirectly by fluoride or the GTP analogues resulted in the dissociation of the oligomer, releasing

the activated α subunit. Preparations of purified βγ were shown to have no effect in activating adenylyl cyclase in *cyc*$^-$ and so the activation appeared to be solely dependent on the activated α subunit. However whilst no stimulatory function was ascribed to it, βγ was shown to exert an inhibitory constraint on adenylyl cyclase activity and, with the availability of purified βγ, it was shown that βγ inhibited the activation of G_S [Northup *et al.*, 1982; Northup *et al.*, 1983]. βγ appeared to interact with only one α subunit, a conclusion based upon the ability of holomeric G_S, containing the αβγ complex, to inactivate fluoride - stimulated G_S. Incubation of G_S at 30 ^oC, and in the presence of divalent cations, resulted in the irreversible dissociation of G_S into α and βγ. Placement into the fluoride activation assay allows for the determination of the stoichiometry of the subunits. Detergent extracts of S49 cells assayed in this way revealed that the amount of βγ released upon incubation at 30 ^oC exceeded the calculated levels for G_S. This suggested that βγ was complexed with one or a number of G - proteins other than G_S. This hypothesis was supported by the finding that the levels of βγ in the *cyc*$^-$ cells were identical to those in the wild type S49 cells and that incubation at 30 ^oC in the presence of a divalent cation was required for *cyc*$^-$ suggesting that βγ was indeed complexed to another G - protein [Northup *et al.*, 1983].

It was suggested that as well as hormone stimulation of adenylyl cyclase via G_S, the enzyme could be inhibited through a ligand coupled to an inhibitory G - protein analagous to G_S. Rodbell and his colleagues were able to show that GTP bimodally regulated adenylyl cyclase activity [Rodbell, 1980]. In the absence of stimulatory hormone, guanine nucleotides are able to inhibit adenylyl cyclase and whilst this inhibition is not so dramatic as the stimulation (usually amounting to some 30 - 70 % diminuation), it is well established for a number of hormones. Like the stimulatory ligands, inhibition requires GTP and divalent cation . In addition, the inhibition is dependent upon the monovalent cation Na^+ [Northup, 1985].

Identification of a G - protein as being involved in the inhibitory arm of hormonal control of adenylyl cyclase was obtained in a similar manner to that for G_s. *Bordetella Pertussis*, the causitive agent of whooping cough, produces a number of exotoxins, one of which islet activating protein (IAP), or more commonly pertussis toxin, was shown to exert its effects by abolishing the hormonal inhibition of adenylyl cyclase. This toxin functions by transfer of an ADP - ribose group to an appropriate acceptor site on the GTP - sensitive element of the inhibitory pathway. Using the toxin in conjunction with radiolabelled NAD^+ revealed specific incorporation of label into a 41 kDa polypeptide in membranes of rat C6 - BU1 glioma cells when analysed using autoradiography. This labelled protein was presumed to be the inhibitory G - protein, G_i [Katada & Ui, 1982].

Purification of G_s from rabbit liver in the absence of fluoride, to prevent subunit dissociation yielded a 41 kDa G - protein associated with $\beta\gamma$ in a 10 x excess over G_s [Northup, 1985]. Katada & Ui had previously shown that pretreatment of membranes from bovine brain with pertussis toxin abolished hormonal inhibition of adenylyl cyclase and that this was associated with a toxin - specific ADP - ribosylation of a 41 kDa polypeptide [**see Section 2. 10.**]. When the 41 kDa protein which co - purified was incubated with pertussis toxin, it too was a substrate for pertussis toxin. So the study into the nature of the $\beta\gamma$ complex led to the isolation of the inhibitory G - protein, G_i.

G_i co - migrates with G_s during the initial chromatographic steps, but can be separated from G_s by either heptylamine - Sepharose or hydroxyapatite column chromatography [Bokoch *et al.*, 1983; Codina *et al.*, 1983]. This protein has subsequently been purified to homogeneity from a number of sources [Northup, 1985].

1. 4. G_o.

1. 4. 1. Isolation, purification and characterization of G_o.

G_o was discovered by a number of groups simultaneously when attempts to purify G_i from brain yielded a doublet of polypeptides with M_r of 39 and 41 kDa [Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Milligan & Klee, 1985]. The initial purification protocol utilised the same procedures used to purify G_s and G_i from rabbit liver [Northup *et al.*, 1980; Sternweis *et al.*, 1981; Bokoch *et al.*, 1983] and G_s from turkey erythrocytes [Hanski *et al.*, 1981]. Essentially, membranes from bovine brain were solubilized and the extract subjected to a series of column separation techniques including anion - exchange chromatography, gel filtration and finally hydrophobic chromatography using heptylamine - Sepharose. The purification was monitored by the ability of the column eluates to bind GTPγS, an indication of the presence of a G - protein. Analysis of this type led to the isolation of two proteins from bovine brain which were responsible for most of the observed GTPγS binding and accounted for 1.5 % of the total membrane protein [Sternweis & Robishaw, 1984]. When the mobilities of the two proteins were compared using high resolving SDS - PAGE analysis, they appeared to differ. These were tentatively identified as the 41 kDa $G_i\alpha$ subunit and the 39 kDa $G_o\alpha$ subunit, designated as such from simply being the " other " G - protein [Sternweis & Robishaw, 1984]. Both of these polypeptides were shown to be excellent substrates for mono - ADP - ribosylation by pertussis toxin with each protein incorporating approximately 1 mol ADP - ribose / mol of α subunit indicating the properties of G_i and G_o to be extremely similar. The possibility that G_o might have been a proteolytic fragment of G_i. was discounted when analysis of the time course of digestion of the two polypeptides with trypsin and of their products gave markedly different results. G_o was digested rapidly to a polypeptide of 38 kDa which was remarkably stable to

further digestion even after 6 hours exposure to trypsin. On the other hand G_i rapidly digested to polypeptides of 39 and 38 kDa. However, these polypeptides did not display the same stability as the 38 kDa fragment from G_o and were digested to polypeptides of about 30 kDa, with total digestion occurring within 6 hours. G_i purified from brain and G_i from liver appeared to be digested with the same trypsin profile [Sternweis & Robishaw, 1984]. Subsequently, Neer was able to demostrate that purified preparations of G_o displayed a measurable GTPase activity and that $G_o\alpha$ was found to be associated with a $\beta\gamma$ complex, which was apparently identical to those isolated with the α subunits of G_i and G_s, confirming it to be one of the ' classical ' G - proteins [Neer *et al.*, 1984].

The identity of G_o as a separate polypeptide, distinct from G_i, was obtained immunologically. Gierschik and his collaborators were able to obtain an antiserum raised against a purified preparation of G - proteins from bovine brain consisting primarily of holomeric G_i and G_o which had been treated with 6M urea. This antiserum, termed RV3, reacted specifically with the α subunit of G_o and with the common β subunit but not with either $G_s\alpha$ or $G_i\alpha$ [Gierschik *et al.*, 1986b]. Previously this group was able to generate a polyclonal antiserum, termed CW6, which was raised against purified transducin and cross - reacted with bovine brain G_i [Pines *et al.*, 1985] however, this antiserum did not recognize brain $G_o\alpha$ providing evidence that G_o could not be derived directly from G_i by proteolytic cleavage. Experiments with mixtures of antisera CW6 and RV3 revealed a doublet on immunoblots, comprising $G_o\alpha$ and $G_i\alpha$, confirming distinct identities for these proteins [Gierschik *et al.*, 1986b].

Antiserum RV3 provided a useful tool for investigating both the distribution of G_o and in its quantitation . Immunoreactivity on western blots with this antiserum was observed in membranes prepared from the brains of a number of vertebrate

species including frog, chicken, human and rat [Gierschik *et al.*, 1986b]. Indeed, antiserum RV3 also identified G_o in membranes of the neuroblastoma x glioma hybrid cell line NG108 - 15 but was unable to detect G_o in membranes from C6 BU1 glioma cells [Milligan *et al.*, 1986]. Assessment of the relative amounts of G_i and G_o in bovine brain cortical membranes from quantitative immunoblotting with antisera RV3 and CW6, showed that the ratio of $G_o\alpha$ to $G_i\alpha$ in these membranes was approximately 5 : 1. Thus using the data obtained from these antisera it would appear that $G_o\alpha$ accounts for almost 1 % of the total membrane protein in bovine brain cortex [Gierschik *et al.*, 1986b]. Applying the same quantitation procedure Milligan was able to assess the levels of G_o in NG108 - 15 cells as 3.8 pmol $G_o\alpha$ / mg membrane protein protein [Milligan *et al.*, 1986].

As well as antibodies generated against the ternary G_o complex, other researchers have produced antisera directed against either the isolated α subunit [Huff *et al.*, 1985; Tsai *et al.*, 1986; Homburger *et al.*, 1987] or against peptide regions deduced from the primary amino acid sequences [Mumby *et al.*, 1986; McArdle *et al.*, 1988; Eide *et al.*, 1987].

As purification protocols have improved, the number of identified G - proteins has increased. Detailed examination of fractions containing pertussis toxin - sensitive G - proteins during purifications from bovine brain have indicated the presence of two distinct populations of $G_o\alpha$ [Goldsmith *et al.*, 1988a]. These forms differed in isoelectric point and as such could be resolved on high performance ion exchange resins. However, a number of different anti - G_o antisera were unable to discriminate between the isoforms. More detailed characterization of these G_o forms has revealed the existence of up to four discrete proteins all displaying G_o immunoreactivity. Lang has reported the isolation of two forms of immunoreactive

G_o from bovine brain which, when subjected to *in situ* peptide mapping, revealed the presence of distinct peptides for each of the isolated proteins [Lang, 1989]. Although it was not possible to distinguish immunologically between the G_o isoforms, they appeared to migrate with different mobilities on high resolving SDS - PAGE gels. Katada was recently able to purify and isolate four proteins from bovine brain which displayed G_o immunoreactivity. Each α subunit was characterized by immunoblot analysis and were distinguishable from each other by their Mono Q column elution profile. To discount the possibilty that the different elution profiles of the $G_o\alpha$ subunits might be due to different $\beta\gamma$ subunits tightly associated with their α subunits, purification was performed in the presence of GTPγS and Mg^{2+}, conditions which allow for subunit dissociation [Kobayashi *et al.*, 1989]. Subsequent analysis of the four $G_o\alpha$ isoforms by means of peptide mappings after their proteolytic digestions and kinetic properties resulted in the proteins being put into two subgroups, each consisting of a pair of similar α subunits [Inanobe *et al.*, 1990]. These two groups of $G_o\alpha$ might correspond to the products of the two types of G_o gene products isolated by Jones & Reed [Jones & Reed, 1987] (see below), the difference within the groups being some form of post - translational modification.

1. 4. 2. The molecular biology of G_o.

Recently much effort has been put on the cloning of cDNAs coding for the various G - protein α subunits. Screening of cDNA libraries from a variety of sources has produced the isolation of a number of $G_o\alpha$ clones. Itoh was able to produce a partial sequence for $G_o\alpha$ by screening a cDNA library from rat C6 BU1 cells with a synthetic probe corresponding to a 17 peptide sequence obtained from amino acid sequence analysis of trypsin - digested purified $G_o\alpha$. This $G_o\alpha$ clone encoded a sequence of 310 amino acid residues that lacked the amino terminal region of the protein [Itoh *et al.*, 1986]. By screening a rat olfactory neuroepithelium cDNA

library with an oligonucleotide probe, Jones & Reed managed to isolate 32 recombinant clones encoding five distinct G - protein α subunits including one which encoded a complete $G_o\alpha$ [Jones & Reed, 1987]. This G_o clone, termed G_{31}, encoded the additional 44 amino - terminal residues absent from the previously reported sequence. Furthermore the comparable protein sequences were identical although the nucleotide sequences did differ at one position. Simultaneous with these studies, the sequence of $G_o\alpha$ was determined from a bovine retinal cDNA clone [Van Meurs *et al.*, 1987]. Comparison of this nucleotide sequence with the partial cDNA clone for rat $G_o\alpha$ revealed a 92 % identity in both the coding and the 3' - untranslated regions. From 67 differences in codons between the species there were only six differences in the corresponding amino acid sequences, five of them being conservative substitutions [Van Meurs *et al.*, 1987].

A number of groups have used oligodeoxynucleotide probes for analysis of $G_o\alpha$ mRNA in different tissues. Jones & Reed found that their $G_o\alpha$ probe hybridized to two messages of 4.1 and 4.5 kilobases [Jones & Reed, 1987]. In contrast Brann and coworkers found that their probe labelled three mRNA species, the most abundant having a size of 3.5 kilobases. As expected, this message was highly expressed in brain although it could be weakly detected in peripheral tissues [Brann *et al.*, 1987]. Using *in situ* hybridization as a probe to map the occurence of G - protein mRNA, Largent and coworkers were able to demonstrate that the mRNA species encoding $G_o\alpha$ had a more limited distribution throughout rat brain than the mRNA for either $G_s\alpha$ or the β subunits [Largent *et al.*, 1988]. Prominent $G_o\alpha$ message expression occurred in only a few areas such as the claustrum, endopiriform nucleus and the Purkinje cells of the cerebellum. These mRNA localisations were consistant with the distribution of the $G_o\alpha$ protein determined by immunohistochemical analysis [Worley *et al.*, 1986].

The development of specific antisera has provided the means whereby the tissue distribution of $G_o\alpha$ can be readily assessed. Early studies indicated $G_o\alpha$ to be primarily located in nervous tissue. Indeed the initial attempts at purifying G_o used bovine brain as the source of the protein [Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Milligan & Klee, 1985]. Using antiserum RV3, raised against purified G - proteins and shown to recognize G_o, Gierschik and coworkers were able to identify this protein in the cerebral cortex from a number of sources including frog, chicken, rat and human [Gierschik *et al.*, 1986b]. However, in accordance with the *in situ* hybridization studies, the concentration of $G_o\alpha$ in the brain is not homogenous. Regional distribution in bovine brain revealed that $G_o\alpha$ is more abundant in the frontal cortex, thalamus and hypothalamus than in medulla and pons [Gierschik *et al.*, 1986b; Asano *et al.*, 1987]. Using a specific $G_o\alpha$ antiserum, Chang and coworkers were able to demonstrate the levels of this protein in rat brain was low before birth but increased after birth and reached the full adult level at four weeks of age [Chang *et al.*, 1988].

Several peripheral neuronal or related tissues also display G_o cross - reactivity with specific antisera. Brain stem and spinal cord, although containing $G_o\alpha$, appear to do so in subtantially lower amounts than do forebrain structures [Gierschik *et al.*, 1986b], and several components of the autonomic nervous system including the enteric nervous system, superior cervical ganglion and adrenal medulla displayed strong G_o immunoreactivity [Terashima *et al.*, 1988].

Although predominantly located in nervous tissue, G_o has been been identified in a number of non - neuronal sources, for example in kidney [Huff *et al.*, 1985], retina [Terashima *et al.*, 1987] and skeletal muscle [Toutant *et al.*, 1990]. A number of groups have also reported $G_o\alpha$ immunoreactivity in heart [Liang *et al.*, 1986; Luetje *et al.*, 1987], however since G_o reactivity has been demonstrated in peripheral nervous tissue this may, in part, be due to tissue innervation.

Immunoreactivity to $G_o\alpha$ is not confined to mammalian systems. It has been detected in tissues such as frog brain [Gierschik *et al.*, 1986b], sea urchin eggs [Oinumura *et al.*, 1986] and *Drosophila* central nervous system [Thambi *et al.*, 1989; de Sousa *et al.*, 1989].

Screening of a *xenopus laevis* oocyte cDNA library with synthetic oligonucleotide probes complementary to conserved regions of different G - protein α subunits detected a number of positive cDNA clones. The determination of a nucleotide sequence of one of the cDNAs included the total sequence coding for a 354 amino acid protein that was 89 % identical to the sequence of rat $G_o\alpha$, the differences occuring in that region postulated to interact with receptors as well as effectors [Olate *et al.*, 1989]. Recently, Yoon and coworkers were able to characterize a *drosophila* gene encoding a G - protein α subunit isolated from a genomic library screened using bovine transducin α subunit cDNA as a probe [Yoon *et al.*, 1989]. The gene encoded for two proteins each of 354 amino acids which differed in seven residues in the amino - terminal region and had deduced amino acid sequences 81 % identical to $G_o\alpha$.The total genomic region encompassed by this gene was very large. The four genomic fragments sequenced, which contained all the coding sequences for the two gene products, were found to span a region of about 26 kilobases. When added to the 3' - noncoding and the 5' - regulatory regions, the gene spanned some 40 kilobases, about twice the size of the largest reported Gα gene, that of $G_s\alpha$ [Kosaza *et al.*, 1988]. The report by Yoon and colleagues contained the first evidence of the heterogeneity of $G_o\alpha$ on a molecular level with the two proteins being generated from alternative splicing of a single gene [Yoon *et al.*, 1989]. More recently, the production of $G_o\alpha$ isoforms produced by differential splicing of mRNA has been observed in a mammalian system. Screening of a hamster insulin - secreting tumor cell cDNA library for $G_o\alpha$ revealed the

presence of two inserts each coding for a distinct $G_o\alpha$. One of the $G_o\alpha$ cDNAs encoded for a protein identical to that cloned previously from rat and bovine tissues, whilst the other encoded for a protein that appeared to be derived from an alternatively spliced mRNA. This novel cDNA coded for a protein that is identical to $G_o\alpha$ in its first two - thirds and differed in the remaining carboxyl third of the polypeptide [Hsu *et al.*, 1990].

1. 4. 3. The function of G_o.

Since its initial purification from bovine brain, particular emphasis has been placed in attempting to assign a role for $G_o\alpha$. Immunohistochemical analysis of the distribution of $G_o\alpha$ in brain slices revealed that the localisation of $G_o\alpha$ corresponded in many areas with those of protein kinase C thus suggesting a major role for this G - protein, as the so called G_p, in the regulation of the phosphatidylinositol pathway [Worley *et al.*, 1986]. However it should be noted that whilst some ligands exert their effects in a pertussis toxin sensitive manner as might be expected if $G_o\alpha$ was the G - proten involved in this system, other ligands appear to interact with this system in a pertussis toxin insensitive manner. For example, the chemoattractant peptide FMLP activates phospholipase C via a G - protein in human leukaemic HL60 cells and furthermore this activation is sensitive to pertussis toxin [Kikuchi *et al.*, 1986]. However the responses of liver cells to vasopressin and angiotensin II [Pobiner *et al.*, 1985] and of 1321N1 human astrocytoma cells to muscarinic agonists such as carbachol [Hepler & Harden, 1986] have been demonstrated to be insensitive to pertussis toxin treatment suggesting that $G_o\alpha$ is not involved in the coupling of receptors to the phosophatidylinositol pathway.

The earliest studies into the function, not only of $G_o\alpha$, but indeed of the all of the pertussis toxin - sensitive G - proteins involved the use of various

reconstitution systems and purified preparations of the G - proteins. Florio & Sternweis were able to demonstrate that preparations of $G_i\alpha$ and $G_o\alpha$ from bovine brain reconstituted into phospholipid vesicles interacted with muscarinic receptors from the same source [Florio & Sternweis, 1985] although no major differences were observed between the G - proteins in this assay. This was taken a step further when Haga observed GTP - sensitive high - affinity binding of acetylcholine to purified porcine brain muscarinic receptors reconstituted into phospholipid vesicle with the three major brain pertussis toxin substrates ($G_i1\alpha$, $G_i2\alpha$, $G_o\alpha$) [Haga *et al.*, 1989]. A number of other receptors have been reconstituted with purified $G_o\alpha$ into phospholipid vesicles including the α_2 - adrenergic [Cerione *et al.*, 1986], μ – opioid [Ueda *et al.*,1988] and prostaglandin E_2 [Negishi *et al.*, 1988] receptors. Pertussis toxin pretreatment of cells abolishes the effects of those receptors which interact with pertussis toxin - sensitive G - proteins. Kurose and coworkers were able to reconstitute purified G_i which had been previously ADP - ribosylated with pertussis toxin and show that any previous interaction with the muscarinic receptor was now lost [Kurose *et al.*, 1986]. Reconstitution of purified G - protein α subunits into membranes prepared from pertussis toxin - treated cells has been shown to restore the effects of ligand activation. In membranes of NG108 - 15 cells, restoration of receptor - stimulated GTPase activity with the δ - opioid DADLE [Milligan & Klee, 1985] and with bradykinin [Higashida *et al.*, 1986] was observed upon reconstitution with purified preparations of G_o and G_i.

Reconstitution assays involving application of purified G - protein α subunits into whole cells pretreated with pertussis toxin have shed most light on the identification of a function for $G_o\alpha$.

Several experiments point to the involvement of a G - protein in the action of certain receptors that inhibit a certain class of voltage - dependent Ca^{2+} channels in a number of neuronal cell types. Chick dorsal root ganglia cells (DRG) display

noradrenaline - and GABA - induced inhibition of voltage - dependent Ca^{2+} channels which, because they are blocked by pertussis toxin and are inhibited by intracellular application of GDPβS, appear to be mediated through a G - protein [Holz *et al.*, 1986]. NG108 - 15 cells also appear to contain such a class of voltage - dependent Ca2+ channels which can be inhibited by noradrenaline [Docherty & McFadzean, 1989] and opioid peptides [Tsunoo *et al.*, 1986; Hescheler *et al.*, 1987; McFadzean & Docherty, 1989]. Hescheler and colleagues were able to show that abolition of DADLE - mediated inhibition of the Ca^{2+} channel with pertussis toxin could be reversed with intracellular application of purified $G_i\alpha$ and $G_o\alpha$. Since $G_o\alpha$ was 10 times more potent than $G_i\alpha$, the authors suggested that the former was more likely to be involved in coupling the opioid receptor to the Ca^{2+} channel [Hescheler *et al.*, 1987]. These findings were strengthened when McFadzean and collaborators were able to demonstrate the ability of a $G_o\alpha$ antiserum to abolish the noradrenaline - induced inhibition of the Ca^{2+} channel in these cells [McFadzean *et al.*, 1989]. Similarly Ewald was able to reverse the pertussis toxin abolition of neuropeptide Y - mediated inhibition of voltage - sensitive Ca^{2+} channels in rat DRG with perfusion of GTP and purified $G_o\alpha$ into the cell [Ewald *et al.*, 1988]. Another approach has been to make use of single identified invertebrate neurones. Harris - Warrick and colleagues recently demonstrated that dopaminergic inhibition of voltage - sensitive Ca^{2+} channels in snail neurones is mediated via a pertussis toxin - sensitive G - protein [Harris - Warrick *et al.*, 1988]. Pertussis toxin - catalysed ADP - ribosylation identified a single substrate for the toxin, which was identified by a polyclonal $G_o\alpha$ - specific antiserum raised against bovine brain $G_o\alpha$. The snail $G_o\alpha$ was not identical to mammalian $G_o\alpha$, as it migrated more slowly in SDS - PAGE but when the $G_o\alpha$ antiserum was microinjected into the neurone it was able to block the effect of dopamine on the Ca^{2+} channels and the effects of pertussis toxin were overcome by the introduction of purified $G_o\alpha$.

The intermediate level of complexity of the fruit fly *D. melanogaster*, its sophisticated genetics and the production of mutants has allowed for its use in dissecting particularlry complex biological processes. In a recent report, Guillen and coworkers studied the expression of a $G_o\alpha$ - like protein in a number of memory and learning *drosophila* mutants, such as *turnip, rutabaga* and *dunce*. They found that fruit fly form of $G_o\alpha$ was overexpressed in these mutants when compared to normal flies, the olfactory mutant *smellblind* and the the visual mutants *sevenless*, *ora* and *norp A*., thus implying a role, at least in *drosophila*, for $G_o\alpha$ in learning and memory. Furthermore, the overexpression appeared to be sex - dependent in at least one of the mutants, with the male *rutabaga* mutant containing twice the levels of $G_o\alpha$ when compared to the female [Guillen *et al.*, 1990].

The subcellular localisation of particular G - proteins may have a role in determining its function. Since the known function of the G - proteins, including G_o, would appear to be the passage of information across the plasma membrane, this requires the physical location of those G - proteins to be at, or near, this site. However, recent studies have indicated a more widespread distribution.

Using affinity - purified polyclonal antibodies to the α subunit of G_o, Gabrion and collaborators have investigated the ultrastructural localisation of this polypeptide in cultured and adult murine neurons [Gabrion *et al.*, 1989]. They were able to demonstrate that G_o was located both on the cytoplasmic face of the plasma membrane lining the cell body, especially in sites of cell to cell contact but was not detected at the inner face of either the pre - or post - synaptic membranes. They suggested that G_o would not be involved in transducing signals at the synapse but more probably modulated synaptic function by controlling the activity of effectors localised outside of the synaptic densities. In a parallel study, the same group was able to demonstrate the presence of $G_o\alpha$ in the choroid plexuses protruding in the third and fourth ventricle of murine brain

which they subsequently localised to the apical membranes and suggested that $G_o\alpha$ might be involved in the transduction of signals from the cerebrospinal fluid to the ependymal cytoplasm [Peraldi *et al.*, 1989]. The distribution of $G_o\alpha$ in this region of the brain appears to correlate well with that of the Na^+ / K^+ ATPase, an enzyme known to play an important role in the production of cerebrospinal fluid.

Neural connection during brain development appears to be extremely complex. The distal tip of neuronal processes, a structure termed the growth cone, is critical for transduction of extracellular siganls into directed growth. A recent report investigated the interaction of $G_o\alpha$ in the growth cone with GAP (Growth Associated Protein) 43, an intraneuronal protein whose expression is closely associated with axonal growth [Strittmatter *et al.*, 1990]. They noted $G_o\alpha$ to be a major growth component and that GAP 43 enhanced GTPγS binding to $G_o\alpha$ and proposed that the sequence homology between GAP 43 and G - protein - linked transmembrane receptors allowed GAP 43 to mimic the receptor activation of $G_o\alpha$, thus promoting the production of some form of intracellular signal.

1. 4. 4. Modifications in the $G_o\alpha$ subunit.

In common with other G - proteins, the α subunit of G_o is able to undergo certain post - translational changes. In astrocytoma cells that had been metabolically labelled with [^{3}H] myristate, Buss and coworkers were able to specifically immunoprecipitate the α subunits of two forms of G_i incorporating [^{3}H] myristate, whereas immunoprecipitated $G_s\alpha$ and the β subunit did not appear to contain the [^{3}H] label. Myristate was also detected by chemical analysis of fatty acid in $G_i\alpha$ and $G_o\alpha$ from bovine brain [Buss *et al.*, 1987].Similarly, Schultz and coworkers identified myristate as a component of purified $G_o\alpha$, postulating the site of attachment to be the amino - terminal glycine residue of the protein [Schultz *et al.*, 1987]. Mumby and collaborators have recently shown myristoylation in a number of G - protein α

subunits, including $G_o\alpha$, in monkey kidney COS cells which had been transfected with cytomegalovirus - based expression vectors encoding these various α subunits [Mumby *et al.*, 1990]. In this study incorporation of [^{3}H] myristate into $G_s\alpha$ was not observed, a reflection perhaps due the lack of a serine residue at position 6 of the primary translation product, which is a common feature of myristoylated proteins. Myristoylation of $G_o\alpha$ was blocked in the COS cells when an alanine was substituted for the amino - terminal glycine, as was the association of the protein with membranes suggesting that a possible role for myristate may be in the targetting of these proteins into the plasma membrane. The observation that overexpressed α subunit in these cells is associated with the membrane if it can be myristoylated may be consistant with this [Mumby *et al.*, 1990].

Although phosphorylation of purified G_i by both protein kinase A [Watanabe *et al.*, 1988] and the tyrosyl kinase activity of the insulin receptor [Zick *et al.*, 1987; O'Brien *et al.*, 1988] has been demonstrated, no information has been forthcoming about the possible phosphorylation of $G_o\alpha$. Close examination of the primary amino acid sequence of $G_o\alpha$ deduced from the cloning studies of Jones & Reed [1987] revealed none of the sequence motifs for phosphorylation by either protein kinase A [XRRXS*X] or protein kinase C [XRXXS*XRX], where **S*** is the phosphate acceptor serine residue, **R** is the specific arginine and X is the less essential residue [for review on protein kinase recognition sequence motifs see Kemp & Pearson, 1990].

1. 5. Recent advances in G - protein methodology.

The extreme homology of the α subunits has led to the development of a number of methodological techniques used to identify and assess the functions of individual G - proteins.

1. 5. 1. Identification and resolution of individual G - proteins.

Early studies characterized the α subunits of the heterotrimeric G - proteins upon their ability to be mono ADP - ribosylated by certain bacterial toxins, the most common being cholera toxin and pertussis toxin. Inability to assign functionality to particular G - proteins because of the lack of specific probes led to the production of antisera against these proteins.

The availabilty of relatively large amount of purified transducin allowed for the generation of the first polyclonal antiserum [Gierschik *et al.*, 1985]. This antiserum was shown to cross - react with a 41 kDa pertussis toxin substrate isolated from bovine brain (now referred to as G_i1) but did not identify a 39kDa pertussis toxin - sensitive protein (G_o) [Pines *et al.*, 1985; Gierschik *et al.*, 1986b]. G_o was susequently identified as a distinct polypeptide when a polyclonal antiserum raised against a mixture of $G_i1\alpha$ and $G_o\alpha$, purified from bovine brain, identified it but not $G_i1\alpha$ [Milligan & Klee, 1985]. Mumby and coworkers were the first to produce a series of antipeptide antisera against synthetic peptides corresponding to sequences of individual G - protein α subunits obtained from corresponding cDNAs [Mumby *et al.*, 1986] and the use of these probes has identified a number of novel G - proteins [Spiegel, 1988; Milligan, 1988; Milligan, 1990].

Measurement of the enhanced rate of GTPase activity of a membrane in response to agonist has, since the early days of research into G - protein interactions, provided a means of direct assessment of the interaction of a receptor with a G- protein. Indeed Cassel & Selinger used this approach in an avian erythrocyte system to demonstrate the interaction of the β - adrenergic receptor with a G - protein [Cassel & Selinger, 1976]. In general, greater success has been achieved using GTPase studies for receptors linked to pertussis toxin - sensitive G - proteins [Koski & Klee, 1981;

Aktories & Jakobs, 1981]. This is presumably due to the higher abundance of these proteins and their greater enzymic activity. As with other methodological techniques to study these proteins, the lack of specificity of pertussis toxin has made it difficult to specify receptor activation of particular pertussis toxin - sensitive G - proteins. The existence of specific antisera has helped address this problem.For example, preincubation of NG108 - 15 cell membranes with an antiserum directed against $G_i2\alpha$ completely abolished the ability of an opioid agonist to stimulate high - affinity GTPase activity [McKenzie & Milligan, 1990]. Clearly the use of such antisera will aid in the determination of G - protein specificity in the future.

Another recently developed assay for determining interactions between receptors and G - proteins is based upon agonist promoted exchange of guanine nucleotide. In the resting state, the α subunit has GDP in the nucleotide - binding site. Occupation of a G - protein - linked receptor is the signal for an exchange of GDP for GTP and subsequent dissociation of the heterotrimeric G - protein allowing the now activated α subunit to interact with its appropriate receptor. However, Gierschik and Jacobs noted that activation of myeloid differentiated HL 60 cells with the chemotatctic peptide FMLP caused the marked stimulation in the cholera toxin - catalysed ADP - ribosylation of a 40 kDa membrane protein in the absence of added exogenous guanine nucleotide [Gierschik & Jacobs, 1987]. This phenomenon was also noted in membranes of NG108 - 15 cells where the addition of saturating amounts of the synthetic enkephalin DADLE also produced a marked increase in the cholera toxin - catalysed ADP - ribosylation of a 40 kDa protein in the absence of guanine nucleotide but had no effect on the cholera toxin - catalysed ADP - ribosylation of $G_s\alpha$ [Milligan & McKenzie, 1988]. A possible explanation for these observations is that the 40 kDa protein observed in each case is that G - protein with which the appropriate receptor interacts. Under the conditions of the assay, as the agonist causes release of GDP from the nucleotide - binding site, there is no available GTP to fill the empty site and the G - protein is in such a conformation that it has become a weak substrate for cholera toxin.

It is pertinent to note that all of the G - proteins identified thus far have an invariant arginine residue in a position in the primary sequence equivalent to that which is the site of cholera toxin - catalysed ADP - ribosylation in $G_s\alpha$. This residue is close to a section of the primary sequence which forms part of the nucleotide - binding site and the maintenance of this arginine is of extreme importance for G - protein GTPase activity [Landis *et al.*, 1989].

1. 5. 2. Identification of G - protein - linked receptors.

At this time about 80 distinct receptors which can be activated by around 40 hormones and neurotransmitters appear to function via a guanine nucleotide - binding protein. It would be reasonable to assume that many more G - protein - coupled receptors will be identified in the fullness of time. A number of these receptors have been purified and cloned including five types of muscarinic receptor, four opsins, two β - and three α - adrenergic receptors, a substance K receptor and two dopamine D2 receptors [for review see Birnbaumer *et al.*, 1990]. Analyses of these sequences show that all of the G - protein - coupled receptors belong to a superfamily of proteins that have seven transmembrane spanning regions and have an extracellular amino terminus and an intracellular carboxyl terminus [Dohlman *et al.*, 1987].

It was the early experiments of Rodbell and coworkers into the effect of guanine nucleotides to decrease the affinity of glucagon - binding to its receptor that led to the proposal of the invovement of G - proteins in mediating receptor - effector coupling [see Rodbell, 1980]. It was susequently noted that guanine nucleotide effects on β - adrenergic receptor binding were confined to agonists and not antagonists [Maguire *et al.*, 1976]. Detailed analysis of ligand binding indicated that agonists were able to recognise two conformations of the receptor with different affinity, whilst antagonist affinity for the two forms appeared to be very similar if not identical. Furthermore,

addition of GTP analogues such as GppNHp or GTPγS reduced the ability of agonists, but not antagonists, to compete for radiolabelled antagonist binding sites. Thus, in the presence of guanine nucleotides a low affinity state of receptor for agonist was adopted, whereas in the absence of guanine nucleotides the receptor demonstrated a higher affinity for agonist.These observations led De Lean and coworkers to propose the ternary complex model, where in the unstimulated state, the inactive G-protein α subunit may interact with receptor, leading to the agonist promoted formation of a high - affinity ternary complex . In the presence of guanine nucleotides,which activate Gα, the ternary complex is destabilised and both agonist and G-protein are able to dissociate from receptor [DeLean *et al.*, 1980]. Although the ternary complex model was originally proposed to account for the effect of guanine nucleotides on agonist binding to receptors linked to stimulation of adenylyl cyclase, ligand binding experiments performed on receptor signalling systems linked to the inhibition of adenylyl cyclase yielded similar results [Koski & Klee, 1981] and led to the general conclusion that if the affinity of receptor for agonist is altered by the presence of guanine nucleotides, then that receptor functions through activation of a G-protein. Thus historically, the guanine nucleotide sensitivity of agonist binding affinity has often provided the initial suggestion that a particular receptor interacts with a G - protein.

Although this approach is limited in as much as it can yield little information as to the molecular nature of the G - protein involved, it can be used in conjunction with other techniques to yield further information. Binding studies performed on membranes from cells which had been pretreated with pertussis toxin [Kurose *et al.*, 1983; Hsia *et al.*, 1984] show that agonist affinity of antagonist displacement was reduced when compared to membranes of untreated cells. Furthermore, addition of non - hydrolysable GTP analogues was not able to further reduce agonist affinity for the receptor, indicating that pertussis toxin pretreatment had modified the relevant G - protein in such a manner that it now appeared to be functionally uncoupled from its receptor. Since

ADP - ribosylation by pertussis toxin allows for the transfer of an ADP - ribose onto a cysteine residue close to the C - terminus of those G - proteins that are substrates, it was proposed that this C - terminal region was likely to represent a key domain for the interaction of the G - protein with its receptor [Masters *et al.*, 1986]. Evidence for this proposal has been forthcoming. Interaction of rhodopsin and Td1 can be attenuated with both an anti - Td1 C - terminal antiserum [Cerione *et al.*, 1988] and with synthetic peptides which form that part of the primary sequence of the α subunit [Hamm *et al.*, 1988]. More recently, McKenzie & Milligan were able to demonstrate that affinity of binding of radiolabelled DADLE, a specific δ - opioid agonist, to membranes of NG108 - 15 cells which had been pretreated with pertussis toxin was reduced when compared to untreated cells, although the number of sites was not affected [McKenzie & Milligan, 1990]. To define further which of the pertussis toxin - sensitive G - proteins was involved in the transduction of the DADLE signal in these cells, membranes were preincubated with antipeptide antisera directed against the C - terminus of a number of G - protein α subunits and their effect on the ability of a single concentration of DADLE to bind specifically to the δ - opioid receptor was assessed. Only the IgG fraction from the antiserum which specifically identified $G_i2\alpha$ in these cells converted the receptor to a state which displayed reduced affinity for agonist.

1. 5. 3. G - protein - effector interactions.

Of the three elements that constitute signal transduction units (receptors, G - proteins, effectors), the effectors are the least understood in molecular terms. These include adenylyl cyclase, the cGMP phosphodiesterase of retinal systems, phospholipase C, phospholipase A_2 and several classes of ion channels including K^+ and Ca^{2+} channels. Thus measurement of the changes brought about by ligand or by direct activation of the G - proteins upon the levels of the various second messengers is a valid method to investigate cellular signalling events.

In common with other methodologies, the main drawback in using changes in the levels of second messengers as an index of G - protein involvement is lack of information about specificity. Although the the involvement of a G - protein may be implicated in the transduction of information from a specific receptor to an effector, the molecular nature of the G - protein involved is still unknown. The use of agents such as pertussis toxin may narrow the choice of involvement. For example, Katada & Ui showed that α_2 - adrenergic receptors were able to mediate inhibition of adenylyl cyclase in pancreatic islet cells and that treatment with pertussis toxin attenuated the inhibition, thus the effect of the toxin was to uncouple the G - protein involved from its receptor [Katada & Ui, 1979; Katada & Ui, 1981]. Knowing that the G - protein involved in this inhibition was a pertussis toxin substrate cut down the number of possible candidates, but still the molecular identity of the adenylyl cyclase inhibitory G - protein was unknown. Opioid inhibition of adenylyl cyclase in NG108 - 15 cells has been shown be mediated via a pertussis toxin - sensitive G - protein [Klee *et al.*, 1985]. NG108 - 15 cells contain three pertussis toxin - sensitive G - proteins ($G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$) [McKenzie & Milligan, 1990] and, in theory, any one or combination thereof of these polypeptides could be the one involved in opioid - mediated inhibition of adenylyl cyclase. McKenzie & Milligan, using specific antipeptide antisera generated against the C - terminus of those G - proteins present in these cells, were able to demonstrate that only the antiserum which recognises $G_i2\alpha$ was able to attenuate the opioid - mediated inhibition of adenylyl cyclase [McKenzie & Milligan, 1990]. This approach is not limited to only adenylyl cyclase. As well as δ - opioid receptors, the NG108 - 15 cell has been shown to expresses α_2 - adrenergic receptors which also mediate the inhibition of adenylyl cylase. Both opioid peptides [Hescheler *et al.*, 1987] and noradrenaline [Docherty & McFadzean, 1989] also act to inhibit voltage - sensitive Ca^{2+} channels in a pertussis toxin - sensitive manner in these cells. McFadzean and coworkers were further able to demonstrate marked attenuation of the noradrenaline - mediated inhibition of the channel upon microinjection of an antipeptide antiserum

raised against the C - terminus of $G_o\alpha$, implying a role for this protein in coupling adrenoreceptors to the calcium channel in these cells [McFadzean *et al.*, 1989].

1. 6. Transcriptional Regulation.

Control at the level of transcription is probably the major way in which gene expression is regulated in eukaryotes. The information encoded in DNA cannot in itself be used as a template for protein synthesis but must be first transcribed into mRNA. In eukaryotes, mRNA is synthesized by the enzyme RNA polymerase II. RNA polymerase II alone cannot accurately initiate transcription in vitro on purified templates unless supplemented with a number of protein transcription factors able to provide recognition of and specificity for minimal promoter DNA sequences.

1. 6. 1. Transcription factors.

Transcription factors can be subdivided into two broad categories, general and promoter specific. General transcription factors have been shown to interact with a promoter sequence which consists of a TATA motif and associated cap site. A number of transcription factors are associated with this promotor including factor TFIID from HeLa cell fractions [Sawadogo & Roeder, 1985], a 38 kDa protein called SII isolated from Ehrlich ascites cells [Sekimizu *et al.*, 1981] and chicken ovalbumin upstream promotor factor [Sagami *et al.*, 1986]. Since these transcription factors recognize a common motif, selectivity of the control of gene expression must be controlled by other more specific factors.

Promotor - specific transcription factors whilst sharing some of the promotors of the more general subtype are able to interact with unique promotor sequences giving a greater control to selective switching - on of particular genes. Included in this family

is the Sp1 transcription factor which binds to a GC - rich promotor region containing the sequence motif GGGCGG [Dynan & Tijan, 1983]. The Sp1 binding site occurs in many other viral and cellular promoters where it may act with other promotor motifs such as the CCAAT box. For example, the herpes simplex virus thymidine kinase promotor has two Sp1 binding sites surrounding a CCAAT box and all three elements are required for maximal promotor activity [Jones *et al.*, 1985]. Both the Sp1 sites and the CCAAT box are usually located in a similar position, about 40 - 100 base pairs from the start of the initiation sequence.

1. 6. 2. Regulation of Gene Expression By cAMP.

An increasing number of cAMP - regulated genes have been isolated and characterized. These genes appear to have a number of common characteristics. They are expressed in tissues which are responsive to hormones or regulatory factors, and their rate of transcription is rapidly altered by cAMP. The promotor region of several of these genes have been isolated and and their cAMP regulatory elements (CRE) defined using functional analysis of detailed deletions through the region and by mutations in specific bases within it. Using such a strategy, Short and his coworkers were able to show that the 5' flanking region of the gene for the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK) (E.C. 4.1.1.32) from the rat contains a CRE. They were able to identify this as a 47 - base pair region with a 12 - base pair core sequence [CTTACGTCAGAG] which had homology with four other cAMP - regulated genes. Deletion in this sequence resulted in the loss of db cAMP - sensitive production of the enzyme [Short *et al.*, 1986].

Similar experiments have identified functional CREs in a growing number of genes incuding those encoding for somatostatin, proenkephalin and vasoactive intestinal peptide [for review see Roesler *et al.*, 1988]. In the pheochromocytoma cell

line PC12 the protein binding to the CRE has a molecular weight of 43 kDa and is phosphorylated *in vitro* by the catalytic domain of cAMP - dependent protein kinase. Treating PC12 cells with forskolin also results in increased phosphorylation and this correlates with transcriptional induction of cAMP - dependent genes [Montminy & Bilezikjian, 1987].

Again it would seem that a number of such regulatory elements acting in cohort is needed in the control of gene expression. The PEPCK promotor - regulatory region contains a CRE and a CCAAT box. Functional analysis of deletions through this region of DNA has shown that removal of the CCAAT element results in a marked reduction in the rate of basal transcription of the enzyme as well as an attenuation in the level of induction by cAMP [Short *et al.*, 19861].

More recently a different cAMP - responsive element was identified. This sequence, termed Activator Protein 2 (AP2) binding site was initially shown to be a basal transcription enhancer in the human metallothionein IIA promotor [Mitchell *et al.*, 1987] and subsequently as an inducible enhancer when addition of forskolin produced elevated levels of transcription of β - globin in transfected HeLa cells [Imagawa *et al.*, 1987]. It should be noted however that transcription from this promotor was also increased in response to phorbol esters which activate protein kinase although addition of both produced an additive effect.

The consensus sequence for the AP2 element [CCCCAGGC] is associated with a number of genes including human growth hormone and rat prolactin [Roesler *et al.*, 1988].

1. 6. 3. Regulation of Gene Expression By Glucocoticoids.

The induction of of gene expression by a number of glucocorticoids is a

common observation. The PEPCK gene contains two glucocorticoid regulatory elements (GRE) within its' promotor region. Both of these regions contain the sequence motif TGTCCTCCCC and the presence of both elements is not required for dexamethasone stimulation of expression of the PEPCK gene [Short *et al.*, 1986]. Up regulation of the β - adrenergic receptor in response to glucocorticoid has been shown as a consequence of an increase in transcription. Using Northern blot analysis, it was demonstrated that the β_2 - receptor mRNA levels increased in a hamster vas deferens cell line exposed to dexamethosone in culture [Collins *et al.*, 1988]. Several putative GREs have been located in the non coding regions of the β_2 - adrenergic receptor gene, one of which in the 5' - untranslated region being obligate for glucocorticoid sensitivity [Malbon & Hadcock ,1988].

There are several instances of glucocorticoid regulation of the levels of G_S. In a rat pituitary cell line, GH_3, exposure to dexamethosone caused a 5 - fold increase in the mRNA for $G_S\alpha$ and a 2 - fold increase in the membrane - bound $G_S\alpha$ polypeptide and that the glucocorticoid - induced enhancement of adenylate cyclase activity in these cells was in part due to the increased expression of $G_S\alpha$. [Chang & Bourne, 1987]. Chronic coricosterone administration to normal rats resulted in the increase in the cerebral cortex of both the mRNA for $G_S\alpha$ and in the amount of the protein itself as assessed by immunoreactivity on immunoblots and ADP - ribosylation by cholera toxin [Saito *et al.*, 1989]. In contrast such treatment tended to decrease both the levels of $G_i\alpha$ mRNA and of the immunoreactivity of the protein itself. The levels of $G_o\alpha$ and β subunit immunoreactivity and mRNA were not affected by the treatment. Although these results are preliminary, they suggest that the role of cAMP and other agents in the control of transcription of the Gα genes may prove to be an important junction at which to regulate the levels of the various G - proteins themselves.

Chapter 2.

MATERIALS AND METHODS.

Chapter 2.

MATERIALS AND METHODS.

2. 1. Materials.

2. 1. 1. Chemicals.

Sigma Chemical Co. Ltd., Poole, Dorset.

KLH, trypsin, ATP, DADLE, DALAMID, arginine hydrochloride, nonidet P - 40, gelatine, Tween 20, 0 - diansidine, hydrogen peroxide, triethanolamine hydrochloride, theophylline, BSA, 7 - deoxycholic acid, bromophenol blue, NAD^+, NEM, TEMED, Amberlite MB - 1, Coomassie Blue R - 250, silver nitrate, formaldehyde, ouabain, cAMP, 8 - Bromo cAMP, PGE_1, Freund's complete adjuvant, Freund's incomplete adjuvant, cholera toxin.

Gibco Life Technology, Paisley, UK.

All tissue culture solutions, with the exception of foetal bovine serum.

Imperial laboratories, West Portway, Andover, Hants, UK.

Foetal bovine serum.

Prolabo, Paris, France.

2 - mercaptoethanol.

Koch Light Ltd, Haverhill, Suffolk, UK.

DMSO.

May & Baker Ltd, Dagenham, UK.

Ammonium persulphate.

Porton Products, Porton Down, Salisbury, Wilts, UK.

Pertussis toxin.

BCL, Lewes, East Sussex, UK.

2-deoxy thymidine, GTP, Db cAMP, DTT, AppNHp, creatine kinase, phosphocreatine.

Calbiochem Corporation, San Diego, California, USA.

Forskolin.

National Diagnostics, Aylesbury, UK.

Ecoscint scintillation fluid.

Pharmacia LKB , Milton Keynes, UK.

Ampholine pH 3 - 10 and ampholine pH 5 - 6.5.

All other laboratory chemicals were of analytical grade and obtained from **BDH Ltd, Poole, Dorset, U.K.**

2. 1. 2. Radiochemicals.

Amersham International plc., Amersham, UK.

[2,8 - ^{3}H] Adenosine 3' : 5' cyclic monophosphate (product no. TRK 559).

Sp. activity 40 - 60 Ci / mmol.

Guanosine 5' - [$\gamma -$ ^{32}P] triphosphate (product no. PB 144).

Sp. activity 10 Ci / mmol.

Rabbit Ig, [^{125}I] - labelled whole antibody (from donkey) (product no. IM 134).

Sp. activity 750 -3000 Ci / mmol.

NEN Research Products, Du Pont (UK) Ltd., Stevenage, UK.

Nicotinamide adenine dinucleotide, di(triethylammonium) salt, [*adenylate* - ^{32}P] (product no. NEG - 023).

Sp. activity 10 - 50 Ci / mmol.

Enkephalin (2 - D - alanine - 5 - D - leucine), [tyrosyl-3,5 - ^{3}H(N)] (product no. NET - 648).

Sp. activity 30 - 50 Ci / mmol.

2. 1. 3. Peptide sources.

A full list of the peptide sequences, their locations on the G - proteins and the resultant antisera is given in **Table 2. 1.**

Peptide NKLEDGISAAKDVK (which resulted in antiserum IM1) was synthesized by Dr. A.I. Magee at NIMR, Mill Hill, London.

Peptides MSELDQLRQE (antiserum BN1) and KNNLKECGLY (antiserum I3B) were synthesized by Biomac Ltd, Glasgow, UK.

All other peptides used were synthesized by Dr C.G. Unson, The Rockerfeller University, New York, USA.

2. 1. 4. Other Antisera

Antiserum HPA, which was raised against a mixture of purified $G_o\alpha$ and β was a kind gift from Dr. H. Ploegh and Dr. L. van der Voorn, Division of Cellular Biochemistry, Netherlands Cancer Institute, Amsterdam, The Netherlands.

Antisera LE2 and AS7 were kind gifts from Dr. A. M. Spiegel, National Institutes of Health, Bethseda , MD, USA.

2. 1. 5. Purified G_i and G_o subunits.

The mixture of purified G_i and G_o from rat brain used in these studies was prepared by the method of [Milligan & Klee, 1985].

2. 2. Animals.

New Zealand White rabbits were purchased commercially and maintained by the Physiology department animal house. Rat and mouse brains were obtained from stock animals in the Physiology department animal house which were sacrificed by means of cervical dislocation.

2. 3. Antibody production.

All the antisera used were prepared according to the method of [Goldsmith *et al.*, 1987]. Pre - immune samples were taken from each of the animals prior to injection. These were checked for any cross reactivity. All the animals used to produce antisera showed no such cross reaction.

2. 3. 1. Peptide conjugation.

10 mg of keyhole limpet haemocyanin and 3 mg of the particular peptide needed were dissolved in 1 ml of 0.1 M Na phosphate buffer (pH 7.0). 0.5 ml of 21 mM glutaraldehyde was then added dropwise with stirring and the combined 1.5 ml was incubated overnight at room temperature. This was then mixed with an equal volume of Freund's complete adjuvant and sonicated for 20 seconds.

2. 3. 2. Immunization of the rabbits.

Immediately after sonication the resultant emulsion was injected in 0.5 ml aliquots into multiple subcutaneous sites in a New Zealand White rabbit. Immunizations were normally performed simultaneously into 2 rabbits in order to maximize successful antibody production. After four weeks each animal received a booster immunization with material prepared identically except that one-half as much peptide and KLH were injected in Freund's incomplete adjuvant.

2. 3. 3. Serum harvesting.

Two weeks after the booster injections the animals were bled from the ear veins and subsequently bled at monthly intervals. The blood was collected into glass universals and allowed to clot overnight at 4 ^{o}C. The serum was removed from the clot and subjected to centrifugation at 1000g for 5 minutes on a Beckman TJ - 6 centrifuge in order to remove any remaining erythrocyte traces. The serum was then aliquoted in 0.25 ml volumes and stored at -80 ^{o}C.

2. 4. Tissue culture.

2. 4. 1. Cell culture conditions.

Neuroblastoma x glioma hybrid cells, NG108 - 15, were a kind gift from Dr W. Klee (NIH, Bethseda, Maryland, US.A.). The cells were grown in continuous culture according to the method of [Hamprecht *et al.*, 1985] in sodium bicarbonate (0.375 % (w/v)) buffered Dulbecco's Modified Eagle's Medium containing foetal bovine serum (10 % (v/v)), 2 mM L - glutamine, 100 I.U./ ml penicillin, 100 µg / ml streptomycin. The medium was further supplemented with HAT (Hypoxanthine, Aminopterin, Thymidine) at final concentrations of 0.1 mM, 10 µM and 16 µM respectively.

The cells were grown in monolayer culture in 75 cm^2 sterile tissue culture flasks (Bibby Science Products, Stone, Staffordshire, UK.) containing 10 ml of the the above medium. The flasks were incubated at 37 ^oC in a VSL incubator (Scotlab), gassed with a final O_2 : CO_2 ratio of 95 % : 5 % and allowed to reach confluency. Medium was changed when deemed necessary, usually every 2 days.

2. 4. 2. Splitting of confluent cell cultures.

Each flask of confluent cells was usually split 1 : 5 into new flasks. The medium was removed and 3 ml of a trypsin solution consisting 0.1 % (w/v) trypsin, 10 mM glucose, 0.67 mM EDTA was added. Trypsin treatment was terminated, when all the cells were detached from the flask, by addition of 7 ml of the 10 % FBS-supplemented DMEM medium. The cells were decanted into a sterile 50 ml conical polypropylene centrifuge tube (Elkay Products Inc., Shrewsbury, Mass., USA.) and spun on a bench top centrifuge at 1000g for 5 minutes. The supernatant was then carefully discarded leaving a tightly packed pellet of cells at the bottom of the tube. The pellet was resuspended thoroughly in 5 ml of the incubation medium and 1 ml added to

new flasks containing 9 ml of fresh medium. The flasks were then placed in the incubator and the medium changed when necessary.

2. 4. 3. Freezing down and storage of cells.

Cells were frozen down and stored in liquid N_2 in order to maintain a stock of cells with as early a passage number as possible. After trypsinization and centrifugation the cell pellet was resuspended in 8 % (v/v) DMSO in foetal bovine serum, 1 ml for every confluent flask, and put in sterile freezing vials (Costar, 205 Broadway, Cambridge, Mass. USA.) in 1 ml aliquots. The cells were frozen slowly to avoid formation of ice crystals in the cells by placing the vials in polystyrene containers with cotton wool and leaving overnight at -80 ^{o}C. The vials were then transferred to liquid N_2 vats and stored until needed.

2. 4. 4. Recovery of cells after freezing.

Vials were removed from liquid N_2 storage and warmed up quickly in a 37 ^{o}C water bath. The whole contents of one vial were put into a 75 cm^2 flask containing 10 ml of the 10 % FBS-supplemented DMEM medium and placed in the incubator. The medium was replaced next day with fresh and the cells were then used as required.

2. 4. 5. Preparation of differentiating agents.

The various agents were made up on the day of use as a 10 x stock, i.e. 10 mM Db cAMP or 8 - Bromo cAMP, 100 µM forskolin or PGE_1 in 2 % FBS-supplemented DMEM. These were filtered through a 0.22 uM filter (Corning Glass Works, Corning, NY, USA.) and collected in a sterile 50 ml centrifuge tube. 1 ml was then added to each flask as described below in **section 2. 4. 6 .**

2. 4. 6. Treatment of cells with differentiating agents.

Cells were differentiated by incubating for 6 days in a variety of agents. These were Db cAMP or 8 - Bromo cAMP at a final concentration of 1 mM and forskolin or PGE_1 at a final concentration of 10 μM.

Confluent flasks of cells were split 1 : 5 using the trypsin method described above. After centrifugation the supernatant was discarded and the pellet resuspended in DMEM medium supplemented with 2 % FBS instead of 10 %. 1 ml of the cell suspension was added to flasks containing 8 ml of 2 % FBS-supplemented DMEM and 1 ml of the particular differentiating agent made up in 2 % FBS-supplemented DMEM as a 10 x stock.

2. 4. 7. Treatment of cells with pertussis toxin

To obtain membranes which had been previously pretreated with pertussis toxin, cells were incubated for 24 hours with 100 ng / ml pertussis toxin and the cells harvested as described in **section 2. 4. 8.**

The toxin was not thiol - activated as is the case for *in vitro* pertussis toxin treatment (see **section 2. 10 .3.**).

2. 4. 8. Harvesting of cells.

Cells were normally harvested 6 days after incubation with the differentiating agent. The cells were gently washed off the surface of the flasks using a pasteur pipette and collected in a 50 ml conical centrifuge tube on ice. The cell suspension was centrifuged at 1000g at 4 °C for 5 min on a Beckman TJ - 6 bench top centrifuge.

The supernatant was discarded and the cell pellet was resuspended in 30 ml ice - cold PBS (0.2 g / L KCl ; 0.2 g / L $KH_2 PO_4$; 8 g / L NaCl ; 2.16 g / L $Na_2HPO_4.7 H_2O$) and spun as before. This procedure was repeated twice. The resultant washed cell paste was stored at -80 °C until needed.

2. 5. Preparation of crude plasma membrane fraction.

Membranes were prepared following the method of [Koski & Klee, 1981]. Frozen cell pastes were thawed and resuspended at 4 °C in 2 ml of the homogenization buffer (10 mM Tris - HCl, pH 7.5, containing 0.1 mM EDTA). All subsequent procedures took place at 4 °C. The cells were then homogenized with 20 strokes of a ground - glass tissue grinder. Homogenates were placed in a Beckman Ti 50 rotor and centrifuged at 500 x g for 10 minutes in a Beckman L5 - 50B ultracentrifuge to remove nuclei and unbroken cells. The supernatant from this spin contained the membrane fraction. The pellet was discarded and the supernatant centrifuged at 48000 x g for 10 minutes using the same rotor and centrifuge. The supernatant was discarded, the pellet resuspended in 5 ml of the homogenization buffer and recentrifuged for 10 minutes at 48000 x g. Again the supernatant was discarded and the pellet resuspended in the homogenization buffer at a protein concentration of 1 - 2 mg / ml. This was then aliquoted into volumes of 100 ul and stored at -80 °C until used. Crude plasma membranes from rat or mouse brain were prepared as above using brain cortex from animals which had been sacrificed by means of cervical dislocation.

2. 6. Determination of protein concentrations.

Protein concentrations were determined by the method of [Lowry *et al.*, 1951] using BSA as a standard. The samples were read at 750 nm on an LKB Biochrom Ultraspec II spectrophotometer.

2. 7. Preparation of samples for SDS - polyacrylamide gel electrophoresis (SDS - PAGE).

2. 7. 1. TCA / deoxycholate precipitation of samples.

An appropriate amount of the crude membrane preparation was taken (between 25 µg and 150 µg of membranes depending on the experiment) and placed on ice in an 1.5 ml eppendorf centrifuge tube. Samples with a volume of over 100 µl were spun for 5 min at 12000 rpm on a Hettich Mikro Rapid / K centrifuge. The supernatant was carefully removed and the pellet resuspended in 20 µl of 10 mM Tris - HCl (pH 7.4), 0.1 mM EDTA. All samples were then treated as follows. 6.5 µl of 2 % (w/v) 7 - deoxycholic acid, sodium salt was added to each tube followed by 750 µl of double distilled H_2O then 250 µl 24 % TCA. The samples were briefly vortex mixed and spun in a Hettich Mikro Rapid / K centrifuge for 20 min at 12000 rpm. After centrifugation the supernatant was carefully discarded and the pellet neutralized with 20 µl 1 M Tris base. Preparation was complete upon addition of 20 µl " Laemmli sample buffer " (50 mM Tris - HCl (pH 8.0) with 30 % (w/v) urea, 5 % (w/v) SDS, 6 % (w/v) DTT and 0.01 % (w/v) bromophenol blue. The sample was then ready for loading onto the gel.

2. 7. 2. NEM treatment

If separation of G - proteins with very similar molecular weights was required on SDS - PAGE then the samples were first alkylated according to the method described in [Sternweis & Robishaw, 1984] then run out on 12.5 % SDS - PAGE gels.

An appropriate amount of the crude membrane preparation was taken (again this was between 25 and 150 µg depending on the experiment being performed) and placed on ice in a 1.5 ml eppendorf centrifuge tube. The samples were spun for 5 min

at 12000g on a Hettich Mikro Rapid / K centrifuge. The supernatant was removed and the pellet resuspended in 20 µl 10 mM Tris - HCl (pH 7.4), 0.1 mM EDTA. 10 µl of 5 % (w/v) SDS, 50 mM DTT was added and each sample was incubated at 90 ^{o}C for 5 min. The samples were briefly cooled on ice, 10 µl of 100 mM NEM added to each tube and the samples left at room temperature for 20 min. 20 µl of " Laemmli sample buffer " (**section 2. 7. 1.**) was added and the samples loaded onto the gel.

2. 8. SDS - Polyacrylamide gel electrophoresis.

2. 8. 1. SDS polyacrylamide gel electrophoresis. Lower resolving gels - 10 % (w/v) polyacrylamide gels.

Gels were performed using the method of [Laemmli, 1970] on slab gels. The gel plates were 180 mm by 160 mm with spacers of 1.5 mm or 2.5 mm when running gels in 2-dimensions. The slabs were run as a part of a Bio-Rad Protean I electrophoresis apparatus (Bio-Rad Laboratories Ltd, Watford, Herts.). Separating gels contained 10 % (w/v) acrylamide and 0.27 % (w/v) bisacrylamide with 0.375 M Tris - HCl (pH 8.8), 0.1 % (w/v) SDS, 0.033 % (v/v) glycerol, 0.0003 % (v/v) TEMED and 0.0004 % (w/v) ammonium persulphate. The final volume used was 24 ml with the 1.5 mm spacers and 36 ml with the 2.5 mm spacers.

2. 8. 2. Lower resolving gels. 12.5 % (w/v) Polyacrylamide gels.

When higher resolution of proteins within a narrow molecular weight range was needed 12. 5 % (w/v) SDS - PAGE gels were run instead of 10 % gels. The gels were also longer in order to maximize detection of molecular weight differences. The dimensions of the gel plates were 160 mm by 200 mm with spacers of 1.5 mm and run as part of a Bio-Rad Protean II electrophoresis system. Separating gels contained 12.5

% (w/v) acrylamide and 0.0625 % (w/v) bisacrylamide with 0.375 M Tris - HCl (pH 8.8), 0.1 % (w/v) SDS, 0.033 % (v/v) glycerol, 0.0003 % (v/v) TEMED and 0.0004 % (w/v) ammonium persulphate. The final volume used was 36 ml per slab. The upper stacking gel was exactly as described in **section 2. 8. 4.** and gels run at the same conditions as for the 10 % slabs.

2. 8. 3. Preparation of SDS - Urea PAGE gels.

SDS - Urea PAGE gels were prepared as described by [Schnefel *et al.*, 1988]. To 50 ml 8 M urea was added 2.5 g of Amberlite MB - 1 monobed resin [wet mesh 16 -50] as a deionizing step. The resultant slurry was allowed to slowly mix on a bench top stirrer for 1 hour after which time the slurry was then filtered through Whatman 3 mm chromatography paper. The deionized urea (final concentration 4 M) was then included as part of a 12. 5 % (w/v) SDS - PAGE gel . The upper stacking gel was exactly as described in **section 2. 8. 4.** and gels run under the same conditions as for the 10 % slabs.

2. 8. 4. Upper Stacking Gels.

The upper stacking gels contained 3 % (w/v) acrylamide and 0.08 % (w/v) bisacrylamide with 0.125 M Tris - HCl (pH 6.8), 0.1 % (w/v) SDS, 0.0005 % (v/v) TEMED and 0.001 % (w/v) ammonium persulphate. Samples were loaded onto the gels using a Hamilton syringe (Hamilton Co., Reno, Nevada.).

2. 8. 5. Electrophoresis Running Buffers.

The running buffer contained 25 mM Tris - HCl (pH 8.5), 0.192 M glycine and 0.1 % (w/v) SDS. Electrophoresis was towards the anode at 25 mA per slab until the bromophenol blue dye fronts were 0.5 cm from the bottom.

2. 9. Staining of SDS - PAGE gels.

2. 9. 1. Coomassie Blue stain.

Gels were soaked for 1 hour in 0.25 % (w/v) Coomassie Blue R - 250, 45 % (v/v) methanol, 10 % (v/v) acetic acid and then destained overnight in 45 % (v/v) methanol, 10 % (v/v) acetic acid.

2. 9. 2. Silver staining.

Gels were silver stained according to the method of [Morrissey, 1981]. Gels were prefixed for 30 min in 50 % (v/v) methanol, 10 % (v/v) acetic acid followed by 5 % (v/v) methanol, 7 % (v/v) acetic acid again for 30 min. Gels were then placed in 10 % (v/v) gluteraldehyde for 30 min after which time they were rinsed in distilled water and left overnight in a large volume of distilled water. Next day the gels were soaked in 5 µg / ml DTT for 30 min. This solution was poured off and, without rinsing, 0.1 % (w/v) silver nitrate was added and left for 30 min. Gels were rinsed rapidly with a small amount of distilled water then with a small amount of developer (50 µl of 37 % (v/v) formaldehyde in 100 ml 3 % (w/v) sodium carbonate) and finally left to soak in the developer. Development was stopped, when the desired level of staining was achieved, upon addition of 12 ml 2.3 M citric acid and agitating for 10 min. The gels were then transferred to distilled water for storage and photography or dried down for autoradiography (see **section 2. 10. 5.**).

2. 10. Mono ADP - ribosylation of membranes by bacterial toxins.

Mono - ADP ribosylation of G - proteins was performed essentially by the

method of [Hudson & Johnson, 1980].

2. 10. 1. Materials

Cholera toxin was purchased from Sigma Chem. Co. Ltd., U. K. and kept as a 1 mg/ml stock at 4 ^{o}C in buffer consisting 50 mM Tris - HCl (pH 7.5), 200 mM NaCl, 3 mM NaN_3, 1 mM Na_2EDTA.

Pertussis toxin was obtained from Porton Products, Ltd., U.K. and stored at - 20 ^{o}C as a 0.44 mg/ml stock in a solution consisting 50 mM phosphate buffer (pH 7.2), 500 mM Nacl with 50 % (v/v) glycerol.

2. 10. 2. Preactivation of cholera toxin.

Cholera toxin was preactivated upon addition of an equal volume of 100 mM DTT. This was gently mixed and allowed to sit at room temperature for 1 hour. 5 µl of this (containing 2.5 ug of the preactivated toxin) was added to each sample to be ADP - ribosylated.

2. 10. 3. Preactivation of pertussis toxin.

Pertussis toxin was preactivated in exactly the same way as cholera toxin. 5 µl (containing 1.1 ug of the preactivated toxin) was added to each incubation tube.

2. 10. 4. Mono - ADP ribosylation of crude membrane fractions by cholera toxin and pertussis toxin.

25 - 50 µg of membrane protein was normally used for each sample to be ADP -ribosylated. To the membranes was added a cocktail containing 20 mM thymidine, 0.1 mM GTP, 250 mM K_2PO_4 (pH 7.0), 1 mM ATP, 20 mM arginine hydrochloride. The cocktail also contained 2 µCi [^{32}P] NAD^+ per incubation tube and the total volume in each tube was 50 µl. The incubation was started upon addition of the appropriate preactivated toxin to each of the tubes. These were placed in a 37 °C water bath for 90 min. The incubation was terminated by placing the tubes on ice and subsequent TCA - deoxycholate precipitation as described in **section 2. 7. 1 .** The samples were then subjected to SDS-PAGE as described in **section 2. 8.**

2. 10. 5. Autoradiography.

Destained gels from toxin - catalysed ADP - ribosylation experiments and containing [^{32}P] labelled proteins were dried onto Whatman 3mm filter paper under suction from an electric vacuum pump attached to a Bio - rad model 583 gel drier. Autoradiography was at -80 °C for an appropriate time on Kodak X - omat S x - ray film in a Kodak X - o - matic cassette with intensifying screens. Films were developed on a Kodak X - o - mat developing machine. Bands corresponding to signals on the films were sometimes excised and Cerenkov - counted in order to quantitate the samples. Immunoblots to autoradiographed were wrapped in cling film and directly placed onto film then autoradiographed and the X - ray film developed as for the gels.

2. 11. Two - dimensional gel electrophoresis.

This was performed essentially according to the method of [O'Farrell *et al.*, 1977] as modified by [Goldsmith *et al.*, 1988a], the first dimension being the

isoelectric focussing step whilst the second dimension was conventional SDS page as described in **section 2. 8.** Both first and second dimensions were run as part of a Bio-Rad Protean I electrophoresis apparatus.

2. 11. 1. Preparation of samples for isoelectric focussing.

150 µg of the crude membrane preparation was spun at 12000 rpm for 5 min on a Hettich Mikro Rapid / K centrifuge. The supernatant was carefully removed and the pellet resuspended in 15 µl of solubilizing buffer consisting of 5 mM K_2CO_3, 20 mM DTT and 1 % (v/v) nonidet P - 40. This was incubated on ice for 60 min after which time the samples were centrifuged as before. 10 µl of each supernatant was removed and added to a tube containing 20 µl of " 2 - D sample buffer " (9.2 M urea, 5 % (v/v) BME, 8 % (v/v) nonidet P - 40, 1.6 % (v/v) ampholine pH 5 - 6.5, 0.4 % (v/v) ampholine pH 3 - 10). Samples were stored at -20 ^{o}C until needed.

2. 11. 2. Casting of isoelectric focussing tube gels.

The IEF first dimension gel were poured to a height of approximately 10 cm in glass tubes of 1 mm diameter. The gel mixture contained 9.2 M urea, 2 % (v/v) nonidet P - 40, 4 % (w/v) acrylamide, 0.24 % (w/v) bisacrylamide, 4 % (v/v) ampholine pH 5 - 6.5, 1 % ampholine pH 3 - 10, 0.05 % (v/v) TEMED and 0.01 % (w/v) ammonium persulphate. Gels were overlaid with double distilled water and allowed to set. Upon setting the water was replaced with an overlay of 30 µl of the " 2 - D sample buffer " (see **section 2. 11. 1.**) and allowed to equilibrate for 30 min.

2. 11. 3. Application of sample to the IEF tube gel.

After equilibration the " 2 - D sample buffer " was replaced with a fresh 30 µl.

The sample was then carefully overlayed onto the gel using a Hamilton syringe. This was followed by a 20 µl layer of " 2 - D sample buffer " which had been diluted 1 : 1 with water. A final overlay with 20 mM NaOH was applied.

2. 11. 4. Running conditions for IEF gels.

Gels were loaded onto a Bio-Rad protean I electrophoresis apparatus. The lower tank was filled 10 mM orthophosphoric acid. The upper chamber contained 20 mM NaOH. Electrophoresis was towards the anode at 500 V for 15 hours. After electrophoresis the gels were removed and equilibrated for 1 hour in 62.5 mM Tris - HCl (pH 6.8) containing 2.3 % (w/v) SDS, 5 % (v/v) BME, 10 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue and then stored at -20 ^{o}C until used. Running the tube gels in the second dimension was as described in **section 2. 8.**

2. 11. 5. Measurement of pH gradients.

An additional first - dimension tube gel was prepared in parallel with those on which the samples were loaded and run at the same time as the experimental gels. After electrophoresis the gel was cut into 1 cm pieces and each placed in a small vial. To each piece was added 1 ml of boiling distilled water and the vials were stoppered tightly. After two hours the vials were vortex - mixed and the pH of the resulting solution was measured with a thin pH electrode.

2. 12. Immunological methods.

2. 12. 1. Electroblotting of proteins onto nitrocellulose.

Transfer of proteins from 1 - dimension or 2 - dimension gels onto nitrocellulose and susequent incubation with antisera was essentially by the method of [Towbin *et al.*, 1979]. Proteins which had been separated on 1 - or 2 - dimension gels were transferred onto nitrocellulose using an LKB 2005 Transphor electroblotting unit. The cassette to hold both the gel and the nitrocellulose during transfer was prepared as follows. A sponge pad was soaked in blotting buffer (25 mM Tris (pH 8.3) containing 192 mM glycine and 20 % (v/v) methanol and placed in the lower part of the cassette. All subsequent loading procedures were done with the cassette totally submerged in blotting buffer to prevent formation of any air bubbles within the cassette which would prevent successful transfer of the proteins from the gel. A piece of Whatman 3mm chromatography filter paper with dimensions slightly larger than the gel was placed on the sponge. The gel was next placed in the cassette followed by the nitrocellulose sheet (Anderman & Co., Ltd., Surrey, U.K.) then a similar piece of Whatman 3mm filter paper. Another sponge completed the sandwich. The cassette was closed and inserted into the electroblotting apparatus. The proteins were then subjected to electrophoresis towards the anode at 1.5 A for 2 hours. 1 - dimension blots were then treated differently from 2 - dimension blots.

2. 12. 2. Incubation with antisera of gels only run in the first dimension.

After electrophoresis the nitrocellulose sheet with the blotted proteins was transferred into a dish and covered with 100 ml blocking buffer consisting of 3 % (w/v) gelatine in TBS (20 mM Tris - HCl (pH 7.5), 500 mM NaCl). The blot was incubated for 2 hours at 30 ^{o}C after which time the gelatine / TBS was completely washed off

using copious amounts of double distilled water and then incubated overnight with antiserum appropriately diluted into TBS containing 1 % gelatine (w/v) at 30 °C. This first antiserum was normally the specific anti - G - protein antiserum. Next day the blots were first washed thoroughly with double distilled water to remove all the unbound antiserum then with two washes each of 5 minutes with TBS containing 0.05 % (v/v) Tween 20 and finally with two washes each of 5 minutes with TBS. The blots were then incubated at 30 °C for 2 hours in the 2nd antisera mix. This consisted of TBS with 1 % (w/v) gelatine and a 1 : 200 dilution of a commercial horseradish peroxidase - conjugated donkey anti - rabbit IgG (Scottish Antibody Production Unit, Carluke, Scotland). After this last incubation the blot was washed with double distilled water, then TBS containing Tween 20 and finally with TBS exactly as before.

2. 12. 3. Incubation with antisera of gels run in 2 dimensions.

After transblotting (**section 2. 12. 1.**) the blots were transferred to a dish, covered with blocking buffer comprising of 5 % (w/v) gelatine in PBS and incubated for 2 hours at 30 °C. The gelatine was then completely washed off using double distilled water. The first antiserum was then added and the blots incubated overnight at 30 °C. This antiserum was the anti - G - protein antiserum and was appropriately diluted in PBS containing 1 % (w/v) gelatine and 0.2 % (v/v) nonidet P - 40. Next day the blots were first washed thoroughly with double distilled water to remove all the unbound antiserum and then with two washes each of 5 minutes with PBS containing 0.2 % (v/v) nonidet P - 40. The blots were then incubated at 30 °C for 2 hours in the 2nd antisera mix consisting of PBS with 1 % (w/v) gelatine, 0.2 % (v/v) nonidet P - 40 and 1 : 200 dilution of the horseradish peroxidase - conjugated donkey anti - rabbit IgG. After this last incubation the blots were washed as before with double distilled water then 2 washes each of 5 minutes with PBS / nonidet P - 40 and finally with 2 washes each of 5 minutes with PBS.

2. 12. 4. Development of Immunoblots.

Detection was identical for both 1 - and 2 - dimension blots. Blots were each placed in a dish containing 40 ml 10 mM Tris - HCl (pH 7.5). To this was added 1 ml 1 % (w/v) newly prepared O - diansidine. Development was initiated upon addition of 20 µl of stock hydrogen peroxide and was terminated by pouring off the developer and adding water. Assessment of the relative intensity of the G - protein subunits as indicated by intensity of immunoblot staining was carried out by analysing scans of a film positive of the blot on a Bio - Rad 620 video densitometer linked to an olivetti M24 personal computer.

2. 12. 5. [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay technique.

After development the blots were placed in a dish containing 50 ml PBS with 1 % (w/v) gelatine and 0.2 % (v/v) nonidet P - 40 spiked with 5 µCi [^{125}I] - labelled donkey anti - rabbit immunoglobulin and incubated for 1 hour at 30 ^{o}C. The blots were then washed thoroughly with double distilled water to remove all the unbound label and then with two washes each of 30 minutes with PBS. The blot was allowed to dry, the immunoreactive bands (and appropriate blanks) were excised and counted on an LKB 1275 minigamma counter.

2. 13. Assay of high - affinity GTPase activity.

High affinity GTPase measurements were obtained using the method of [Cassel & Selinger, 1976]. Preparation of the assay tubes was carried out at 4 ^{o}C and each incubation was done in quadruplicate. 5 µg of crude membrane was resuspended in 100 µl of an incubation cocktail with final concentrations as follows : 1 mM ATP, 1

mM ouabain, 10 mM AppNHp, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 20 mM Tris - HCl (pH 7.5), 1μM GTP, 5 mM phosphocreatine and 5 units of creatine kinase. Each assay tube also contained 50000 cpm of [γ - ^{32}P] GTP and drugs as appropriate. Incubations were for 10 min at 37 °C after which time the incubation was terminated by the addition of 900 μl of ice cold 5 % (w/v) activated charcoal in 20 mM orthophosphoric acid. Each sample was immediately vortex - mixed and centrifuged at 3000g for 20 min on a Beckman TJ - 6 bench top centrifuge. 0.5 ml of the supernatant was removed and Cerenkov counted in an LKB counter using a wide open channel setting.

2. 14. Assay of adenylyl cyclase activity.

2. 14. 1. Sample incubation.

Adenylyl cyclase activity was assayed by the method of [Houslay *et al.*, 1976]. All preparatory work was carried out at 4 °C and the assay was performed in triplicate. 20 μg of crude membrane preparation was placed in a 1.5 ml eppendorf centrifuge tube. To this was added 50 μl of an assay cocktail comprising 50 mM triethanolamine hydrochloride (pH 7.4), 2 mM EDTA, 10 mM $MgSO_4$, 20 mM theophylline, 2 mM DTT, 14.8 mg/ml phosphocreatine, 0.5 mg/ml creatine kinase, 1.25 mg/ml BSA, 0.3 mM ATP, 0.2 mM GTP. Since the final assay volume was 100 μl for each tube this cocktail was essentially a 2 x stock. Forskolin at a final concentration of 10 μM was added as well as the appropriate drug to be studied. Double distilled water was added to give a final volume of 100 μl.

The samples were then incubated at 30 °C for 10 minutes and the reaction terminated by placing the tubes in a boiling bath for 5 minutes. Precipitated protein was pelleted by centrifugation at 12000g for 5 minutes on a Hettich Mikro Rapid / K centrifuge. 50 μl of the supernatant was removed for cAMP determination. These

samples were stored at -20 °C until needed.

2. 14. 2. cAMP determination.

Determination of cAMP content in samples was based on the competition binding assay of [Brown *et al.*, 1972] as modified by [Tovey *et al.*, 1974]. All subsequent procedures were performed at 4 °C.

A series of unlabelled cAMP standards were prepared in assay buffer consisting 50 mM Tris - HCl (pH 7.4 at 4 °C), 4 mM EDTA. These ranged from 0 - 320 pmoles / ml. Stock [^{3}H] - cAMP (5, 8 - ^{3}H - adenosine 3' : 5' - cyclic monophosphate) in ethanol was diluted in assay buffer to give approximately 500000 cpm / ml. 100 µl of this was placed in a 1.5 ml eppendorf centrifuge tube containing 50 µl assay buffer. To each tube was added 50 µl of either appropriate cAMP standard or sample prepared as described in **section 2. 14. 1.** 100 µl cAMP binding protein (prepared from bovine heart by the method of [Rubin *et al.*, 1974] and a kind gift from Professor M.D. Houslay) diluted 1 : 70 in assay buffer was then added to each tube. The tubes were gently vortex mixed and incubated at 4 °C for 2 - 3 hours. 10 minutes before the end of the incubation a charcoal suspension consisting 2% (w/v) GSX-100 charcoal, 1%(w/v) BSA in ice cold assay buffer was prepared. This suspension was gently mixed at 4 °C until needed.

0.25 ml of the charcoal suspension was added to each tube at the end of the incubation period. The tubes were vortex mixed and the charcoal pelleted by centrifuging at 4 °C for 4 minutes at 12000 rpm on a Hettich Mikro Rapid / K centrifuge. 0.4 ml of the supernatant was removed, placed in vials containing 7 ml ecoscint scintillation fluid and counted in an LKB 1219 Rackbeta scintillation counter with an RIA curve fitting facility. This automatically constructed a standard curve,

calculated the cAMP content of each sample and expressed it in pmoles / ml sample. Routinely the sensitivity of the binding assay was between 0.25 and 8 pmoles / ml sample.

2. 15. Assay of opiate receptor binding in crude plasma membrane fractions.

Saturation binding studies were performed by the filtration method of [Pert & Snyder, 1973] as modified by [McKenzie & Milligan, 1990].

150 µg of membrane fraction was resuspended at 4 ^{o}C in a cocktail consisting 10 mM Tris - HCl (pH 7.5), 50 mM sucrose, 20 mM $MgCl_2$ and an appropriate amount of [^{3}H] - DADLE to a final volume of 250 µl. Studies were carried out with the concentrations of the radioligand of 0 - 20 nM. Non-specific binding of the ligand to the opiate receptor was assessed using parallel tubes containing an excess (10 µM) non-labelled DADLE or DALAMID. Appropriate blank values were determined using buffer instead of membrane fraction. All assay points were performed in quadruplicate. Incubations were carried out at 25 ^{o}C for 30 minutes after which time the tubes were transferred onto ice and quickly filtered through Whatman GF / C glassfibre filters. Each filter was washed 3 times with ice-cold buffer. The filters were soaked overnight in 10 ml ecoscint scintillation fluid prior to liquid scintillation counting on an LKB 1219 rackbeta counter.

2. 16. Treatment of NG108-15 membranes with alkaline phosphatase.

Alkaline phosphatase treatment of membranes were performed essentially by the method described by [Stadel *et al.*, 1988].

800 units / ml of this enzyme (Sigma) was added to membranes (150 µg) of dibutyryl cAMP differentiated NG108-15 cells in 10 mM Tris / HCl (pH 9.8), 0.1 mM EDTA, 10mM $MgCl_2$ to a final volume of 10 µl and incubated for 30 minutes at 37 °C. The incubation was stopped upon addition of an equal volume of a solubilizing buffer consisting 10 mM K_2CO_3, 40 mM DTT, 2 % (v/v) Nonidet P - 40 (2 x normal solubilizing buffer) and left on ice for 1 hour. The samples were then subjected to 2 - D immunoblot analysis as described in **section 2. 11. 1.**

Table 2. 1.

The peptide sequences used to generate a series of antipeptide antisera directed against the various subunits of the G - proteins.

Antiserum	Peptide Used	G-protein sequence	Antiserum Identifies
)C1	ANNLRGCGLY	$G_o\alpha$ 345-354	$G_o\alpha$
)N1	GCTLSAEERAALERSK	$G_o\alpha$ 1-16	$G_o\alpha$
M1	NLKEDGISAAKDVK	$G_o\alpha$ 22-35	$G_o\alpha$
3B	KNNLKECGLY	$G_i3\alpha$ 345-354	$G_i3\alpha$
.S7	KENLKDCGLF	Td1α 341-350	Td1α, Td2α $G_i1\alpha$, $G_i2\alpha$
N1	MSELDQLRQE	β 1-10	β1
E2	LERIAQSDYI	$G_i2\alpha$ 160-169	$G_i2\alpha$

All the above antisera were produced acccording to the method of Goldsmith *et al.* 987 as described in **section 2. 3.** Amino acids are represented using the one letter ode.

Chapter 3.

CHARACTERIZATION OF ANTIPEPTIDE ANTISERA DIRECTED AGAINST A FAMILY OF G - PROTEINS.

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CHARACTERIZATION OF ANTIPEPTIDE ANTISERA DIRECTED AGAINST A FAMILY OF G - PROTEINS.

3. 1. Introduction.

The extreme homology of the primary amino acid sequences of the α subunits of the G - proteins has required the need for more selective probes to be employed in their definitive identification and for subsequent investigation into their function. The ability of bacterial toxins to catalyze the ADP - ribosylation of the G - protein α subunits was, for some time, the only method for the specific identification of the α subunits in membranes. This method has a number of limitations, not the least of which is the relative lack of specificity of pertussis toxin for its G - protein substrates.

Currently the most convenient approach for the identification of the G - proteins involves the use of specific antisera raised against either isolated proteins or more recently against peptide sequences unique to that particular G - protein as deduced from conventional protein sequencing or analysis of cDNA clones. Mumby *et al.* were the first to produce such a series of antipeptide antisera and were able to demonstrate that these antisera displayed greater specificity for particular G - proteins on immunoblots than did antisera raised against purified G - protein α - subunits [Mumby *et al.*, 1986].

Such information has made it possible to generate a series of specific antipeptide antisera directed against regions of known importance in a number of G - proteins such that not only can these antisera be used to identify a particular G - protein, they can also be used to probe their function.

This study involved use of such a series of specific antisera to investigate the expression of particular G - proteins during morphological differentiation of NG108 - 15 cells and functional changes resulting as a consequence. A number of the antisera used were kind gifts from other laboratories but several were produced ' in house '. As such it is important that the antisera be fully characterized. The results presented in **chapter 3** detail the generation of these antisera and determination of their specificity.

3. 2. Results.

3. 2. 1. Characterization of donated antisera.

The antisera used in the experiments to be described in this study can be conveniently placed into two main groups for the purposes of their characterization.The first group comprised those antisera generated in other laboratories and which were kind gifts. As a consequence these antisera have been fully characterized elsewhere in a series of published articles [see below]. The antisera in this group consist of HPA, AS7 and LE2.

The second group contained those antisera which were produced in our laboratory specifically for this and other studies by our research group. These antisera were raised in New Zealand White rabbits against a conjugate of the appropriate peptide coupled to keyhole limpet haemocyanin with glutaraldehyde using the method of Goldsmith *et al.*, 1987 as described in **section 2. 3.** Included in this group are antisera IM1, OC1, ON1, SG1 and I3B.

A full list of the antisera used, the peptide sequences which they were raised to and the G - proteins that they recognize is given in **Table 2. 1.**

3. 2. 1. (a). Antiserum AS7.

Antiserum AS7, although raised against the C - terminal decapeptide of Td1α, has been shown to recognize $G_i1\alpha$ and $G_i2\alpha$ [Goldsmith *et al.*, 1987]. This is because these two G - proteins share an identical C - terminal sequence and this sequence differs by only one conservative change from that of the C - terminus of Td1 [Milligan, 1988]. Antiserum AS7 has been fully characterized and used previously in a number of studies. [Goldsmith *et al.*, 1987; Gawler *et al.*, 1987 ; Falloon *et al.*,

1986; Milligan *et al.*, 1987b].

Our laboratory has also produced an antiserum against this peptide sequence. This antiserum named, SG1, has been shown to recognize $G_i1\alpha$ and $G_i2\alpha$ in both rat adipocytes and rat cerebral cortex [Mitchell *et al.*, 1989] and is, in general, equivalent to antiserum AS7. Recent work in our laboratory has used this antiserum instead of AS7, however all of the work on G_i 2α in NG108 - 15 cells described in this study used antiserum AS7.

3. 2. 1. (b) Antiserum LE2.

Antiserum LE2 was produced when it became clear from cDNA studies that there was more than one form of " G_i " [Goldsmith *et al.*, 1987]. This second cDNA, corresponding to $G_i2\alpha$, was cloned from a variety of cDNA libraries including those produced from rat C6 BU1 glioma [Itoh *et al.*, 1986], mouse macrophage [Sullivan *et al.*, 1986] and human monocyte [Didsbury *et al.*, 1987] cells. The sequence encoded a 355 amino acid protein which at the time of publication had not been identified. This antiserum was raised against an internal sequence (residues 160 -169) in the predicted primary structure of $G_i2\alpha$. This sequence differed in three residues from the corresponding region in $G_i1\alpha$ and the resultant antiserum reacted stongly with a 40 kDa protein highly abundant in membranes of human neutrophils andpresent in lower levels in brain [Goldsmith *et al.*, 1987]. This antiserum is specific in its identification of $G_i2\alpha$ and was later used to identify this protein as the major pertussis toxin substrate in C6 BU1 rat glioma cells [Milligan *et al.*, 1988].

3. 2. 2. Characterization of antiserum I3B.

Antiserum I3B was produced in our laboratory against a peptide sequence

corresponding to the carboxyl terminal decapeptide of $G_i3\alpha$. This antiserum been fully characterized [Mitchell *et al.*, 1989; McKenzie & Milligan, 1990; Milligan, 1990].

3. 2. 3. Production of antisera directed against the α subunit of G_o.

The cDNA sequence for the α subunit of G_o has been obtained from a number of sources including rat olfactory neuroepithelium [Jones & Reed, 1987], bovine retina [Van Meurs *et al.*, 1987] and rat glioma C6 cells [Itoh *et al.*, 1986; Itoh *et al.*, 1988]. These studies revealed the primary sequence of $G_o\alpha$ to consist of 354 amino acid residues which have 82 % identity with $G_i1\alpha$. Using this sequence information it was possible to generate three distinct antisera raised against peptide sequences in $G_o\alpha$ of supposed importance in G - protein function The first antiserum produced was IM1 which was raised against amino acids 22 -35 of $G_o\alpha$. This antiserum was assessed by its ability to discriminate between the α subunits of " G_i " and G_o in a mixture of these polypeptides purified from bovine brain, a tissue known to be rich in these particular proteins. This was achieved by performing mixing experiments with antiserum AS7 and comparing the mobility of immunoreactive polypeptides on immunoblots. It should be noted that antiserum AS7 is able to recognize $G_i1\alpha$, the predominant form of $G_i\alpha$ in brain, and $G_i2\alpha$ equally well. However under the conditions of the experiments to be described these proteins were not separated from each other and appear as a single immunoreactive band on the blots whereas $G_o\alpha$ is quite clearly separable from the $G_i\alpha$ mixture under the conditions used.

3. 2. 3. (a). Characterization of antiserum IM1.

A mixture of $G_o\alpha$ and $G_i\alpha$ purified from bovine brain [Milligan & Klee, 1985], a kind gift from Dr. G. Milligan, was resolved by SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted with antisera AS7, IM1 or a mixture of the two as

described in **sections 2. 7. & 2. 8.** The mixture of the two antisera identified two polypeptides of 39 and 40 kDa [**Fig. 3. 1.**]. However each antiserum individually identified only a single immunoreactive species. Antiserum AS7 recognized the 40 kDa polypeptide ($G_i\alpha$) whereas IM1 recognized the 39 kDa species ($G_o\alpha$).

The above experiment was essentially repeated using membranes prepared from rat brain cortex. 25 μg of a crude membrane fraction was first alkylated using NEM to maximize the separation between $G_i\alpha$ and $G_o\alpha$ [Sternweis & Robishaw, 1984] and was again resolved by SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted with antisera AS7, IM1 or a mixture of the two [**Fig. 3. 2.**]. The mixture of the two antisera identified two polypeptides of 39 and 40 kDa respectively with antiserum AS7 identifying a single band at 40 kDa and IM1 recognizing a polypeptide at 39 kDa.

Antiserum IM1 has been further characterized [Goldsmith *et al.*, 1988a] where it was used in conjunction with a number of other antisera to identify $G_o\alpha$ in two dimensional analyses of the purified protein and of the *in vitro* translated products of cDNAs encoding $G_o\alpha$.

3. 2. 3. (b). Characterization of antiserum OC1.

Antiserum OC1 was raised against a peptide sequence comprising the last ten amino acids of the C -terminus of $G_o\alpha$. The resulting antiserum was screened using the same procedures as noted above for antiserum IM1. A sample of crude membrane fraction from rat brain cortex was first alkylated with NEM, resolved by SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted with antisera AS7, OC1 or a mixture of the two [**Fig. 3. 3.**]. The mixture of the two antisera again identified two polypeptides of 39 and 40 kDa with antiserum AS7 identifying a single band of 40 kDa and OC1 recognizing a polypeptide of 39 kDa.

Figure 3. 1.

Antisera AS7 and IM1 identify mutually exclusive polypeptides within a mixture of purified G - proteins.

A mixture of purified G_i and G_o (330 ng) was subjected to NEM treatment resolved on SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum AS7 (A), IM1 (C), or a mixture of these two antisera (B) [**sections 2. 7. 2., 2. 8. & 2. 12.**]. Both antisera were used at 1 : 200 dilution. Similar results were obtained in 3 other experiments.

Figure 3. 2.

Antisera AS7 and IM1 identify mutually exclusive polypeptides in rat brain membranes.

Membranes (25 µg) from rat brain cortex were subjected to NEM treatment resolved on SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum AS7 (A), IM1 (C), or a mixture of these two antisera (B) [**sections 2. 7. 2., 2. 8. & 2. 12.**]. Both antisera were used at 1 : 200 dilution. Similar results were obtained in 3 other experiments.

Figure 3. 1.

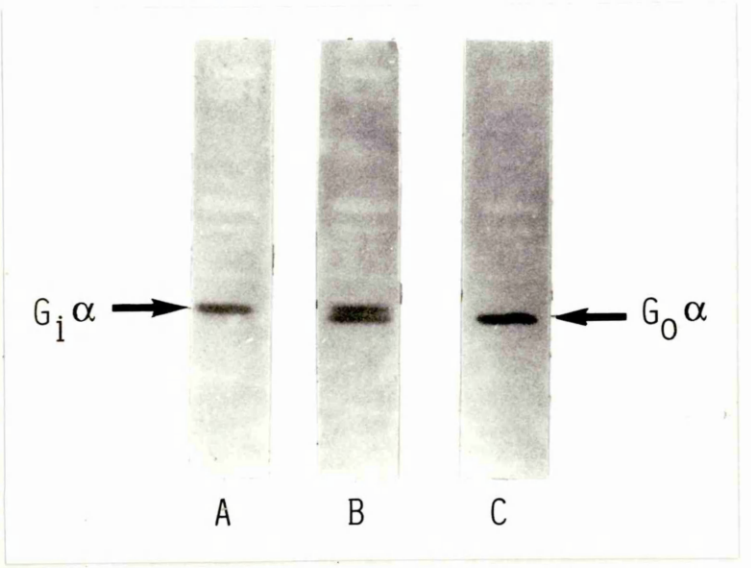


Figure 3. 2.

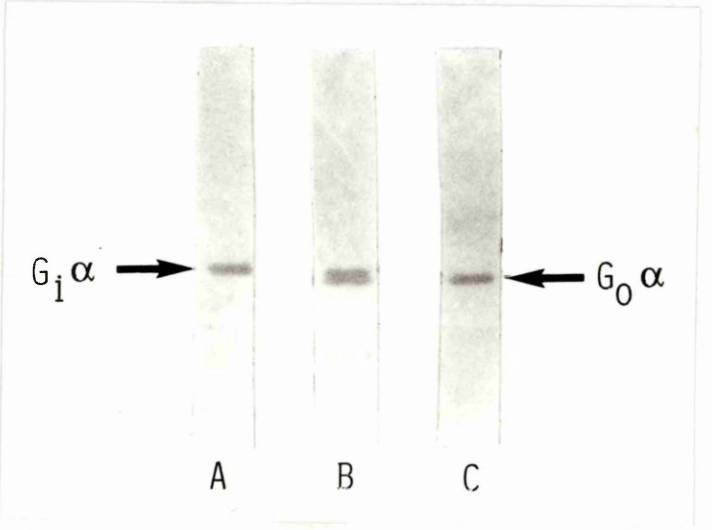
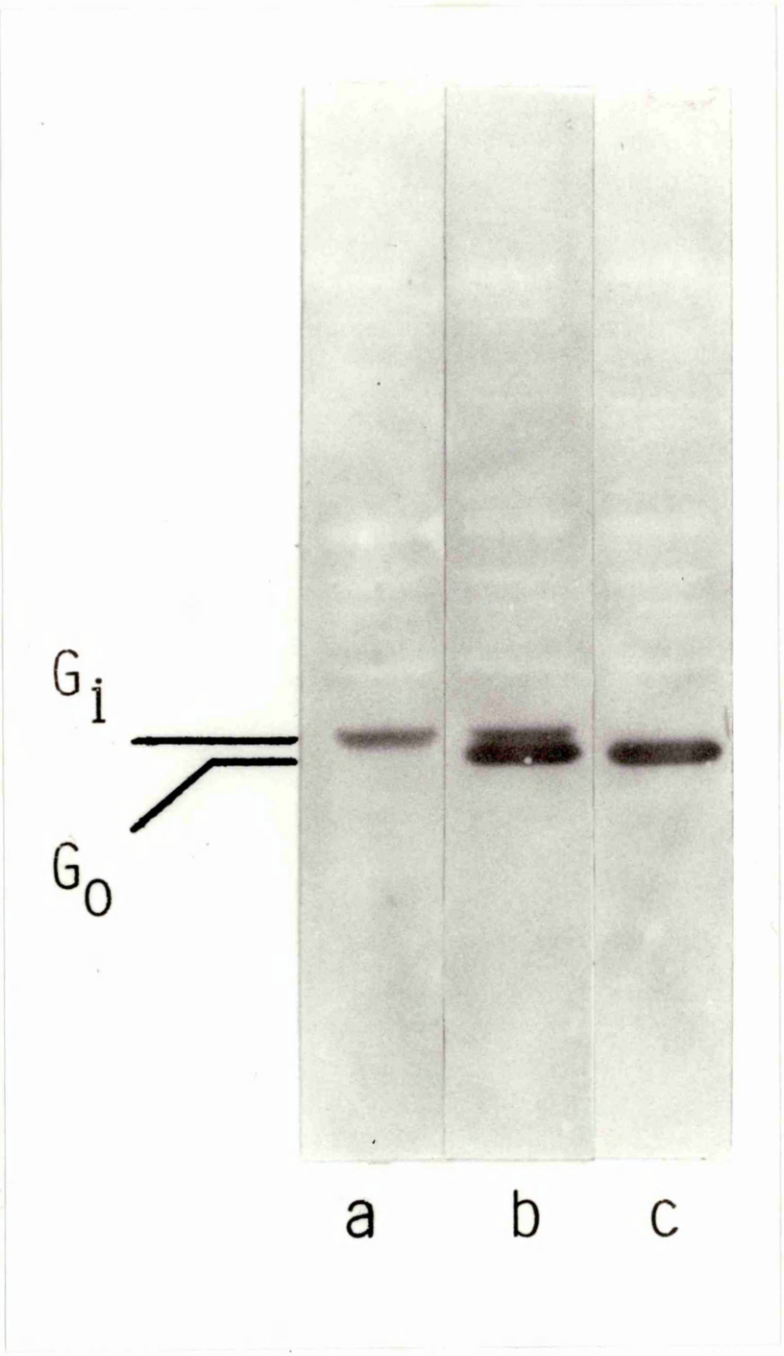


Figure 3. 3.

Antisera AS7 and OC1 identify mutually exclusive polypeptides in rat brain membranes.

Membranes (25 μg) from rat brain cortex were subjected to NEM treatment resolved on SDS PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum AS7 (**a**) (1 : 300 dilution), OC1 (**c**) (1 : 1500 dilution), or a mixture of these two antisera (**b**) [**sections 2. 7. 2., 2. 8.** & **2. 12.**]. Similar results were obtained in 3 other experiments.

Figure 3. 3.



3. 2. 3. (c). Characterization of antiserum ON1.

Antiserum ON1 was raised against a peptide sequence corresponding to the first 16 amino acids of the N - terminus of $G_o\alpha$. This antiserum was again screened with the mixing experiments described above for antisera IM1 and OC1.

Samples from a crude membrane preparation were first treated with NEM, resolved by SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted with antisera AS7, ON1 or a mixture of the two [**Fig. 3. 4.**]. The mixture of the two antisera again identified two polypeptides of 39 and 40 kDa respectively with antiserum AS7 identifying a single band at 40 kDa and ON1 recognizing a polypeptide at 39 kDa.

3. 2. 3. (d). Antisera IM1, OC1 and ON1 recognize a single 39 kDa polypeptide.

Samples from a crude rat brain membrane preparation were subjected to NEM treatment resolved on SDS - PAGE on a single gel and immunoblotted using antiserum IM1 (**1**), OC1 (**2**), or ON1 (**3**). From **Fig. 3. 5.** it can be seen that each of the antisera recognized a single immunoreactive species which had the same mobility on this gel.

These mixing experiments demonstrate that each of the presumed $G_o\alpha$ antisera recognizes a single polypeptide of 39 kDa and that this immunoreactivity is distinct from that seen with antiserum AS7 which recognizes the α subunits of G_i1 and G_i2. Immunoblotting with a purified preparation of G_i / G_o showed that antiserum IM1 reacted with $G_o\alpha$. Analysis of the immunoreactive species seen with antisera IM1, ON1 and OC1 on a single gel shows the mobility of each reactive species to be

Figure 3. 4.

Antisera AS7 and ON1 identify mutually exclusive polypeptides in rat brain membranes.

Membranes (25 μg) from rat brain cortex were subjected to NEM treatment resolved on SDS PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum AS7 (**a**) (1 : 300 dilution), ON1 (**c**) (1 : 1500 dilution), or a mixture of these two antisera (**b**) [**sections 2. 7. 2., 2. 8. & 2. 12.**]. Similar results were obtained in 3 other experiments.

Figure 3. 4.

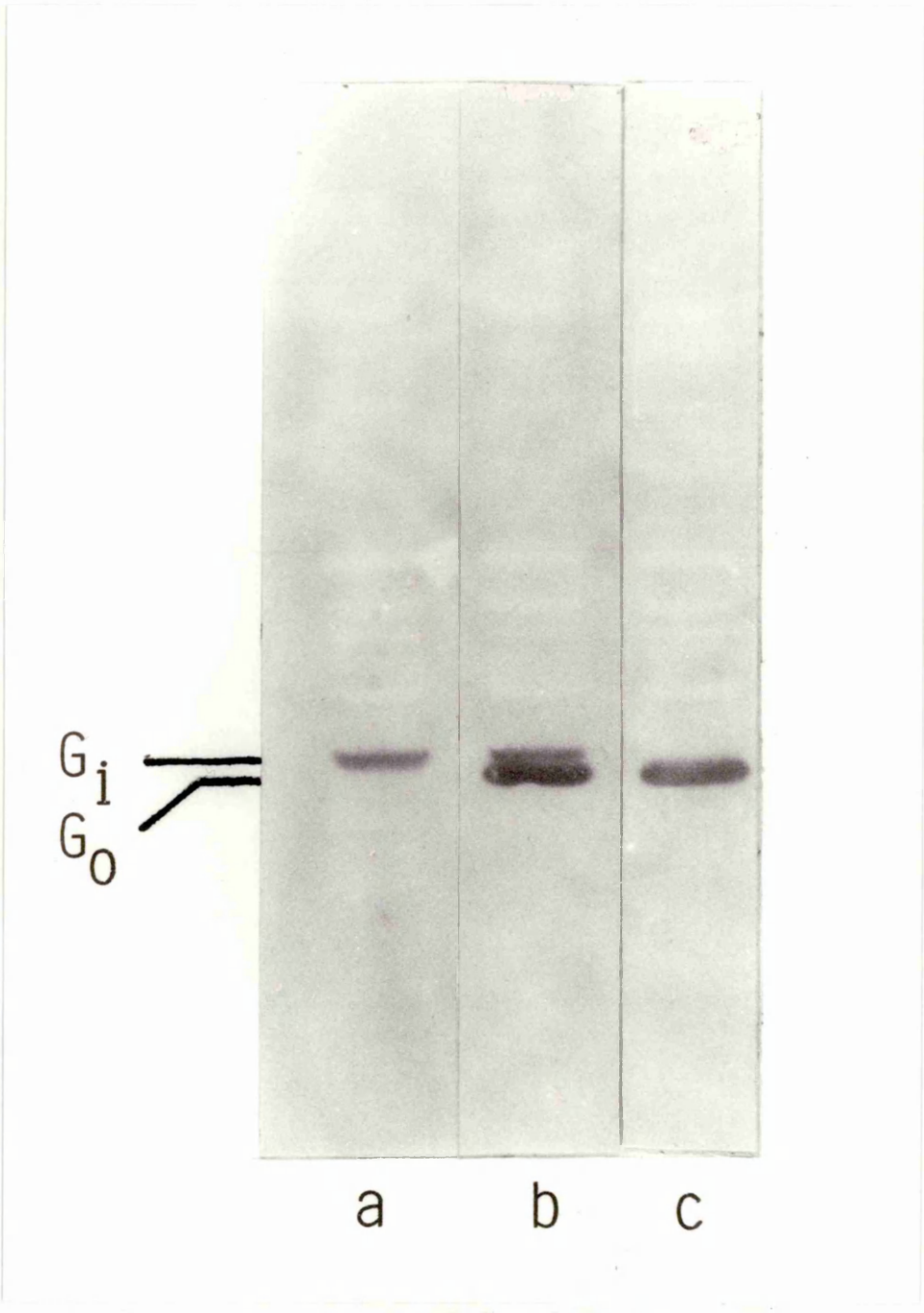
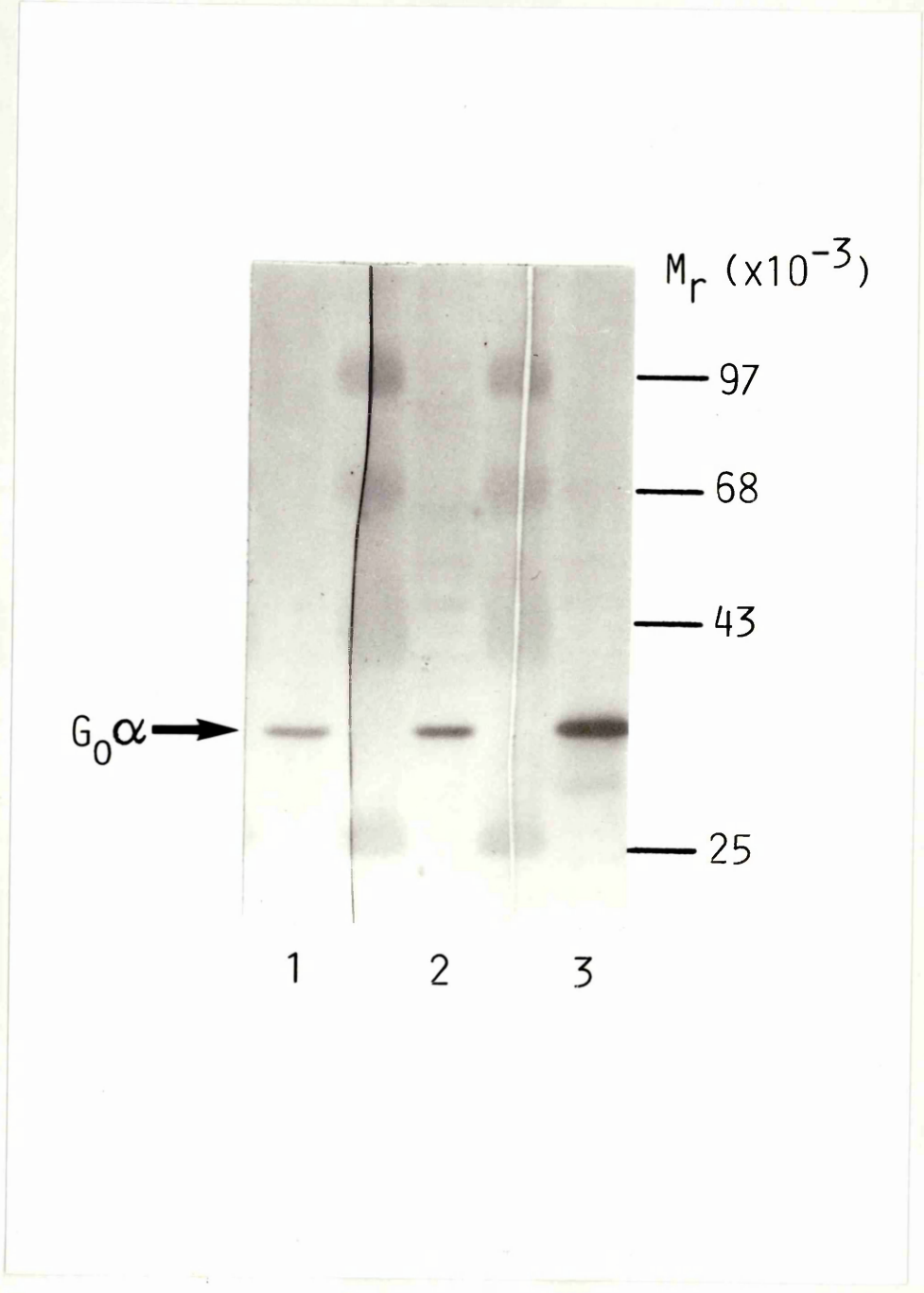


Figure 3. 5.

Antisera IM1, OC1 and ON1 recognize a single 39 kDa polypeptide.

Membranes (25 μg) from rat brain cortex were subjected to NEM treatment resolved on SDS PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum IM1 (**1**) (1 : 200 dilution), OC1 (**2**) (1 : 1500 dilution), or ON1 (**3**) (1 : 1500 dilution) [**sections 2. 7. 2., 2. 8. & 2. 12.**]. Similar results were obtained in 2 other experiments.

Figure 3. 5.



identical.

3. 2. 3. (e). Characterization of antiserum HPA.

Antiserum HPA was the only antiserum used in this study which was not raised against a peptide sequence. This antiserum was raised by immunization of a rabbit with a mixture of holomeric pertussis toxin - sensitive G - proteins which were purified from bovine brain. It contains populations of antibodies which recognize the β subunit associated with G - proteins as well as the α subunit of G_o.

Membranes from rat brain were resolved upon SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using either antisera IM1, AS7 or HPA or a combination of mixtures of these antisera [**Fig. 3. 6.**]. The mixtures of antisera HPA and AS7 [**lane a**] and IMI and AS7 [**lane c**] identified two distinct polypeptides in the region of 40 kDa. By contrast the mixture of antisera HPA and IM1 [**lane b**] identified but a single polypeptide in this region. Antiserum HPA also identified a band at 36 kDa corresponding to the β - subunit.

3. 2. 4. Generation of the β subunit antiserum, BN1.

Classical G - proteins exist as heterotrimers consisting of α,β and γ subunits. Although the β and γ subunits are not covalently linked to each other, the native proteins cannot be dissociated without denaturation and therefore form a single functional unit under all physiological conditions. As such the levels of the β subunits can be taken as indicative of the βγ complex.

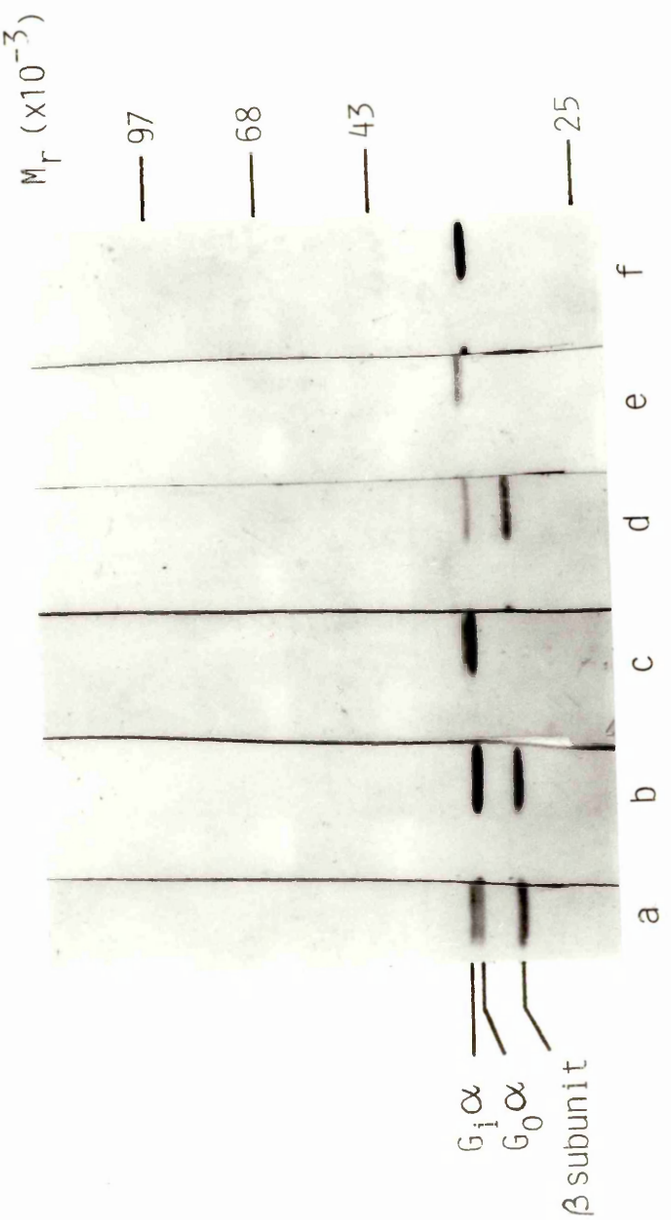
There are at least two very similar forms of the β subunit one of 36 kDa (termed β1) and one of 35 kDa (β2) in mammalian cells [Sternweis & Robishaw,

Figure 3. 6.

Antiserum HPA specifically identifies $G_o\alpha$.

Membranes (25 μg) from rat brain cortex were subjected to NEM treatment resolved on SDS PAGE (10 % (w/v) acrylamide) and immunoblotted with (a) a mixture of antiserum HPA (1 : 2000 dilution) and antiserum AS7 (1 : 200 dilution), (b) a mixture of antiserum HPA (1 : 2000 dilution) and antiserum IM1 (1 : 200 dilution), (c) a mixture of antiserum AS7 (1 : 200 dilution) and antiserum IM1 (1 : 200 dilution), (d) antiserum HPA (1 : 2000 dilution), (e) antiserum AS7 (1 : 200 dilution), (f) antiserum IM1 (1 : 200 dilution) [**sections 2. 7. 2., 2. 8. & 2. 12.**]. Similar results were obtained in 3 other experiments.

Figure 3. 6.



1984; Winslow *et al.*, 1986]. In most tissues the β1 form is the predominant form, while the β2 is a minor component [Woolkalis & Manning, 1987]. The amino acid sequence of both forms has been deduced from cDNA analysis and have been shown to be some 90 % identical [Fong *et al.*, 1987; Gao *et al.*, 1987]. Using these sequences it was decided to produce an antipeptide antisera raised against residues 1 - 10 from the N - terminus of the β1. The resulting antiserum BN1 was compared against antiserum HPA which has been shown to contain a population of antibodies directed against the β subunit.

Samples from a crude rat brain membrane preparation were subjected to NEM treatment, resolved on SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum HPA (A) or BN1 (B) [**Fig. 3. 7.**]. Both antisera recognized a single band of 36 kDa corresponding to the β subunit and HPA also recognized $G_o\alpha$.

3. 2. 5. Cross - reactivity of G - protein antisera.

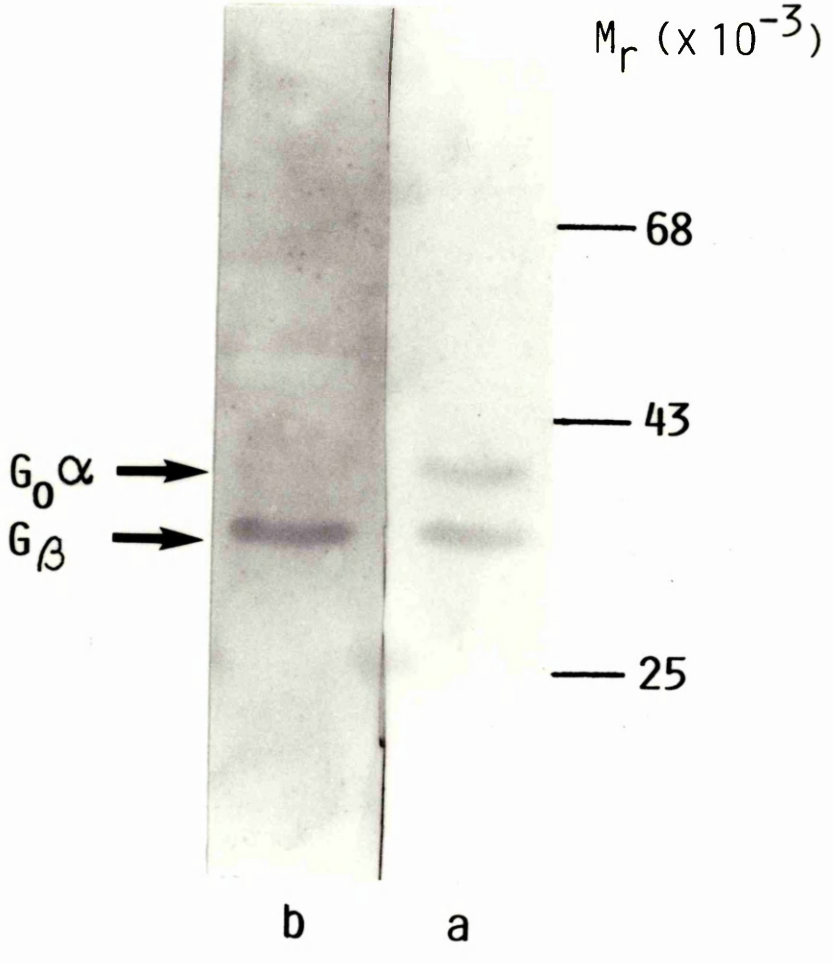
Possible cross - reactivity of the G - protein antisera must be considered carefully. As more G - proteins are identified the possibility of these proteins having sufficient identity with those already defined cannot be discounted. With two exceptions, the antisera used in this study do not, to the best of current knowledge, cross - react with any G - protein other than the polypeptide for which it was designed to identify. Antiserum I3B has been shown to identify a single polypeptide of some 41 kDa in membranes of rat adipose tissue and rat glioma C6 BU1 cells. Furthermore when northern blots of total RNA from adipose tissue were probed with a synthetic deoxyoligonucleotide complementary to $G_i3\alpha$, the probe hybridized to a single single RNA of 3.4 kilobases [Mitchell *et al.*, 1989] thus confirming the presence in this tissue of $G_i3\alpha$. However when membranes from rat cerebral cortex were

Figure 3. 7.

Antisera HPA and BN1 recognize the same 36 kDa polypeptide.

Membranes (25 μg) from rat brain cortex were subjected to NEM treatment, resolved on SDS PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum HPA (**a**) (1 : 2000 dilution) or BN1 (**b**) (1 : 200 dilution) [**sections 2. 7. 2., 2. 8. & 2. 12.**]. Similar results were obtained in 2 other experiments.

Figure 3. 7.



immunoblotted with antiserum I3B, two immunoreactive species were noted [Milligan, 1990]. The cross - reactivity of this antiserum was further examined.

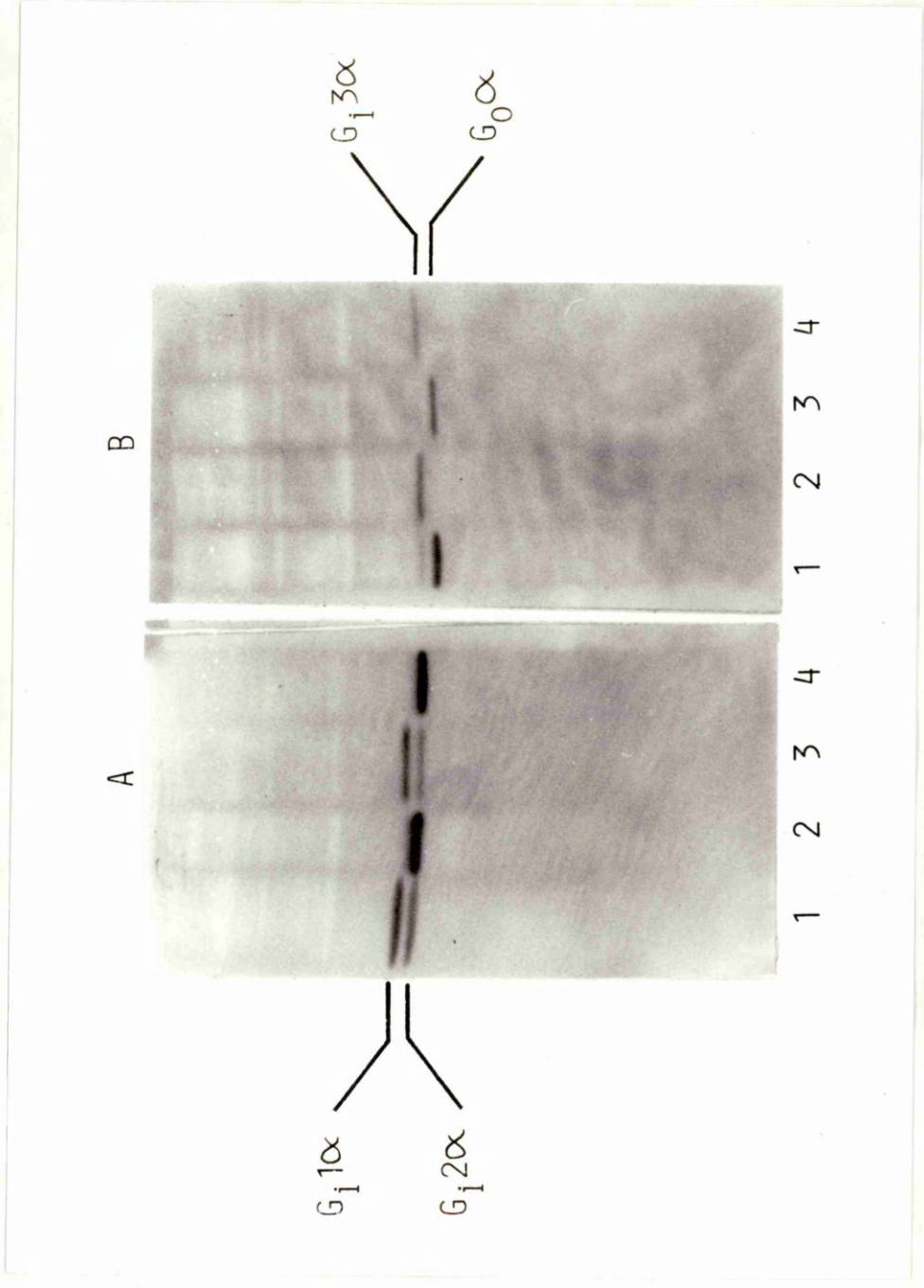
Fig. 3. 8. shows membranes of rat glioma C6 BU1 cells (**lanes 2, 4**) which express high levels of $G_i2\alpha$ and substantial quantities of $G_i3\alpha$ but not $G_o\alpha$ and membranes of rat cerebral cortex (**lanes 1, 3**) which have high levels of $G_i1\alpha$ and $G_o\alpha$ and lower levels of $G_i2\alpha$ and $G_i3\alpha$ which were resolved on SDS - PAGE and immunoblotted using either antiserum SG1 (**panel A**) or antiserum I3B (**panel B**). Antiserum I3B identified a 41 kDa polypeptide in C6 cells ($G_i3\alpha$) but in cerebral cortex, while low levels of $G_i3\alpha$ were noted (**panel B, lane 1**), the antiserum also identified a 39 kDa protein ($G_o\alpha$) which was not detected in C6 BU1 membranes showing that I3B cross - reacts with both $G_i3\alpha$ and $G_o\alpha$ but not with $G_i1\alpha$ or $G_i2\alpha$. The converse is also true, antiserum OC1 which was raised against the corresponding C - terminal sequence of $G_o\alpha$ displayed a slight cross - reactivity with $G_i3\alpha$ under conditions which are able to resolve $G_i3\alpha$ from $G_o\alpha$. Since all of the experiments to be described in this study rely on corroboration with other $G_o\alpha$ antisera which do not recognize $G_i3\alpha$, this cross - reactivity, although noted, can be disregarded.

Figure 3. 8.

Antiserum I3B cross - reacts with G_i3 and G_o but not with G_i1 or G_i2.

Membranes of rat glioma C6 BU1 cells (lane 2 : 100 µg, lane 4 : 50 µg) and of rat cerebral cortex (lane 1 : 100 µg, lane 3 : 50 µg) were subjected to NEM treatment, resolved on SDS PAGE (12.5 % (w/v) acrylamide) and immunoblotted using either antiserum SG1 (panel A) or antiserum I3B (panel B).

Figure 3. 8.



3. 3. Discussion.

Recognition by specific antisera remains the best approach in the identification of individual G - proteins. Originally these antisera were produced using purified preparations of holomeric G - proteins. However these attempts were not always successful and relied upon the protein purification protocols available, purification to homogeneity of the individual G - proteins being technically very demanding. Indeed the use of a mixture of pertussis toxin - sensitive G - proteins isolated from brain resulted in the generation of antisera which recognized the β subunit and $G_o\alpha$ but showed no cross - reactivity against $G_i\alpha$ [Gierschik *et al.*, 1986b; Huff *et al.*, 1985], although recently the generation of a polyclonal anti - G_i antiserum using purified brain G_i as an antigen has been reported [Katada *et al.*, 1987].

An alternative approach has been to utilise the tools afforded by recent advances in molecular biology. Information from cDNAs corresponding to the various G - proteins has led to the production of a series of antipeptide antisera directed against short synthetic peptides which can be predicted to be present in particular G - proteins.

The results detailed in this chapter describe the production of several antipeptide antisera directed against a number of G - proteins using distinct peptide sequences determined from analysis of cDNA studies from a number of sources [Jones & Reed, 1987; Lochrie & Simon, 1988]. The resultant antisera were characterized by their ability to recognize purified preparations of G - protein α subunits, by comparison of mobility of various G - proteins on high - resolving SDS - PAGE in membranes with defined G - protein status and by agreement of recognition with other well characterized antisera. Those antisera raised against sequences found in the α subunit of G_o, namely IM1, OC1 and ON1, were shown to immunoreact on western blots with a single polypeptide of 39 kDa which could be identified as being $G_o\alpha$.

It was also possible to generate such an antipeptide antiserum directed aganst the β subunit. The resulting antiserum, BN1, was raised against a peptide sequence of 10 amino acids found at the amino terminal end of the β1 form of this protein. This sequence contains only one conservative difference between β1 and β2, β1 having an aspartate residue in position 5 as opposed to a glutamate in the same position of β2. Immunochemical differences between the β subunits have been noted. Some transducin β antisera have been shown to cross react preferentially with β1 [Roof *et al.*, 1985; Evans *et al.*, 1987] and an antiserum raised against a synthetic peptide representing residues 130 - 145 of β1 was shown to identify β1 but not β2 [Mumby *et al.*, 1986].

Although the antisera displayed very little cross - reactivity between them, antiserum I3B, which identified $G_i3\alpha$, was shown to cross - react to some degree with $G_o\alpha$. $G_i3\alpha$ has a predicted protein sequence from cDNA analysis which shows that it is more closely related to $G_i1\alpha$ (94 % identity) than is $G_i2\alpha$. However although antiserum AS7 recognizes $G_i1\alpha$ and $G_i2\alpha$ equally well, it does not react with $G_i3\alpha$ [McKenzie & Milligan, 1990] even though its' C - terminal ten amino acids are highly similar to those of $G_i1\alpha$ and $G_i2\alpha$ having only two conservative changes. Furthermore antiserum I3B does not react with $G_i1\alpha$ or $G_i2\alpha$ but does cross - react with $G_o\alpha$which is probably a reflection of the antigenic importance of the aromatic C - terminal tyrosine residue which $G_i3\alpha$ and $G_o\alpha$, but not $G_i1\alpha$ and $G_i2\alpha$, have as a common feature [Milligan, 1990]. Antiserum OC1 which was raised against the carboxyl decapeptide of $G_o\alpha$ and contains this same immunogenic tyrosine residue displayed a slight cross - reactivity with $G_i3\alpha$. This cross - reactivity and its implications are further discussed in **chapter 6.**

Chapter 4.

ELEVATED LEVELS OF THE GUANINE NUCLEOTIDE - BINDING PROTEIN $G_{o}\alpha$ ARE ASSOCIATED WITH cAMP - INDUCED DIFFERENTIATION OF NG108 - 15 CELLS.

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4. 1. Introduction.

Modulation in the levels of G - proteins can be achieved under a variety of conditions. A number of clinical disorders appear to display changes in the amounts of G - proteins and this can be manifest in altered signal transduction. Levine and coworkers have been able to show that in type Ia - pseudohypoparathyroidism there is a reduced expression of $G_s\alpha$ as indicated by the reduced concentrations of mRNA encoding for this protein in affected fibroblasts [Levine *et al.*, 1988] and in plasma membranes of hepatocytes isolated from streptozotocin - induced diabetic rats markedly lower levels of $G_i\alpha$ were recorded in comparison to control animals [Gawler *et al.*, 1987].

A number of immunological and biochemical investigations have clearly indicated that the distribution of $G_o\alpha$ is essentially restricted to nervous and endocrine tissue [Homburger *et al.*, 1987; Asano *et al.*, 1988a]. By employing a highly sensitive immunoassay, Murayama and colleagues were able to demonstrate that levels of $G_o\alpha$ were enhanced in the cerebrospinal fluid of patients with a variety of neurological disorders. These included meningitis which showed a 48 % increase in $G_o\alpha$ and encephalitis which displayed a 100 % enhancement [Murayama *et al.*, 1989].

Alterations in the amounts of various G - proteins in developing tissues has also been observed. An increase in $G_o\alpha$ occurs during the onset of functional

parasympathetic innervation of the chick heart. This increase, of up to 50 %, was noted, first in whole heart and then in membranes prepared from atrial and ventricular tissue, between days 10 to 20 of embryonic development [Luetje *et al.*, 1987; Liang *et al.*, 1986].

Differentiation of 3T3 - L1 fibroblasts into adipocytes by treatment with IBMX and dexamethasone is accompanied by increases of up to 48 % in the levels of $G_o\alpha$ in membranes prepared from these cells [Gierschik *et al.*, 1986a; Watkins *et al.*, 1987] which led to the suggestion that G_o may be involved in the control of cell proliferation.

The neuroblastoma x glioma hybrid cell line, NG108 - 15, a commonly used system in neurobiology, was generated by cell fusion of the 6 - thioguanine - resistant clonal mouse neuroblastoma cell, N18TG2 and the bromodeoxyuridine - resistant rat glioma cell, C6 BU1, selection and cloning. These cells have been shown to express a wide range of receptors for various neurotransmitters and hormones, which can be demonstrated to couple to a number of distinct second messenger effector systems and ion channels [for review see Hamprecht *et al.*, 1985]. Amongst the transmembrane signalling systems studied in most detail are the stimulation and inhibition of adenylyl cyclase [Klee *et al.*, 1985] and the regulation of voltage - sensitive calcium channels [Tsunoo *et al.*, 1986].

NG108 - 15 cells are able to undergo morphological differentiation upon the addition of agents which function to elevate intracellular concentrations of cAMP. Under such conditions the cells withdraw from the cell cycle, take on a more rounded appearence and produce a number of neurite outgrowths. Differentiated NG108 - 15 cells are commonly used for electrophysiological experimentation because they are more amenable to to penetration by microelectrodes and are more electrically excitable than the undifferentiated cells. Differentiation also results in a number of biochemical

changes in these cells. Vallano and Beaman - Hall noted an increase in the levels of a type II calcium / calmodulin - dependent protein kinase, implicated in a number of cellular events, in NG108 - 15 cells differentiated with db cAMP [Vallano & Beaman - Hall, 1989] and Carrithers and collaborators observed the up - regulation of an angiotensin III selective receptor that mediated phosphatidylinositol breakdown in NG108 - 15 cells that had been differentiated with DMSO [Carrithers *et al.*, 1990]

NG108 - 15 cells have been shown to contain a variety of G - proteins including G_s and a number of the pertussis toxin substrates including G_i2, G_i3 and G_o [Milligan *et al.*, 1989; Milligan *et al.*, 1986; McKenzie *et al.*, 1988; McKenzie & Milligan, 1990].

The work described in **chapter 4** outlines an investigation into alterations in the levels of $G_o\alpha$ which occur with cAMP - induced differentiation of NG108 - 15 cells

4. 2. Results.

4. 2. 1. Pharmacological treatment of NG108 - 15 cells.

The neuroblastoma x glioma hybrid cell line, NG108 - 15, is able to undergo morphological differentiation when subjected to prolonged exposure to a variety of pharmacological agents which, as a common mode of action, elevate the levels of intracellular cAMP. **Fig. 4. 1. (a)** shows some subconfluent, non - differentiated NG108 - 15 cells which were photographed 3 days after splitting from a confluent culture. These cells, which have been grown in a reduced foetal bovine serum concentration of 2 % (v/v) as a parallel control to the differentiated cells, will be termed control cells in the series of experiments to be described. **Fig. 4. 1. (b)** shows cells which have been incubated for 6 days with 1 mM db cAMP, a membrane permeable cAMP analogue and one of the most commonly used differentiating agents.These cells can be seen to have undergone pronounced morphological changes and will be termed the differentiated cells in the work to be described. The cells have stopped dividing, the cell bodies have grown and there has been the production of a network of neurite - like outgrowths. Failure to reduce the concentration of foetal bovine serum, in this case from 10 % (v/v) to 2 % results in the loss in the ability of db cAMP to cause differentiation of the cells. The reasons for this are unknown at present. No observable changes in the levels of any of the G - proteins studied were seen on simply reducing the concentration of serum without the addition of the cAMP analogue

The morphological changes seen upon differentiation were not exclusive to db cAMP. Cells grown in 8 - bromo cAMP, another cAMP analogue; forskolin, which directly activates adenylyl cyclase or prostaglandin E_1, which acts via a cell surface receptor and G_s to stimulate adenylyl cyclase all produced these morphological changes [see **Table 4. 1.** for a list of agents and the concentrations used to differentiate the cells].

Figure 4. 1. (a).

Morphological appearance of control NG108 - 15 cells in tissue culture.

Control NG108 - 15 neuroblastoma x glioma hybrid cells were grown as described in **section 2. 4. 1.** The cells had been maintained in tissue culture for 3 days when these photographs were taken. The scale bar is equivalent to 10 μm.

Figure 4. 1. (a).

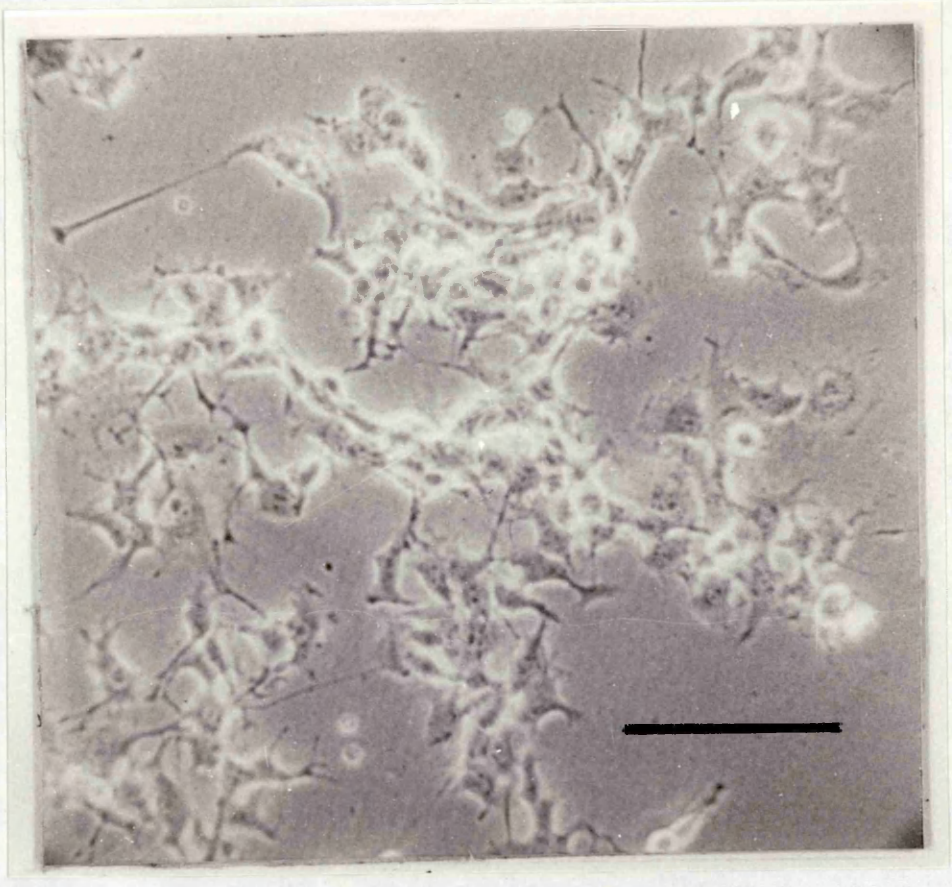


Figure 4. 1.(b).

Morphological appearance of db cAMP - differentiated NG108 - 15 cells in tissue culture.

Db cAMP - differentiated NG108 - 15 neuroblastoma x glioma hybrid cells were grown as described in **section 2. 4. 6.** The differentiated cells had been maintained in the presence of db cAMP (1mM) for 6 days when these photographs were taken. The scale bar is equivalent to 10 μm.

Figure 4. 1.(b).

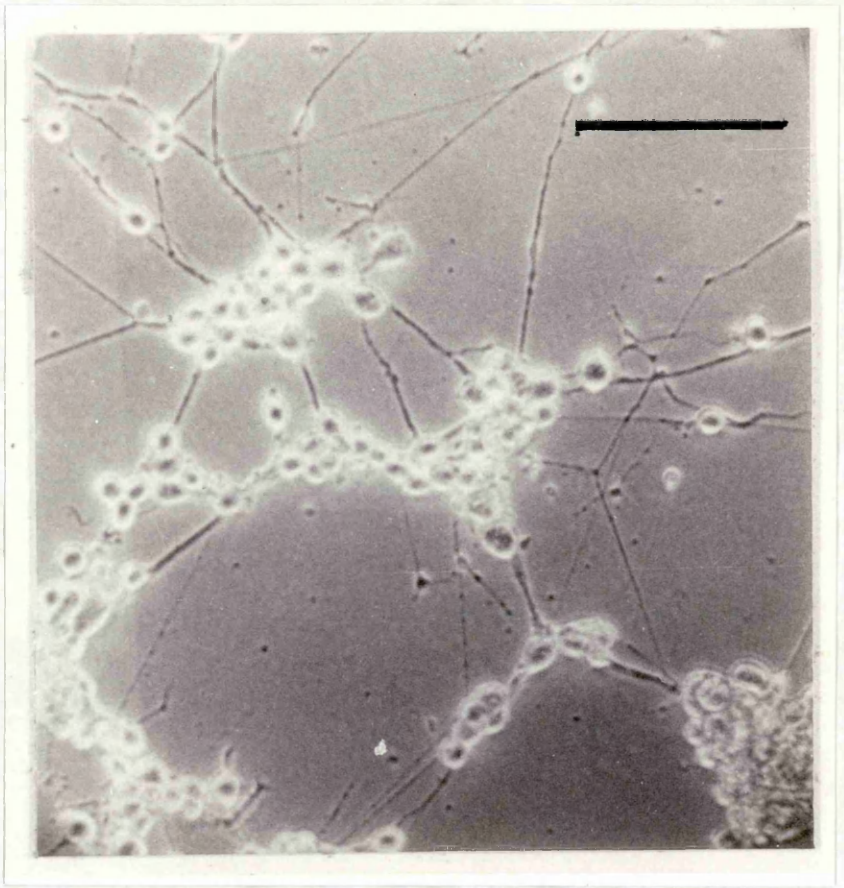


Table 4. 1.

List of agents and the concentrations used to differentiate NG108 - 15 cells.

Treatment	Final concentration
Db cAMP	1 mM
Forskolin	10 μM
Prostaglandin E1	10 μM
8-bromo cAMP	1 mM

NG108 - 15 cells were maintained for 6 days in the absence or presence of agents which elevate intracellular cAMP levels, as detailed in **sections 2. 4. 1.** and **2. 4. 6.**

All the variously treated cells were harvested and crude plasma membrane fractions were prepared as described in **section 2. 5.** When subjected to resolution by SDS - PAGE and subsequent staining with Coomassie Blue, the membranes from the control cells and from cells from each of the treatments appeared to be extremely similar [**Fig. 4. 2.**] suggesting there to be no gross changes in the major protein constituents of the plasma membrane upon differentiation.

4. 2. 2. Mono ADP - ribosylation of NG108 - 15 cell membranes using pertussis toxin.

Bordetella pertussis, the causitive agent of whooping cough is able to produce a number of exotoxins one of which, islet activating protein or more commonly pertussis toxin, has been shown to modulate the receptor - mediated control of cAMP production [Katada & Ui, 1979; Katada & Ui, 1981]. Pertussis toxin contains an ADP - ribosyltransferase activity which is now known to catalyse the transfer of an ADP - ribose moiety from an NAD^+ substrate onto the α subunit of a number of G - proteins including the various G_i's and G_o. The site of pertussis - toxin catalysed ADP - ribosylation in all of these G - proteins is a conserved cysteine residue located four amino acid residues from the C - terminus [Milligan, 1988]. The use of [^{32}P] NAD^+ as a substrate allows for the visualisation, when subjected to gel electrophoresis followed by autoradiography, of polypeptides which are specifically ADP - ribosylated by this toxin .

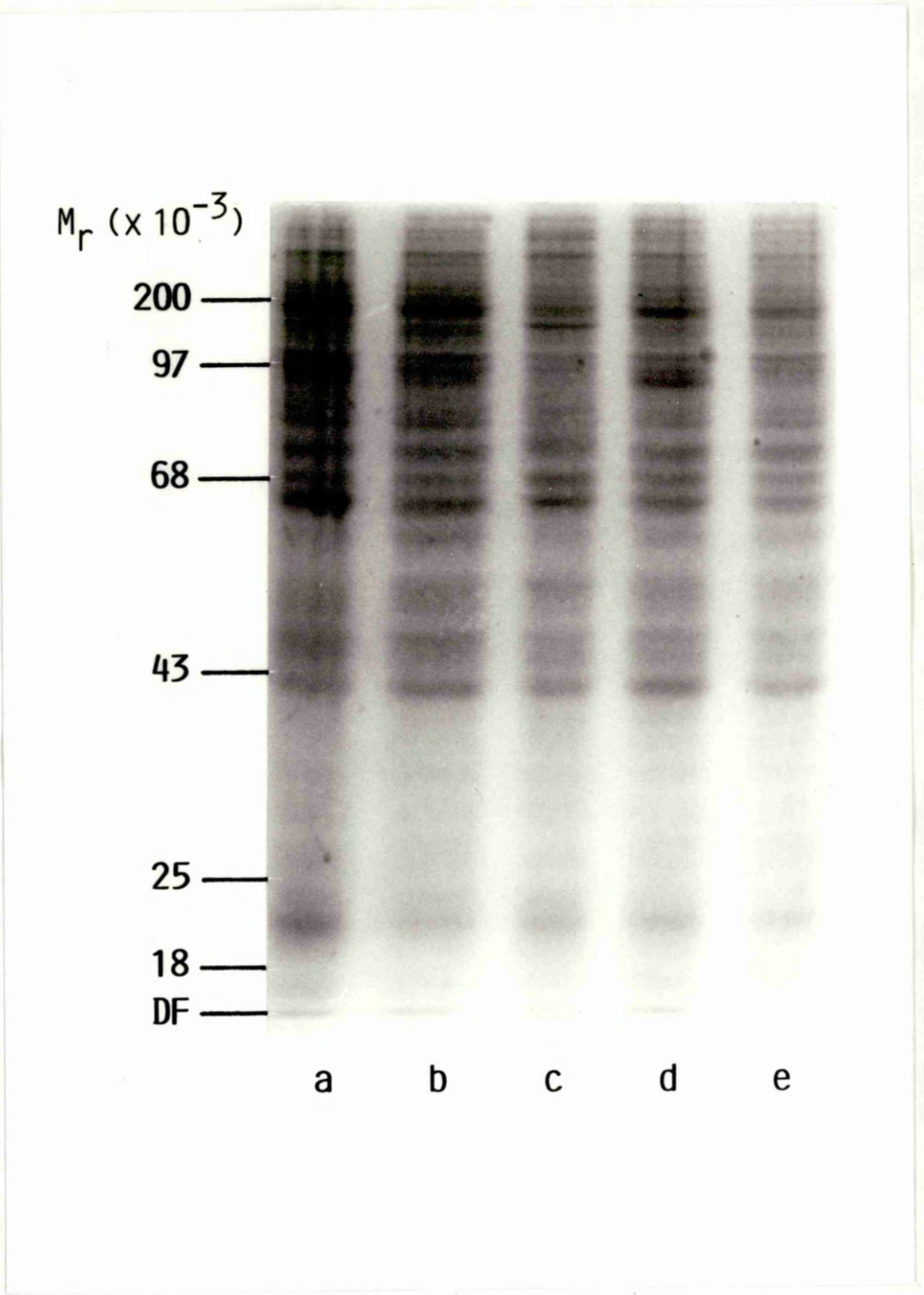
Membranes from control cells and from cells differentiated with the various agents were subjected to such an analysis using pertussis toxin and [^{32}P] NAD^+, the rationale being that any changes in the G - protein profile brought about by differentiation might manifest themselves as changes in the amount of radioactive label incorporated into the membrane. Autoradiography of the resultant gel demonstrated

Figure 4. 2.

SDS - PAGE analysis of membranes from control and cAMP - differentiated NG108 - 15 cells.

Membranes (25 μg) of control (a) and cAMP - differentiated (b - e) cells were subjected to deoxycholate / TCA precipitation followed by resolution under denaturing conditions by SDS - PAGE (10 % (w/v) acrylamide) as described in **section 2. 8. 1.** The resultant gel was stained with Coomassie Brilliant Blue [**section 2. 9. 1.**]. The position of marker proteins of known molecular weights are indicated. All cells were differentiated for 6 days with 1 mM db cAMP (lane b); 1 mM 8 - bromo cAMP (c); 10 uM forskolin (d); 10 uM PGE_1 (e).

Figure 4. 2.



markedly elevated incorporation of radioactive label over the control membranes into the 39 - 40 kDa region with each of the treatments [**Fig. 4. 3.**]. The 39 - 40 kDa region being that in which the G - proteins of interest are known to occur. This increase was quantified by excision of the relevant areas of the gel and subsequent scintillation counting. **Table 4. 2.** summarizes the results obtained from ADP - ribosylation of control and differentiated membranes from 4 different samples assessed in this way. There was a 2 - 3 fold increase in incorporation of label with each treatment. Since this data represents incorporation of radioactivity into a broad band with Mr of 39 - 40 kDa , it must be taken as indicative of a combination of $G_i\alpha$ and $G_o\alpha$ as their separation by molecular weight is not possible using 10 % (w/v) acrylamide SDS - PAGE gels.

Separation of the major pertussis toxin substrates is however possible using a modified gel system consisting of a SDS - PAGE resolving gel containing 12.5 % (w/v) acrylamide with 0.0625 % (w/v) bisacrylamide and with samples which had first been alkylated with NEM as described in **sections 2. 7. 2. & 2. 8. 2.**

Control and db cAMP differentiated NG108 - 15 membranes were treated with pertussis toxin and [^{32}P] NAD^+ , subjected to alkylation with NEM then resolved on the 12.5 % gel and autoradiographed for forty eight hours. Two polypeptides of apparent M_r of 39 kDa and 40 kDa were labelled in the presence of pertussis toxin [**Fig. 4. 4.**]. These are likely to represent the α subunits of G_i2 and G_o.

From **Fig. 4. 4.** it can be seen that there was relatively little difference in the incorporation of radioactivity into the 40 kDa polypeptide between the control and differentiated membranes. However, a marked increase in incorporation was noted in the 39 kDa polypeptide in membranes of the db cAMP - treated cells when compared to membranes of untreated cells. Densitometric analysis of the autoradiograph showed there to be a 4.6 fold [from 7.9 to 36.0 arbitary units] increase in optical density in the

Figure 4. 3.

SDS - PAGE analysis of membranes of untreated and cAMP - differentiated NG108 - 15 cells subjected to mono ADP - ribosylation with pertussis toxin.

Membranes (25 μg) of untreated (A,D) and cAMP - differentiated NG108-15 (B,C,E,F) cells were ADP - ribosylated with [^{32}P] NAD^+ and pertussis toxin for 90 min as described in **section 2. 10. 5.** Samples were recovered by deoxycholate / TCA precipitation, resolved using SDS - PAGE (10 % (w/v) acrylamide) and autoradiographed as described in **section 2. 8.** Lanes A, D, untreated cells, lane B, cells treated with 8 - bromo cAMP (1 mM), lane C, with prostaglandin E_1 (10 μM), lane E, with db cAMP (1 mM), lane F, with forskolin (10 μM). Similar results were obtained in 3 other experiments.

Figure 4. 3.

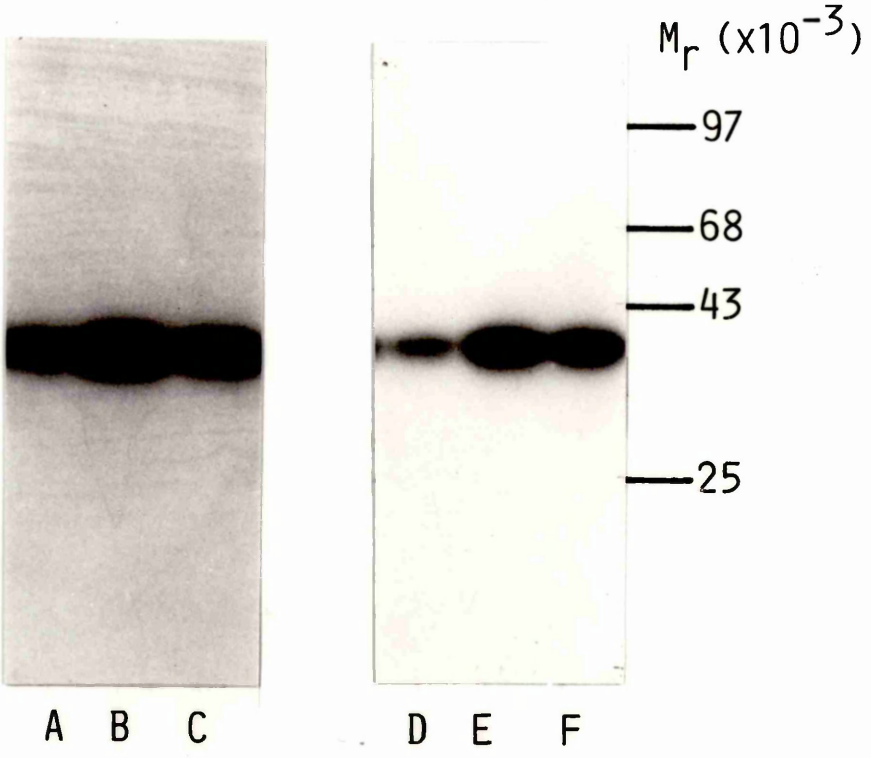


Table 4. 2.

Pertussis toxin - catalysed ADP - ribosylation of membranes of untreated and cAMP - differentiated NG108 - 15 cells.

Treatment	[32P] ADP-ribose incorporated into 39 +40 kDa band (cpm)	% of cpm in untreated membranes
Experiment 1		
Untreated	912 ± 22	100
Dibutyryl cAMP (1 mM)	2215 ± 236	243
Forskolin (10 μM)	1804 ± 160	198
Experiment 2		
Untreated	504 ± 22	100
Prostaglandin E1 (10 μM)	1212 ± 30	276
8-bromo cAMP (1 mM)	1393 ± 26	240

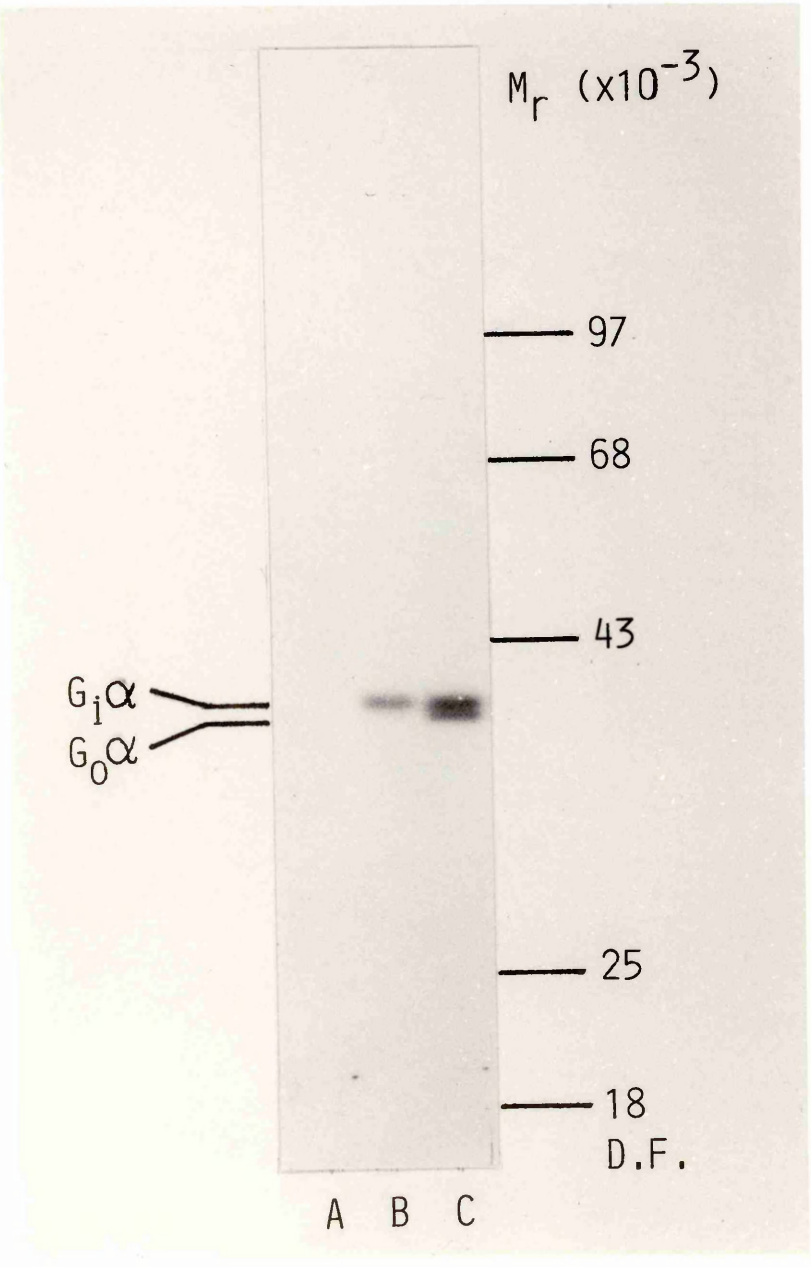
The autoradiograph described as **Fig. 4. 3.** was used as a template to locate the radioactivity at approximately 40 kDa which was excised from the dried gel and determined by liquid scintillation counting. No radioactivity was incorporated into polypeptides of this approximate size in the absence of pertussis toxin. Data for the cpm are means ± S.E. (n=4). The cells were maintained in the differentiating agents for 6 days.

Figure 4. 4.

High - resolution SDS - PAGE analysis of membranes from control and db cAMP - differentiated NG108 - 15 cells subjected to mono ADP - ribosylation with pertussis toxin .

Membranes (25 μg) of control (A, B) or db cAMP - differentiated (1mM, 6 days) (C) NG108-15 cells were incubated with [^{32}P] NAD^+ and in the absence (A) or presence (B,C) of pertussis toxin for 90 min as described in **section 2. 10. 5.** Samples were subjected to NEM treatment [**section 2. 7. 2.**], recovered by deoxycholate / TCA precipitation and finally resolved using SDS - PAGE (12.5 % (w/v) acrylamide) as described in **section 2. 8.** and autoradiographed. Similar results were obtained in 2 other experiments.

Figure 4. 4.



db cAMP - treated cells perhaps reflecting a significant increase in the levels of the 39 kDa polypeptide.

4. 2. 3. Pertussis toxin treatment of NG108 - 15 membranes *in vitro* results in the total pool of G_o being ADP - ribosylated.

There are particular difficulties in using data from ADP - ribosylation studies as a means of quantitatively assessing changes in the amounts of the G - proteins in the cells. It has been noted that, depending on the tissue under investigation, less than the theoretical maximal degree of ADP - ribosylation may occur. In brain for example, the substrate NAD^+ can be degraded by NAD^+ - glycohydrolases present and hence is not available as a substrate for the toxin [Milligan *et al.*, 1987a]. Although no attempt has been made to directly assess this enzymic activity in NG108 - 15 cells, the possibility that the alterations observed in labelling of the G - proteins were artifactual was investigated. Cell membranes prepared from control cells were treated with pertussis toxin and [^{32}P] NAD^+ as described in **section 2. 10. 4.** These samples and others which had not been pertussis toxin - treated were resolved on SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted with antiserum OC1 which recognizes $G_o\alpha$ [**Fig. 4. 5.**]. It can be seen from this experiment that the immunoreactive species migrated more slowly in membranes that had been treated with pertussis toxin. This reduction in mobility is likely to be due to the increase in molecular weight caused by the addition of the ADP - ribose moiety by the toxin.

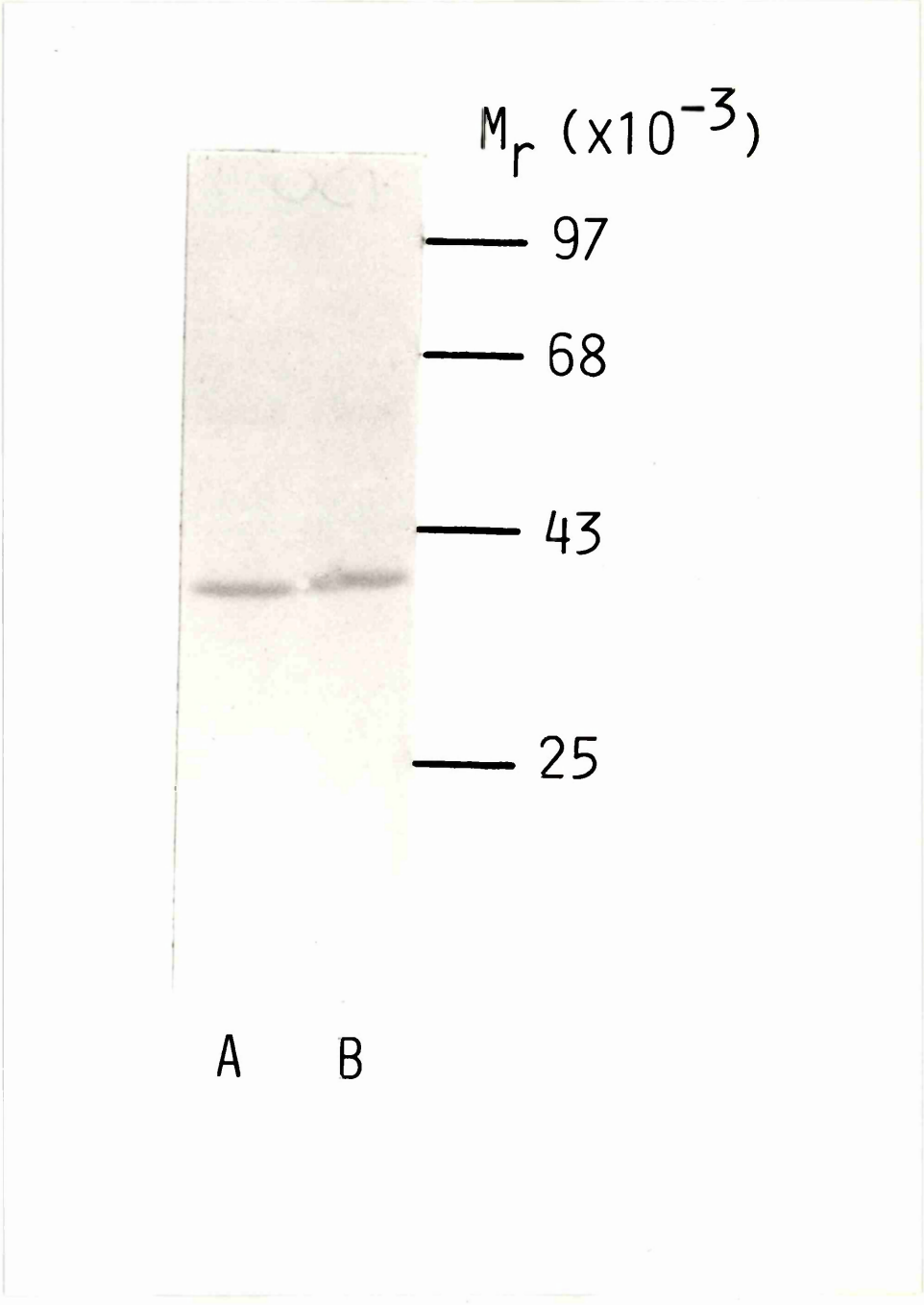
Another conclusion to draw from this experiment is that since there is a single immunoreactive band of $G_o\alpha$ in the pertussis toxin - treated samples then it implies that all of the available $G_o\alpha$ in these membranes has indeed become ADP - ribosylated thus validating attempts to quantify changes in their levels during differentiation by excision

Figure 4. 5.

Immunoblotting of membranes from control and pertussis toxin - treated NG108 - 15 cells with antiserum OC1.

Control cell membranes (A) and membranes which had been ADP - ribosylated with pertussis toxin and [^{32}P] NAD^+ for 90 min (B) as described in **section 2. 10. 5.** were subjected to deoxycholate / TCA precipitation followed resolution by SDS - PAGE (10 % (w/v) acrylamide) under denaturing conditions and subjected to immunoblotting as described in **sections 2. 7. 1. , 2. 8. & 2. 12.** using antiserum OC1 (1 : 5000) as the primary agent. 100 µg of membrane protein was used in each lane. Similar results were obtained in 2 other experiments.

Figure 4. 5.



of the labelled bands and their subsequent liquid scintillation counting.

Although the site for ADP - ribosylation for $G_o\alpha$ is within the epitope identifiedby antiserum OC1, pertussis toxin treatment of the membranes did not reduce the ability of this or any of the other anti - $G_o\alpha$ antisera used, to recognize the protein. Immunoreactivity before and after pertussis toxin treatment appears to be identical

The nature of the guanine nucleotide bound to a G - protein can substantially alter the rate of pertussis toxin - catalysed ADP - ribosylation. In the presence of the GDP analogue GDPβS, the rate of ADP - ribosylation of $G_i\alpha$ in rat glioma C6 cells was some four times greater than in an equivalent experiment with the GTP analogue GTPγS [Milligan, 1987], the implication being that pertussis toxin is better able to interact with the holomeric GDP - bound form of the G - protein than with the monomeric GTP - bound form. This conclusion has been supported by the observation that $G_o\alpha$ is a poorer substrate for pertussis toxin when it is separated from its $\beta\gamma$ subunit complex. This is reversed upon addition of purified $\beta\gamma$ back into the system [Neer *et al.*, 1984]. This problem of potential changes in the levels of $\beta\gamma$ subunits during differentiation is addressed below.

4. 2. 4. Construction of standard curves for quantitation of immunoblots with the various $G_o\alpha$ antisera.

Since pertussis toxin catalysed ADP - ribosylation of G - proteins can be modulated by a number of factors other than the absolute levels of the α subunit, other means of assessing G - protein identity and their status within the plasma membrane were utilised.

The primary amino acid sequences of at least 10 G - protein α subunits have been identified by cDNA cloning although not all of the corresponding proteins have yet been purified [Jones & Reed, 1987; Tanabe, 1985; Lochrie *et al.*, 1985]. Using the information gained from these analyses it is possible to generate a variety of selective probes which can be used in the identification of the individual G - proteins. A series of such probes have been developed, namely antipeptide antisera, which have been shown to be mono - specific in their identifcation of several G - proteins. These antisera were raised by the method described by Goldsmith *et al.*, [1987] against a number of peptide sequences known to be unique for particular G - proteins and have been characterized extensively in **Chapter 3.** Amongst these antisera are a number which were raised against particular sequences of $G_o\alpha$ [see **Table 2. 1.**]. These antisera, namely IM1, OC1 and ON1, were used in determining the degree of modulation of $G_o\alpha$ observed when NG108 - 15 cells are morphologically differentiated. This involved subjecting the membranes to the western or immunoblotting technique [Towbin *et al.*, 1979] described in **section 2. 12.** This procedure involves resolving membranes on a conventional SDS - PAGE gel and electroblotting the proteins onto a sheet of nitrocellulose. The nitrocellulose is first incubated with the anti - G - protein antisera then with a second antisera which can recognize the first antisera. This second antibody normally contains a detection apparatus of some kind. In the experiments to be described the second antisera used was a commercial donkey anti - rabbit IgG which is linked to a horseradish peroxidase activity. Visualization of the G - protein is achieved using orthodiansidine as a substrate and hydrogen peroxide as the reaction catalyst.

It was elected to try and overcome the problem of quantitation of protein with the antisera in two ways. Firstly by densitometric analysis of the resulting immunoblots and comparing changes in immunoreactivity relative to an arbitrary value for the control and secondly to charge the immunoblots with [^{125}I] - labelled donkey anti - rabbit

immunoglobulin, excise the bands and count on a Gamma counter. However for any of these techniques to be valid it was first necessary to construct a standard curve using a range of amounts of membranes in order to find the optimum amount of protein to be used in subsequent experiments.

4. 2. 4. (a). Antiserum IM1.

A range of amounts of membrane protein, from 10 - 250 μg, was subjected to analysis by immunoblotting with antiserum IM1 [**Fig. 4. 6. (a)**]. IM1 was raised against amino acids 22 - 35 from the amino terminus of the α subunit of G_o [see **Table 2. 1.**] and is specific in its recognition of this G - protein.

This blot shows increasing immunoreactivity with increasing amounts of membranes with a maximum signal occurring at between 200 - 250 μg of protein resolved. It should also be noted that there was hardly any immunoreactivity noticeable below 100 ug. This preliminary analysis suggests that the optimal amount of protein to see any changes in the levels of $G_o\alpha$ on an immunoblot using this antiserum lies between 100 and 200 ug.

It is possible however to get a more quantitive analysis of immunoblot data by densitometrically scanning a film positive taken from the blot [see **section 2. 12. 4.**]. Computer analysis of the scan can be used to give an indication of the relative amounts of $G_o\alpha$. **Fig. 4. 6. (a)** was subjected to densiometric scanning and computer analysis. The results are plotted graphically as **Fig. 4. 6. (b)**. This figure also appears to give an optimal range of between 100 and 200 μg protein in which to see any differentiation - induced changes in the levels of $G_o\alpha$. This blot was then probed with [^{125}I] - labelled donkey anti - rabbit immunoglobulin as described in **section 2. 12. 5.** and counted. The results were plotted as **Fig. 4. 6. (c)**. This data appears to correlate with that obtained from the densiometric analysis of the blot. It

Figure 4. 6. (a).

Immunoblotting of NG108 - 15 membranes with antiserum IM1 to determine optimal loading for detection of modulations in $G_o\alpha$.

Various amounts of control NG108 - 15 membranes were subjected to deoxycholate / TCA precipitation followed resolution by SDS - PAGE (10 % (w/v) acrylamide) under denaturing conditions and subjected to immunoblotting as described in **sections 2. 7. 1. , 2. 8. & 2. 12.** Antiserum IM1 (1 : 200 dilution) was used as the primary reagent. Lane **a** contained 10 µg membrane protein; lane **b**, 25 µg; lane **c**, 50 µg; lane **d**, 100 µg; lane **e**, 150 µg; lane **f**, 200 µg; lane **g**, 250 µg. Similar results were obtained in 3 other experiments.

Figure 4. 6. (a).

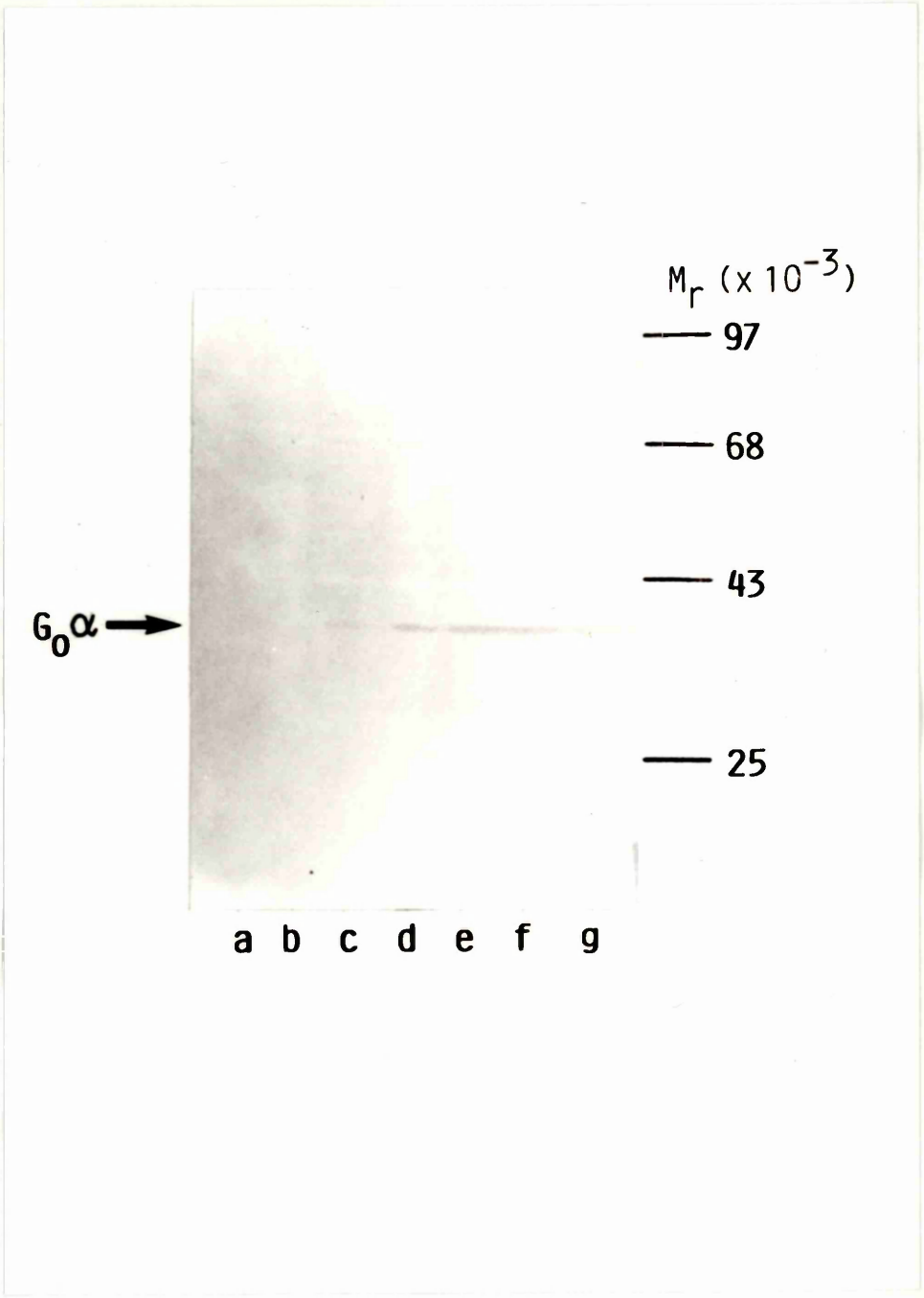


Figure 4. 6. (b).

Quantitative analysis of immunoblot with antiserum IM1 showing optimal loading of NG108 - 15 membranes: Densitometric scanning.

The immunoblot shown previously as **Fig. 4. 6. (a).** was analysed densitometrically. A film positive of the blot was scanned on a Bio - Rad video densitometer as described in **section 2. 12. 4.** The results are presented as changes in immunoreactivity relative to an arbitrary control value.

Inset. Results were expressed graphically as a percentage of the value obtained for the sample corresponding to 100 ug membrane protein and are the mean of three separate experiments ± SEM.

Figure 4. 6. (b).

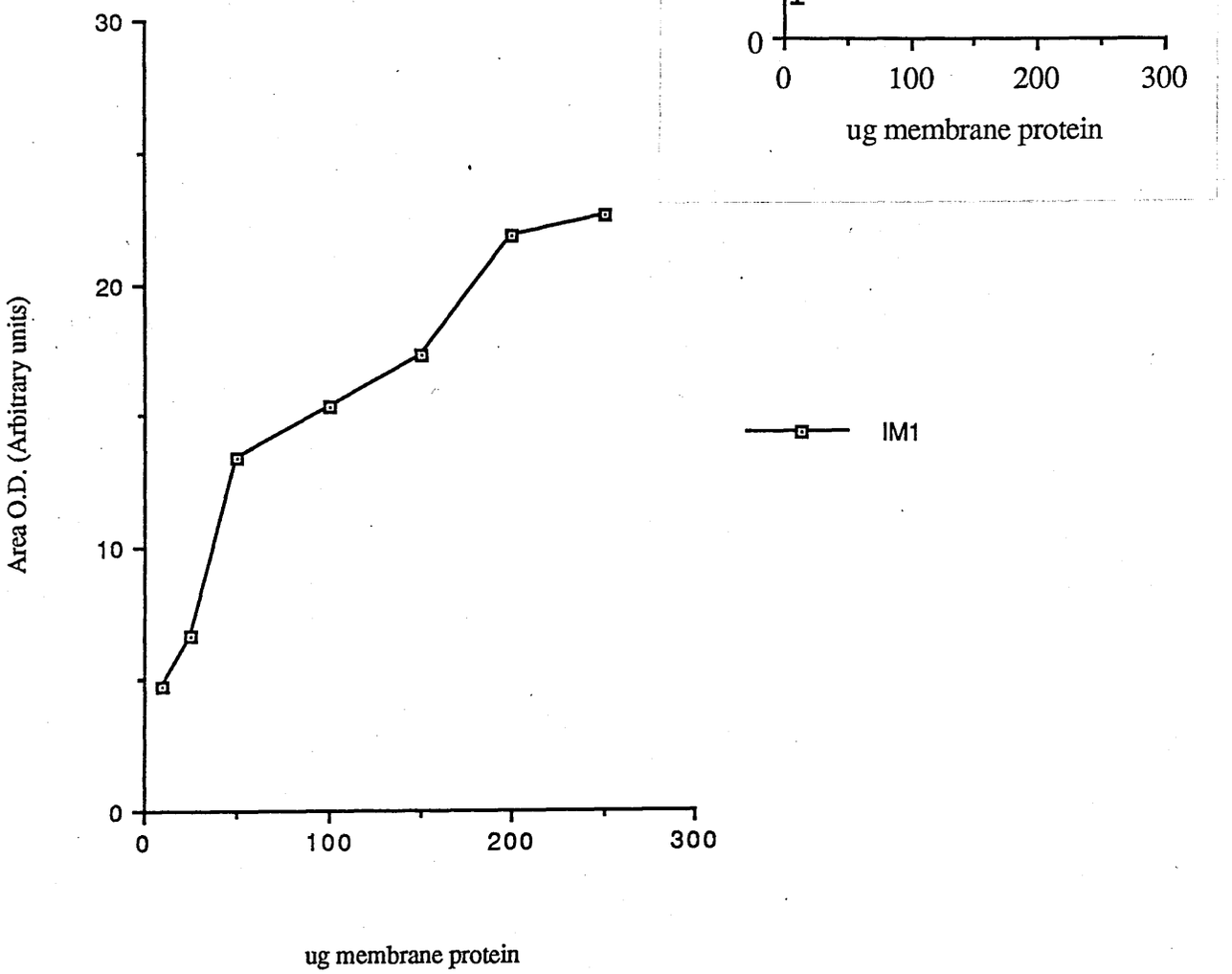

200
100
0
% control
0
100
200
300
ug membrane protein
30
20
10
0
Area O.D. (Arbitrary units)
0
100
200
300
ug membrane protein
IM1

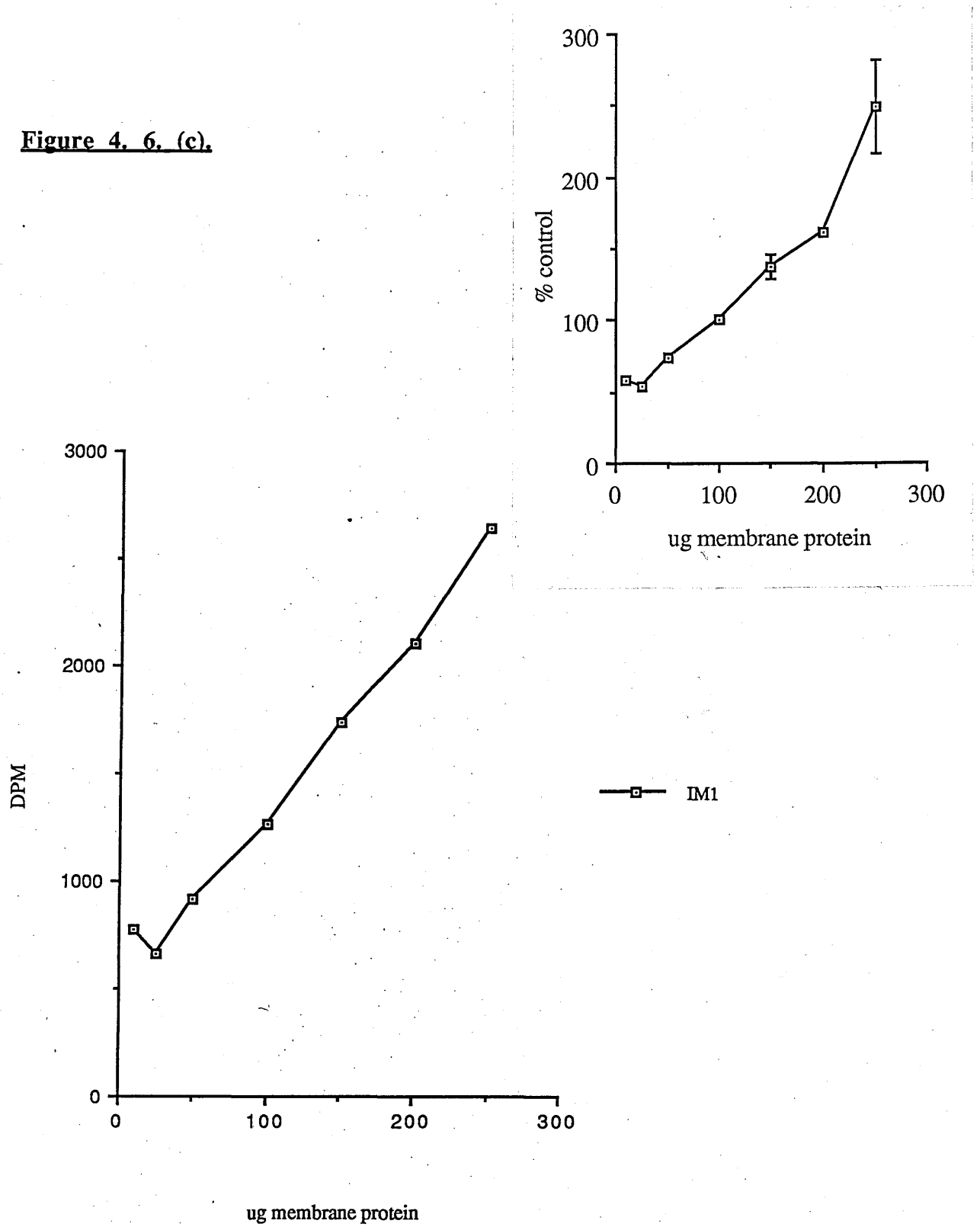
Figure. 4. 6. (c).

Quantitative analysis of immunoblot with antiserum IM1 showing optimal loading of NG108 - 15 membranes : [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay.

The immunoblot shown previously as **Fig. 4. 6. (a).** was analysed using the [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay technique as described in **section 2. 12. 5.** The immunoreactive bands were excised as counted on a gamma counter. The results are presented graphically as the DPM obtained with each of the different amounts of membrane protein analysed.

Inset. Results were expressed graphically as a percentage of the value obtained for the sample corresponding to 100 ug membrane protein and are the mean of three separate experiments ± SEM.

Figure 4. 6. (c).


300
200
100
0
% control
0
100
200
300
ug membrane protein
3000
2000
1000
0
DPM
0
100
200
300
ug membrane protein
IM1

gives an optimal range in which to see any modulations in the levels in $G_o\alpha$ caused by morphological differentiation of between 100 and 200 μg protein.

Although antiserum IM1 has been characterized and is specific in its identification of $G_o\alpha$, it was important to corroborate any results obtained with this antiserum with the other available $G_o\alpha$ antipeptide antisera . The antisera used were OC1 which was raised against the extreme carboxy terminal sequence of $G_o\alpha$ and ON1 which was raised against the sequence at its amino terminus [see **Table 2. 1.**]. Both of these antisera have been characterized in **chapter 3.**

4. 2. 4. (b). Antisera OC1 & ON1.

Antisera OC1 and ON1 were subjected to the same standard curve analysis as IM1. **Figs. 4. 7. (a)** and **4. 7. (b)** show the blots and subsequent densitometric analysis for antiserum OC1 whereas **Fig. 4. 7. (c)** shows the results from the [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay for this antiserum. **Figs. 4. 8. (a), 4. 8. (b)** and **4. 8. (c)** shows the same analysis for antiserum ON1. Increasing amounts (10 - 250 μg) of control membranes were used and immunoblotting was identical to that for IM1. Both of these antisera appeared to fall within the range of protein amounts obtained from the results with IM1 although it should be noted that both OC1 and ON1 show greater immunoreactivity for $G_o\alpha$ than IM1 and as such were used at a greater dilution (1 : 5000 compared to 1 : 200 for IM1).

From the above experiments it was decided to investigate any changes in $G_o\alpha$ immunoreactivity with differentiation using 100 μg of plasma membrane fraction in a series of immunoblots with the various $G_o\alpha$ - specific antisera.

Figure 4. 7. (a).

Immunoblotting of NG108 - 15 membranes with antiserum OC1 to determine optimal loading for detection of modulations in $G_o\alpha$.

Various amounts of control NG108 - 15 membranes were subjected to deoxycholate / TCA precipitation followed resolution by SDS - PAGE (10 % (w/v) acrylamide) under denaturing conditions and subjected to immunoblotting as described in **sections 2. 7. 1. , 2. 8. & 2. 12.** Antiserum OC1 (1 : 5000 dilution) was used as the primary reagent. Lane A contained 10 µg membrane protein; lane B, 25 µg; lane C, 50 µg; lane D, 100 µg; lane E, 150 µg; lane F, 200 µg; lane G, 250 ug. Similar results were obtained in 3 other experiments.

Figure 4. 7. (a).

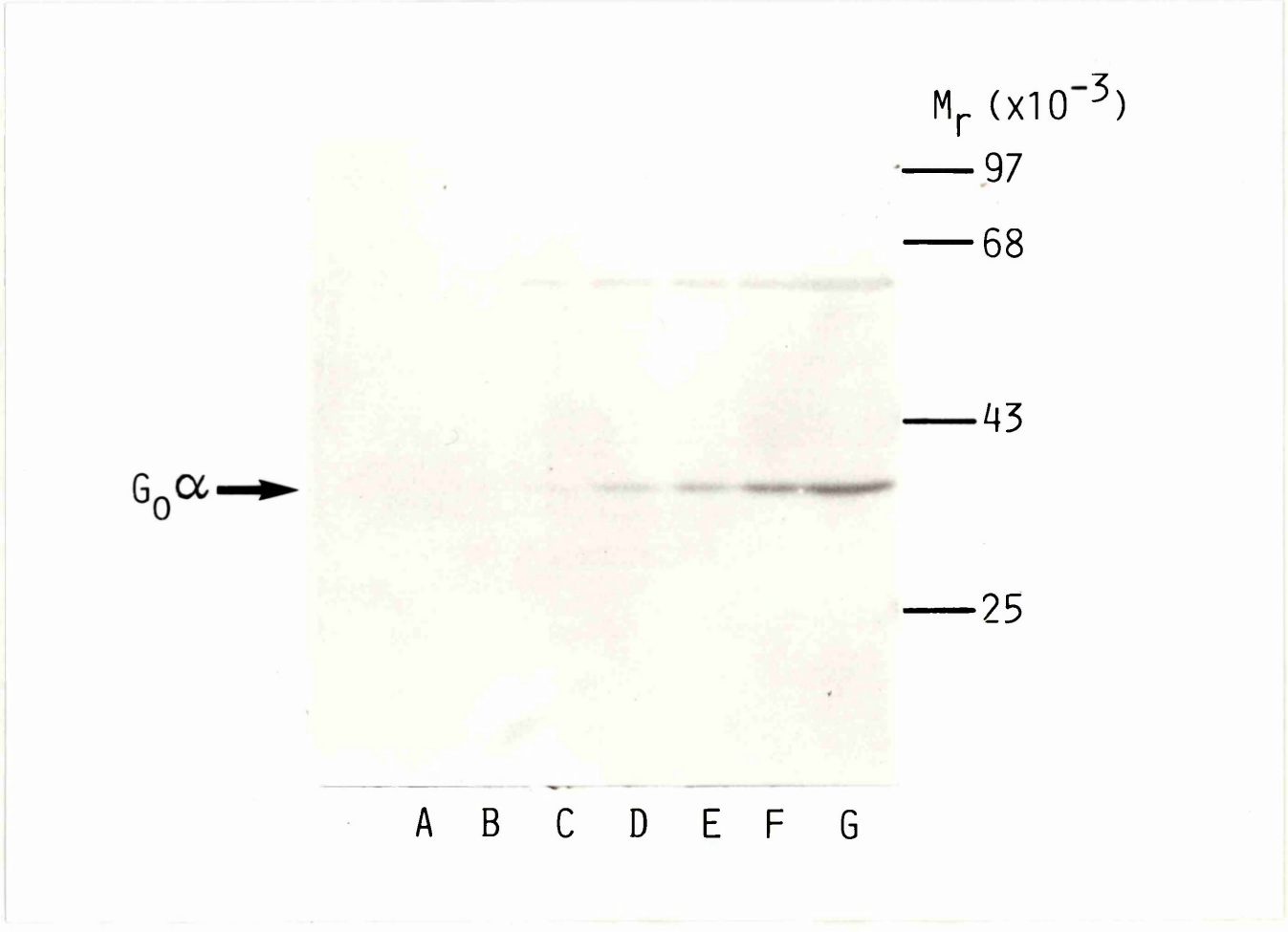


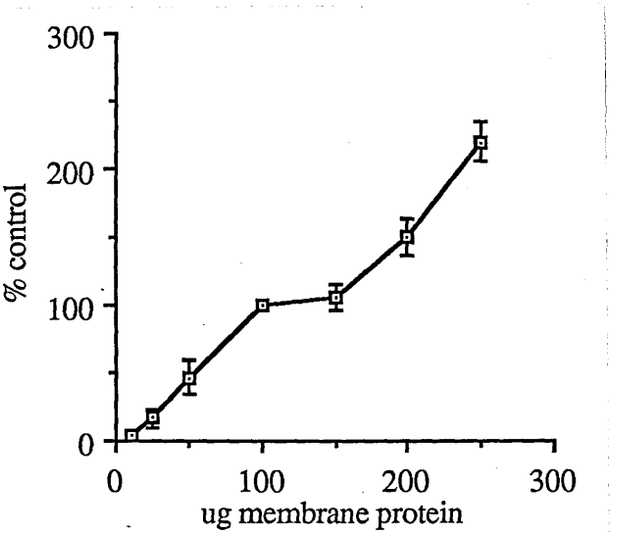
Figure 4. 7. (b).

Quantitative analysis of immunoblot with antiserum OC1 showing optimal loading of NG108 - 15 membranes : Densitometric scanning.

The immunoblot shown previously as **Fig. 4. 7. (a).** was analysed densitometrically. A film positive of the blot was scanned on a Bio - Rad video densitometer as described in **section 2. 12. 4.** The results are presented as changes in immunoreactivity relative to an arbitrary control value.

Inset. Results were expressed graphically as a percentage of the value obtained for the sample corresponding to 100 ug membrane protein and are the mean of three separate experiments ± SEM.

Figure 4. 7. (b).


300
200
100
0
% control
0
100
200
300
ug membrane protein

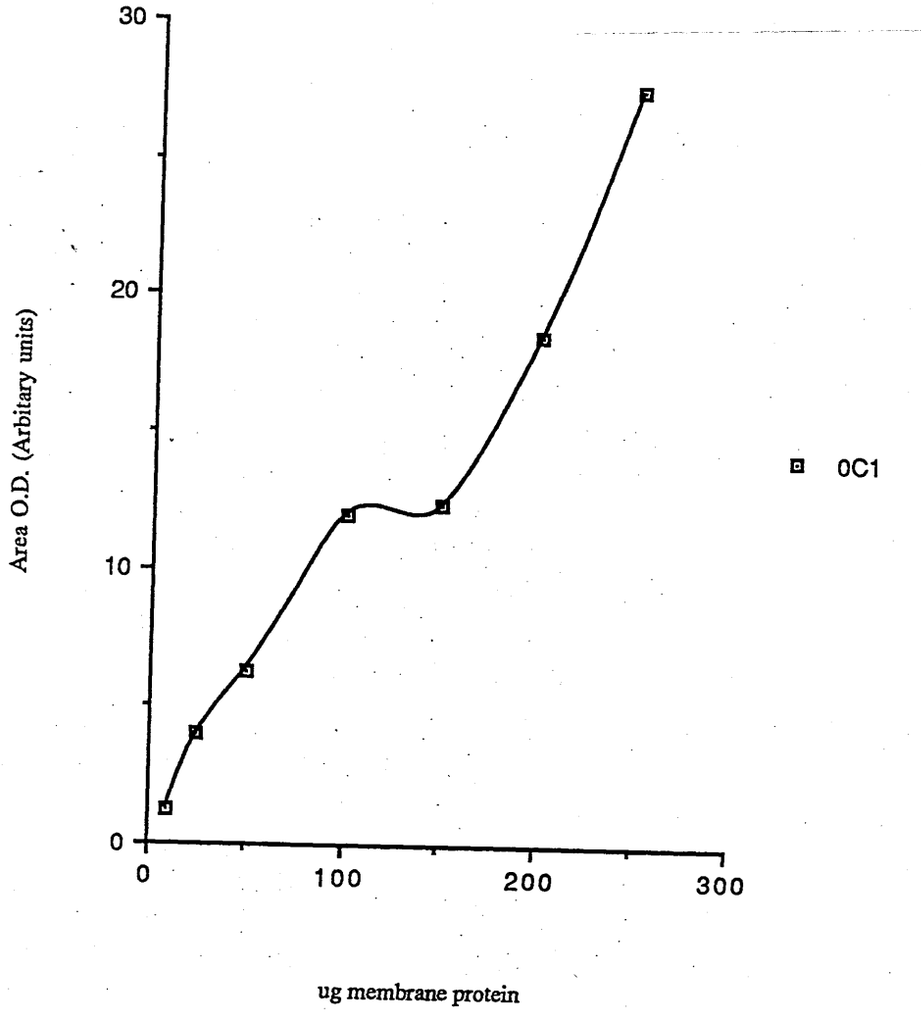

30
20
10
0
Area O.D. (Arbitary units)
0
100
200
300
ug membrane protein
0C1

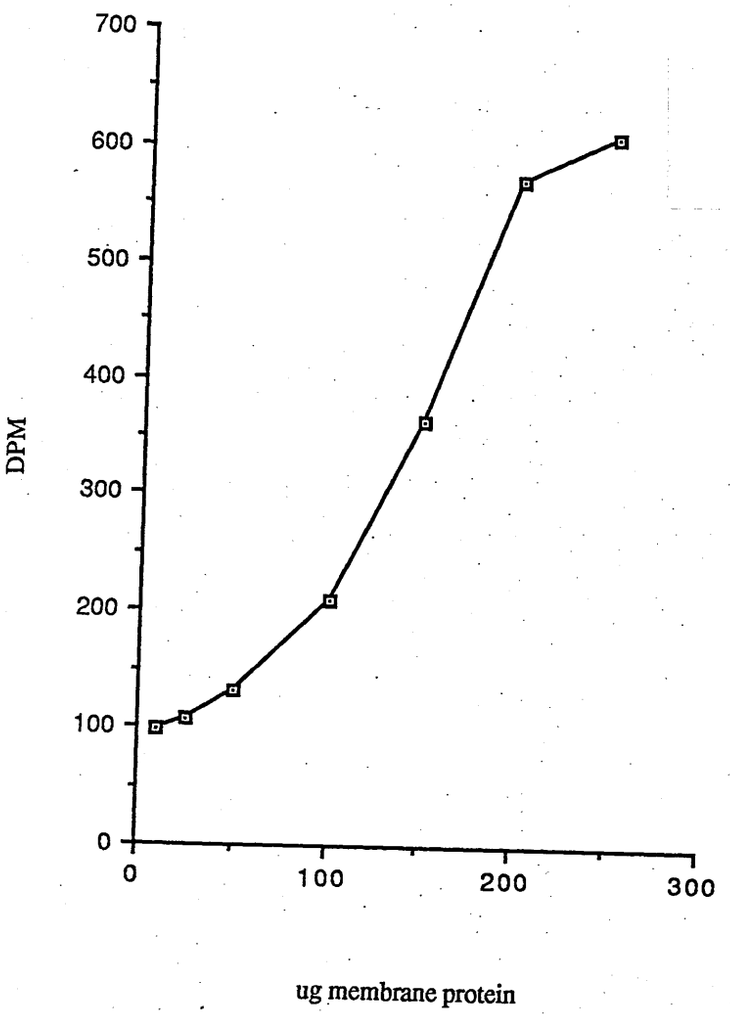
Figure 4. 7. (c).

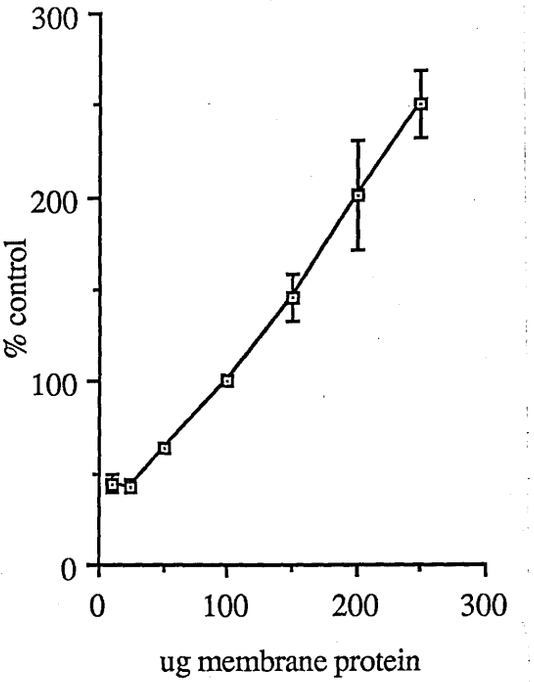
Quantitative analysis of immunoblot with antiserum OC1 showing optimal loading of NG108 - 15 membranes : [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay.

The immunoblot shown previously as **Fig. 4. 7. (a).** was analysed using the [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay technique as described in **section 2. 12. 5.** The immunoreactive bands were excised as counted on a gamma counter. The results are presented graphically as the DPM obtained with each of the different amounts of membrane protein analysed.

Inset. Results were expressed graphically as a percentage of the value obtained for the sample corresponding to 100 ug membrane protein and are the mean of three separate experiments ± SEM.

Figure 4. 7. (c).


700
600
500
400
300
200
100
0
DPM
0
100
200
300
ug membrane protein


300
200
100
0
% control
0
100
200
300
ug membrane protein


OC1

Figure 4. 8. (a).

Immunoblotting of NG108 - 15 membranes with antiserum ON1 to determine optimal loading for detection of modulations in $G_o\alpha$.

Various amounts of control NG108 - 15 membranes were subjected to deoxycholate / TCA precipitation followed resolution by SDS - PAGE (10 % (w/v) acrylamide) under denaturing conditions and subjected to immunoblotting as described in **sections 2. 7. 1. , 2. 8. & 2. 12.** Antiserum ON1 (1 : 5000 dilution) was used as the primary reagent. Lane A contained 10 µg membrane protein; lane B, 25 µg; lane C, 50 µg; lane D, 100 µg; lane E, 150 µg; lane F, 200 µg; lane G, 250 µg. Similar results were obtained in 3 other experiments.

Figure 4. 8. (a).

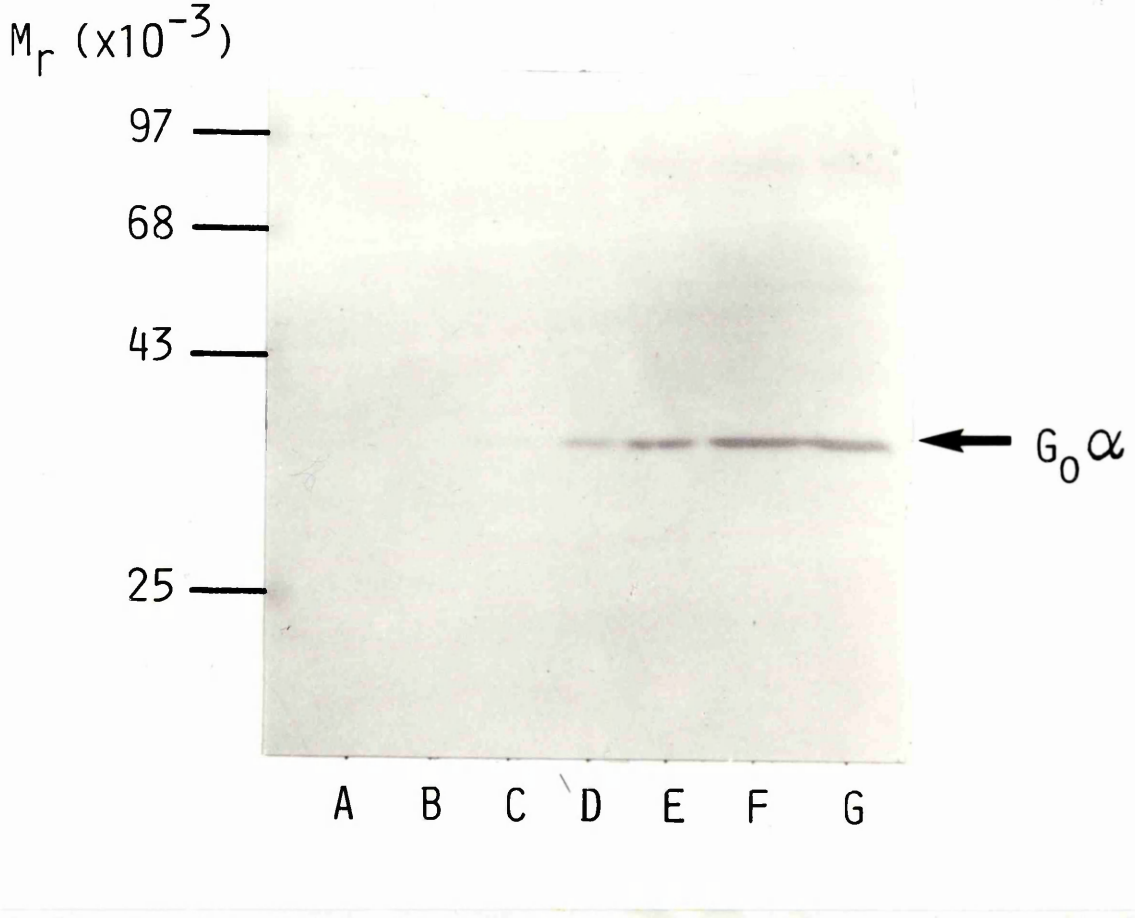


Figure 4. 8. (b).

Quantitative analysis of immunoblot with antiserum ON1 showing optimal loading of NG108 - 15 membranes with : Densitometric scanning.

The immunoblot shown previously as **Fig. 4. 8. (a).** was analysed densitometrically. A film positive of the blot was scanned on a Bio - Rad video densitometer as described in **section 2. 12. 4.** The results are presented as changes in immunoreactivity relative to an arbitrary control value.

Inset. Results were expressed graphically as a percentage of the value obtained for the sample corresponding to 100 ug membrane protein and are the mean of three separate experiments ± SEM.

Figure 4. 8. (b).

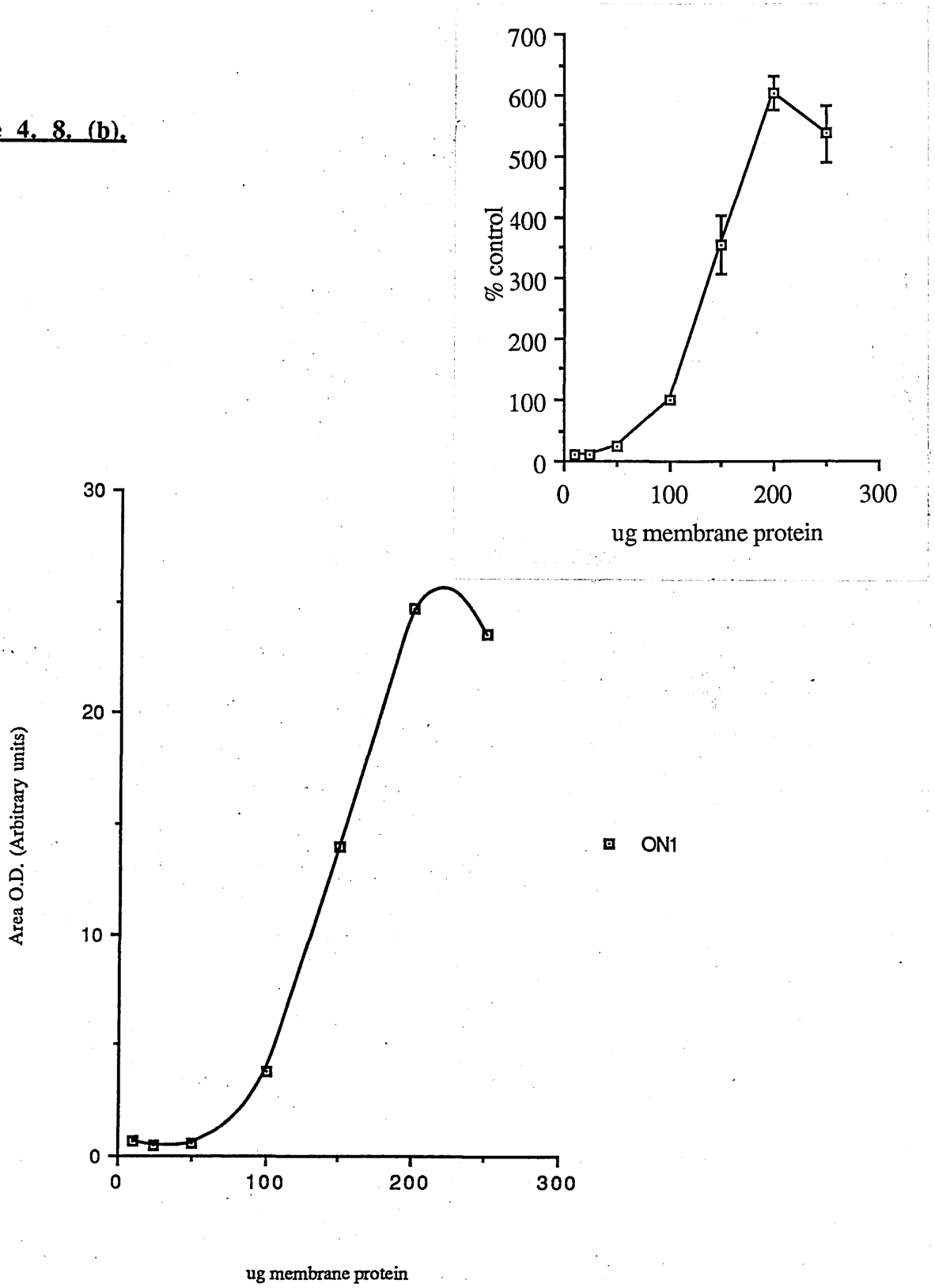

700
600
500
400
300
200
100
0
% control
0
100
200
300
ug membrane protein
30
20
10
0
Area O.D. (Arbitrary units)
0
100
200
300
ug membrane protein
ON1

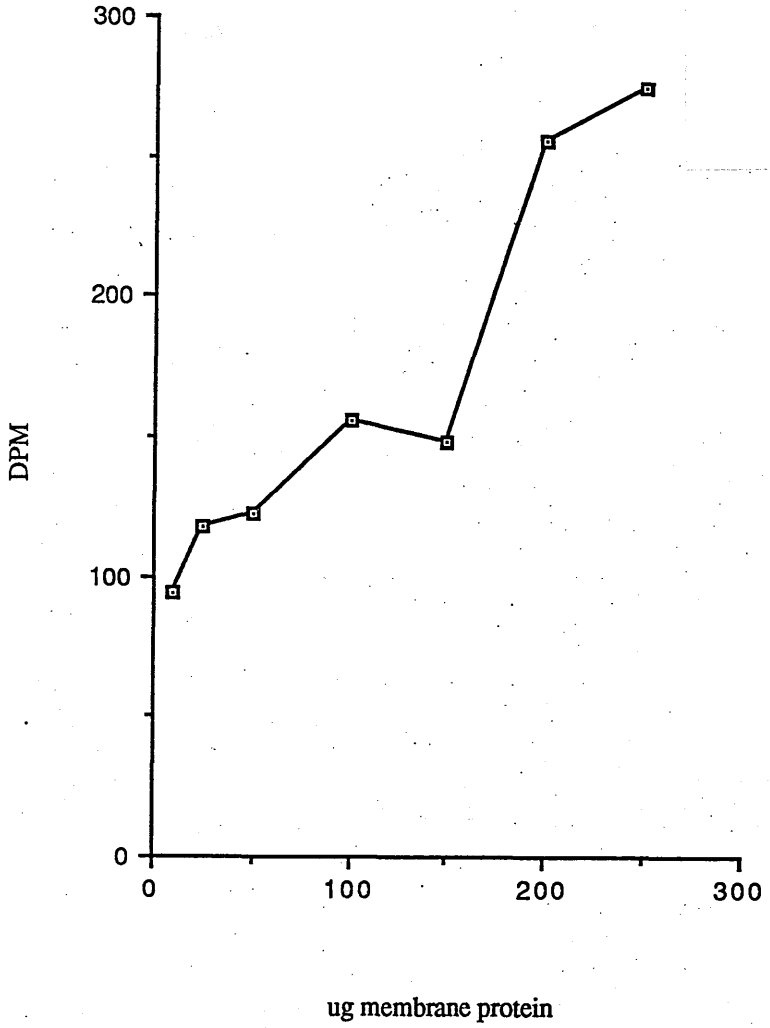
Figure 4. 8. (c).

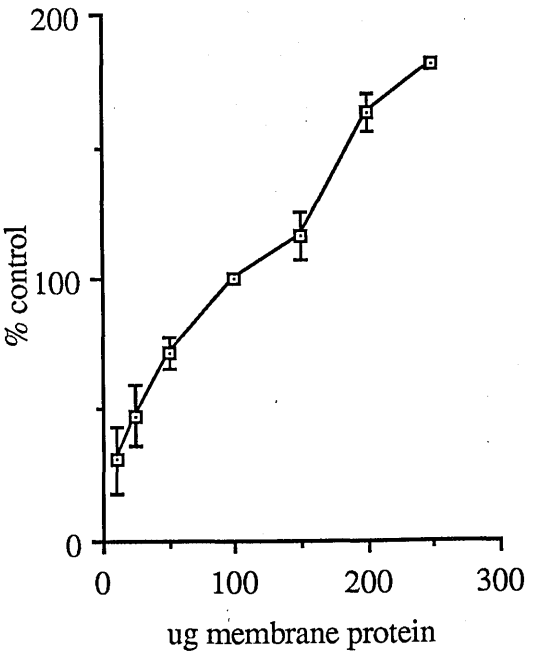
Quantitative analysis of immunoblot with antiserum ON1 showing optimal loading of NG108 - 15 membranes : [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay.

The immunoblot shown previously as **Fig. 4. 8. (a).** was analysed using the [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay technique as described in **section 2. 12. 5.** The immunoreactive bands were excised as counted on a gamma counter. The results are presented graphically as the DPM obtained with each of the different amounts of membrane protein analysed.

Inset. Results were expressed graphically as a percentage of the value obtained for the sample corresponding to 100 ug membrane protein and are the mean of three separate experiments ± SEM.

Figure 4. 8. (c).





ON1

4. 2. 5. Immunoblot analysis in the modulations in the levels of $G_o\alpha$ upon morphological differentiation.

Initial experiments were performed using antiserum IM1. **Fig. 4. 9. (a).** shows the results of one such immunoblot. In this experiment 100 μg of plasma membrane fraction prepared from the control cells and from each of the treatments were immunoblotted with 1 : 200 dilution of antiserum IM1. It can be seen that there is a marked increase in immunoreactivity with this antiserum in the membranes from all of the cells treated with the various differentiating agents when compared with the control. Densitometric scanning analysis of this blot revealed there to be at least a doubling in immunoreactivity in each of the treated cells [**Fig. 4. 9. (b).**]. This confirms the data obtained from the ADP - ribosylation studies which suggested that the increase in labelling observed upon differentiation was indeed due to an increase in the amounts of $G_o\alpha$ in the plasma membrane. Immunoblots of membranes from cells treated with the various agents with the other available $G_o\alpha$ antisera revealed a similar picture. The control membranes and the cAMP - differentiated membranes were immunoblotted using antiserum OC1 [**Fig. 4. 10. (a).**] and antiserum ON1 [**Fig. 4. 11. (a).**].Densitometric analysis of the immunoblots obtained in both of these experiments revealed there to be a marked increase in immunoreactivity with each of the treatments thus confirming with these other antisera that there is indeed an increase in the levels of $G_o\alpha$ in these cells upon differentiation [**Figs. 4. 10. (b). & 4. 11. (b).**].

4. 2. 6. Construction of standard curve analysis for immunoblot quantitation with the antisera raised against the β subunit.

G - proteins exist as heterotrimeric complexes consisting of α and β / γ subunits. Since in all physiological situations the γ subunit remains tightly associated with β, detection of the β subunit can be taken as indicative of the βγ complex. The βγ

Figure 4. 9. (a).

Immunoblotting of membranes of untreated and cAMP - differentiated NG108-15 cells with antiserum IM1 to detect $G_o\alpha$.

Membranes (100 μg) of control (A,D) and cAMP - differentiated NG108 - 15 (B,C,E,F) cells were resolved by SDS - PAGE (10 % (w/v) acrylamide), transferred to nitrocellulose and immunoblotted using antiserum 1 : 200 dilution of IM1. Lanes A, D, untreated cells, lane B, cells treated with 8 - bromo cAMP (1 mM); lane C, with prostaglandin E_1 (10 μM); lane E, with db cAMP (1 mM); lane F, with forskolin (10 μM). Similar results were seen in 4 further experiments.

Figure 4. 9. (a).

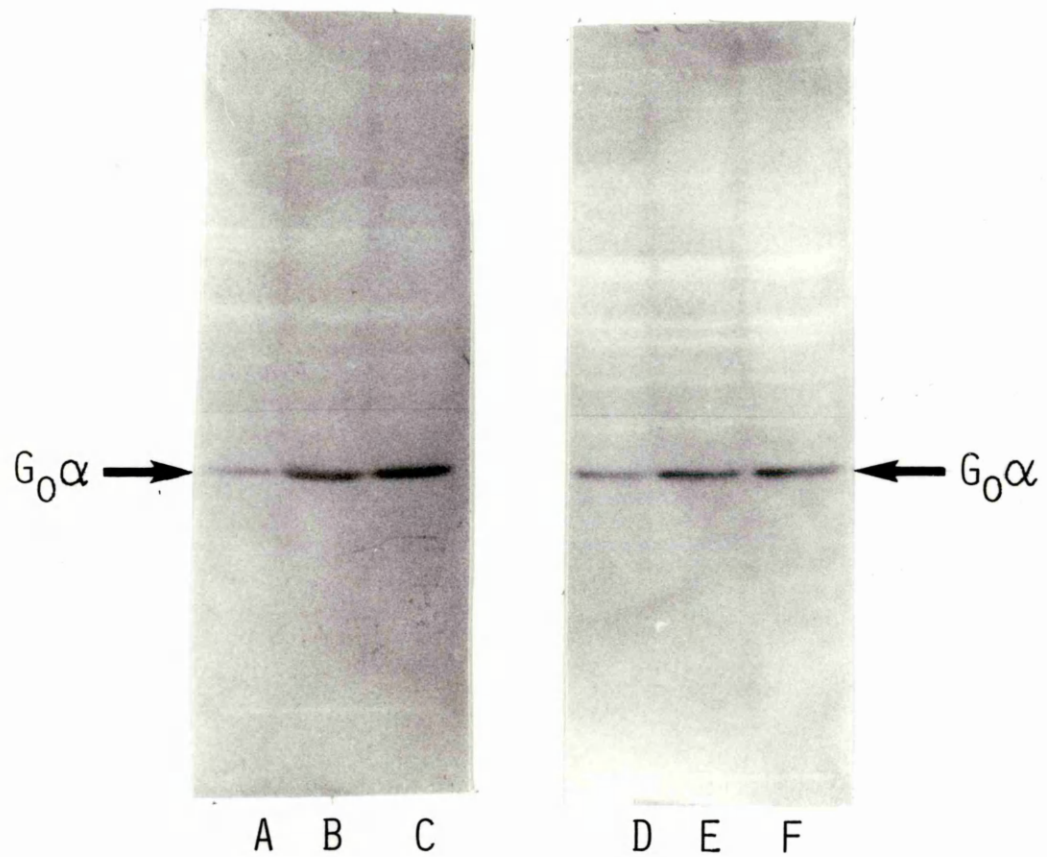


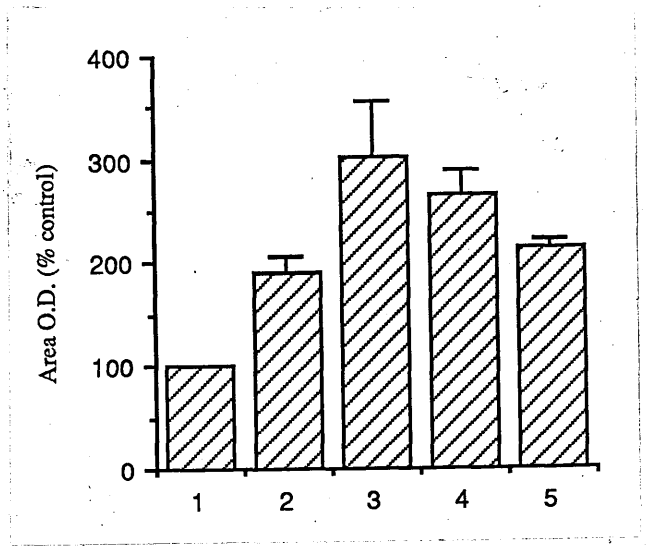
Figure 4. 9. (b).

Quantitative analysis of control and cAMP - differentiated NG108 - 15 cells with antiserum IM1: Densitometric scanning.

The immunoblot shown previously as **Fig. 4. 9. (a).** was analysed densitometrically. A film positive of the blot was scanned on a Bio - Rad video densitometer as described in **section 2. 12. 4.** The results are presented as changes in immunoreactivity relative to an arbitrary control value. Lanes A, D contain membranes from untreated cells. The other lanes represent cells differentiated with 8 - bromo cAMP (1 mM), lane B; PGE_1 (10 μM), lane C; db cAMP (1 mM), lane E; forskolin (10 μM), lane F.

Inset. Densitometric analysis of membranes from each of the variously treated NG108 - 15 cells immunoblotted with antiserum IM1 was performed. Results are expressed as a percentage of an arbitrary control value of 100% given to untreated cells and represent the mean of 4 separate experiments ± S.E.M. Lane 1 contains membranes from untreated cells; lane 2, cells treated with 1mM db cAMP; lane 3, 1 mM 8 - bromo cAMP; lane 4, 10 uM PGE_1; lane 5, 10 uM forskolin.

Figure 4. 9. (b).


Area O.D. (% control)
400
300
200
100
0
1
2
3
4
5

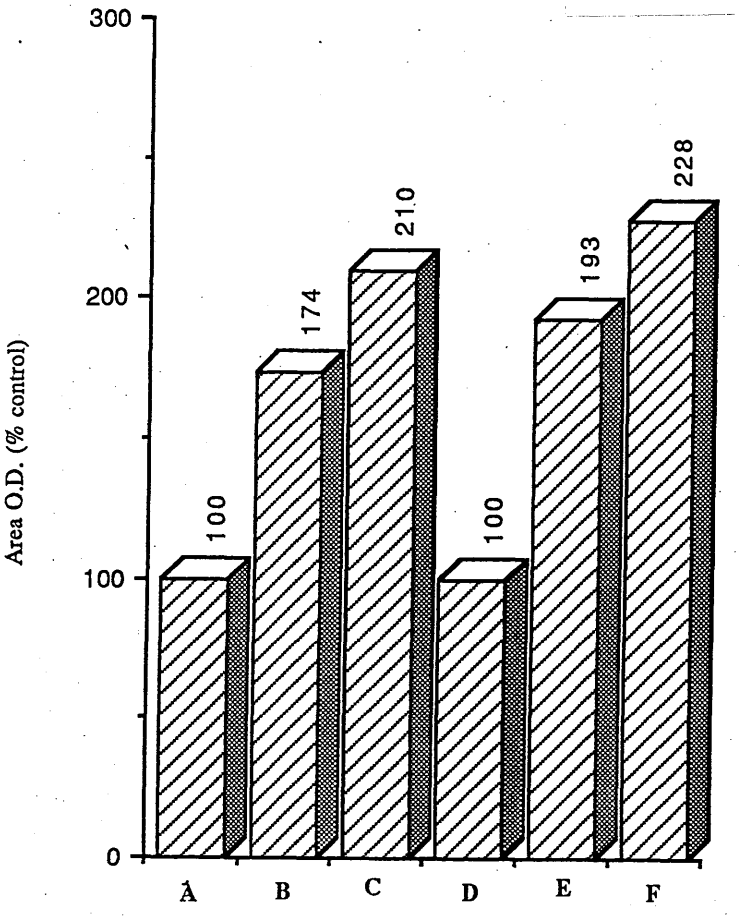

Area O.D. (% control)
300
200
100
0
100
174
210
100
193
228
A
B
C
D
E
F

Figure 4. 10. (a).

Immunoblotting of membranes of untreated and cAMP - differentiated NG108 - 15 cells with antiserum OC1 to detect $G_o\alpha$.

Membranes (100 μg) of control (A,D) and cAMP - differentiated NG108 - 15 (B,C,E,F) cells were resolved by SDS - PAGE (10 % (w/v) acrylamide), transferred to nitrocellulose and immunoblotted using antiserum OC1 (1 : 5000 dilution) as the primary reagent. Lane B, cells differentiated with db cAMP (1 mM); lane C, with forskolin (10 μM); lane E, with 8 - bromo cAMP (1 mM); lane F, with prostaglandin E_1 (10 μM). Similar results were seen in 4 further experiments.

Figure 4. 10. (a).

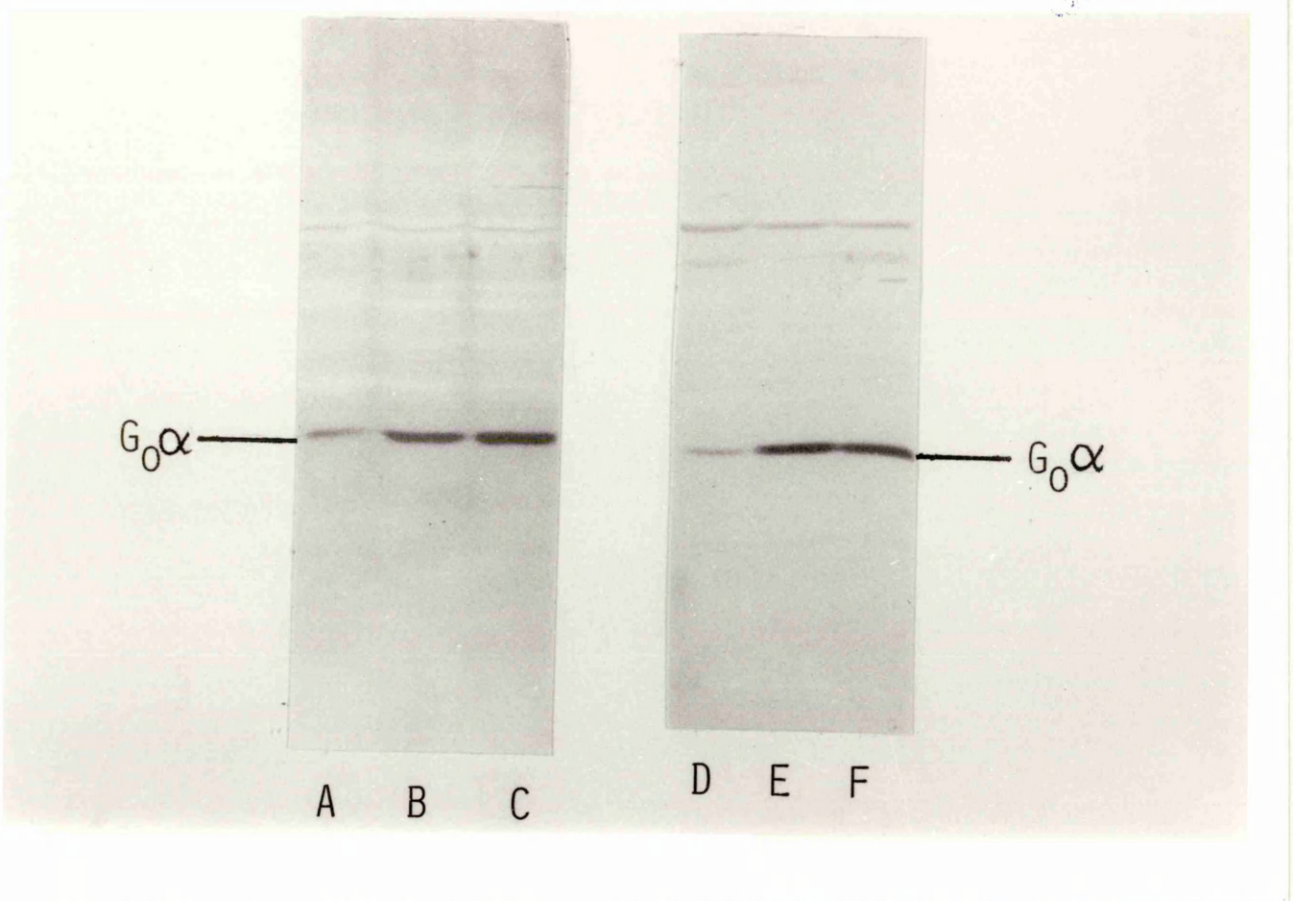


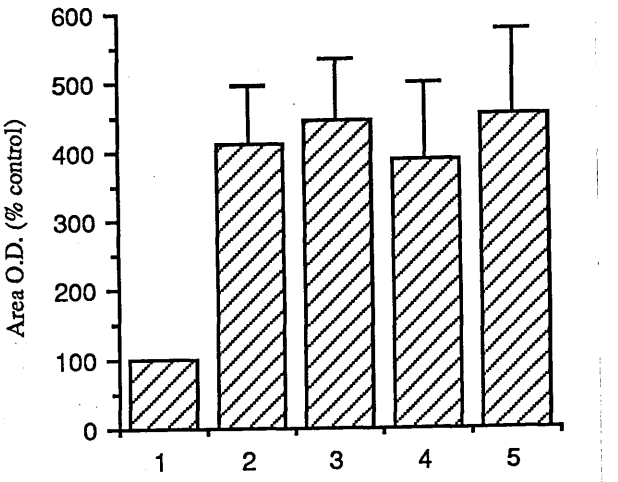
Figure 4. 10. (b).

Quantitative analysis of control and cAMP - differentiated NG108 - 15 cells with antiserum OC1: Densitometric scanning.

The immunoblot shown previously as **Fig. 4. 10. (a).** was analysed densitometrically. A film positive of the blot was scanned on a Bio - Rad video densitometer as described in **section 2. 12. 4.** The results are presented as changes in immunoreactivity relative to an arbitrary control value. Lanes A, D contain membranes from untreated cells. The other lanes represent cells differentiated with db cAMP (1 mM), lane B; forskolin (10 μM), lane C; 8 - bromo cAMP (1 mM), lane E; PGE_1 (10 μM), lane F.

Inset. Densitometric analysis of membranes from each of the variously treated NG108 - 15 cells immunoblotted with antiserum OC1 was performed. Results are expressed as a percentage of an arbitrary control value of 100% given to untreated cells and represent the mean of 4 separate experiments ± S.E.M. Lane 1 contains membranes from untreated cells; lane 2, cells treated with 1mM db cAMP; lane 3, 1 mM 8 - bromo cAMP; lane 4, 10 uM PGE_1; lane 5, 10 uM forskolin.

Figure 4. 10. (b).


Area O.D. (% control)
600
500
400
300
200
100
0
1
2
3
4
5

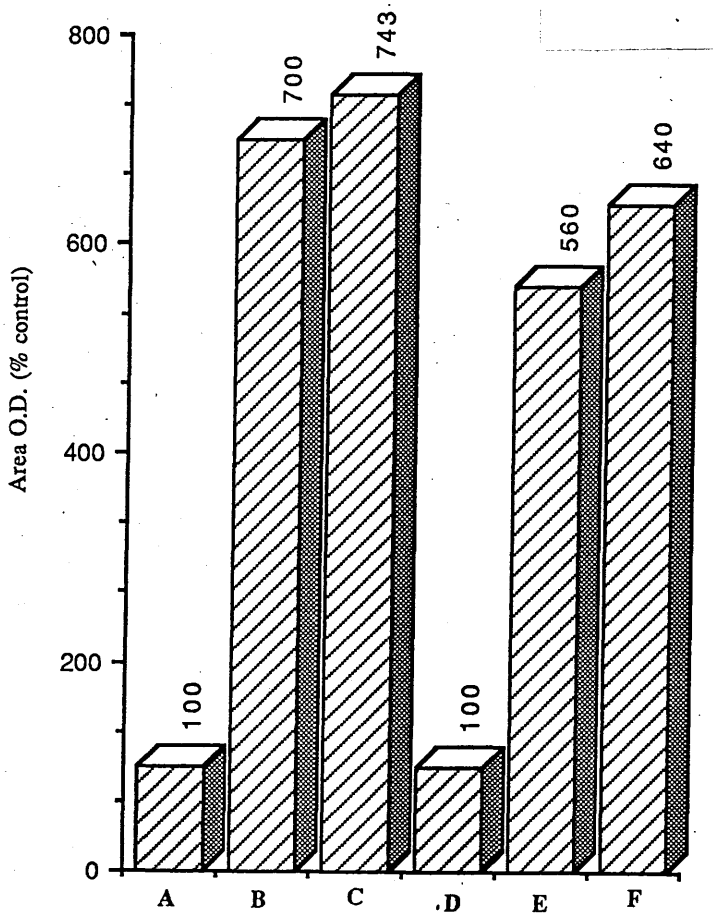

Area O.D. (% control)
800
600
400
200
0
100
700
743
100
560
640
A
B
C
D
E
F

Figure 4. 11. (a).

Immunoblotting of membranes of untreated and cAMP - differentiated NG108 - 15 cells with antiserum ON1 to detect $G_o\alpha$.

Membranes (100 μg) of control (1) and cAMP - differentiated NG108 - 15 (2,3,4,5) cells were resolved by SDS - PAGE (10 % (w/v) acrylamide), transferred to nitrocellulose and immunoblotted using antiserum ON1 (1 : 5000 dilution) as the primary reagent. Lane 2, cells differentiated with db cAMP (1 mM); lane 3,with 8 - bromo cAMP (1 mM); lane 4, with forskolin (10 μM); lane 5, with prostaglandin E_1 (10 μM). Similar results were seen in 4 further experiments.

Figure 4. 11. (a).

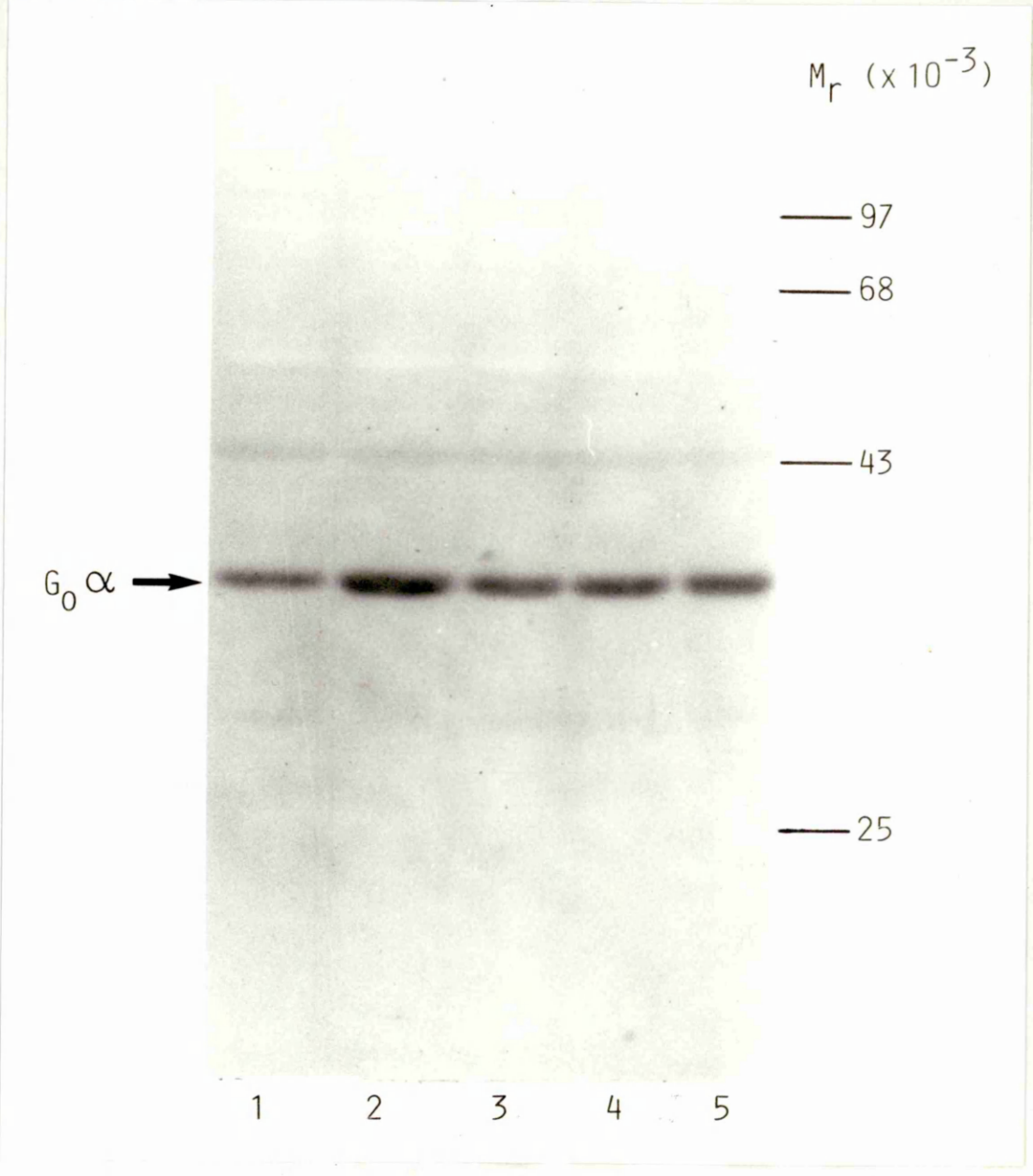


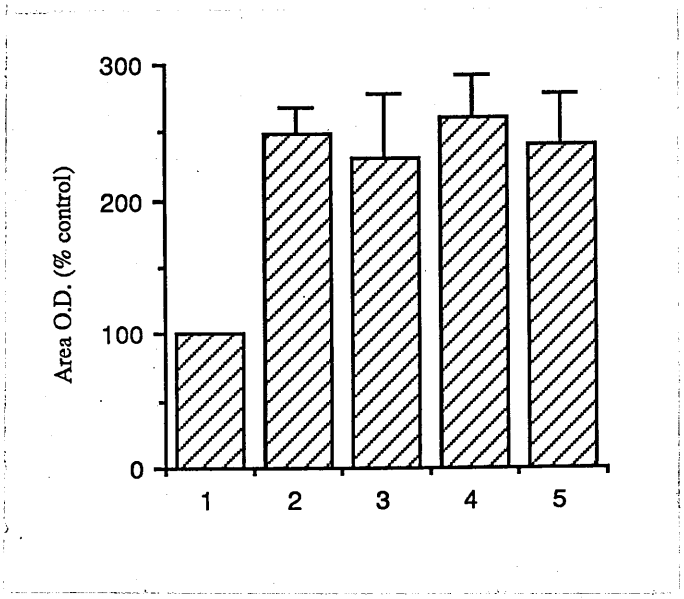
Figure 4. 11. (b).

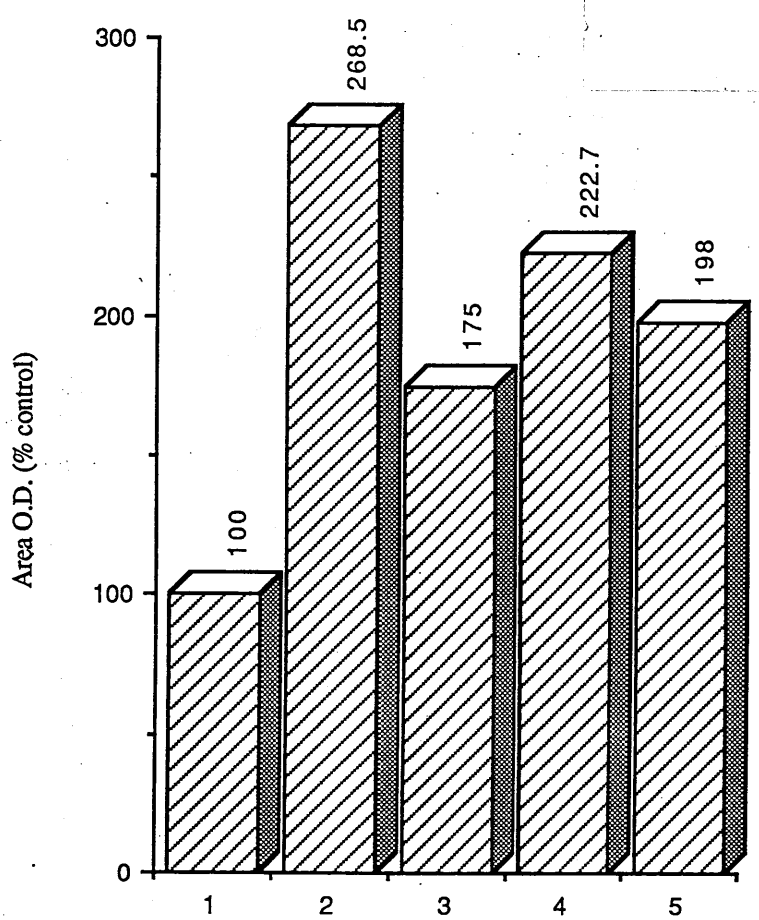
Quantitative analysis of control and cAMP - differentiated NG108 - 15 cells with antiserum ON1: Densitometric scanning.

The immunoblot shown previously as **Fig. 4. 11. (a).** was analysed densitometrically. A film positive of the blot was scanned on a Bio - Rad video densitometer as described in **section 2. 12. 4.** The results are presented as changes in immunoreactivity relative to an arbitrary control value. Lane 1 contains membranes from untreated cells. The other lanes represent cells differentiated with db cAMP (1 mM), lane 2; 8 - bromo cAMP (1 mM), lane 3; forskolin (10 μM), lane 4; PGE_1 (10 μM), lane 5.

Inset. Densitometric analysis of membranes from each of the variously treated NG108 - 15 cells immunoblotted with antiserum ON1 was performed. Results are expressed as a percentage of an arbitrary control value of 100% given to untreated cells and represent the mean of 4 separate experiments ± S.E.M. Lane 1 contains membranes from untreated cells; lane 2, cells treated with 1mM db cAMP; lane 3, 1 mM 8 - bromo cAMP; lane 4, 10 uM PGE_1; lane 5, 10 uM forskolin.

Figure 4. 11. (b).


Area O.D. (% control)
300
200
100
0
1
2
3
4
5


Area O.D. (% control)
300
200
100
0
100
268.5
175
222.7
198
1
2
3
4
5

complex has been shown to be necessary for pertussis toxin to ADP - ribosylate $G_o\alpha$ and as such examination of the levels of β subunit and on any observed changes in those levels upon differentiation must be considered. The approach used was again to utilise the immunoblotting technique with antiserum BN1 as the primary reagent. BN1 was raised against a peptide sequence at the extreme N - terminus of the β1 subtype of the β subunit [see **Table 2. 1**]. The standard curve approach used with the other antisera was again employed in order to ascertain the optimal amount of membrane to be used in subsequent immunoblots. 10 - 250 μg of protein was immunoblotted using antiserum BN1 [**Fig. 4. 12. (a)**] and subjected to densitometric analysis exactly as before [**Fig. 4. 12. (b)**]. This yielded a range of protein values of between 100 and 200 μg in which any modulation in the levels of β subunit upon differentiation would be observed. This was confirmed using the [^{125}I] - labelled donkey anti - rabbit immunoglobulin method [**Fig. 4. 12. (c)**] .

4. 2. 7. Immunoblot analysis of modulation in the levels of the β subunit upon morphological differentiation.

Modulation in the levels of the β subunit has been shown to occur in a number of instances. In rat adipose tissue, hypothyroidism has been shown to be associated with an increase in the steady state levels this protein [Milligan *et al.*, 1987b]. More recently Ros and coworkers have shown that adrenalectomy results in a loss of the β subunit by up to 50 % in rat fat cells and that dexamethosone treatment reverses the decline as assessed by quantitaive immunoblotting and northern analysis [Ros *et al.*, 1989a; Ros *et al.*, 1989b]. However as no genomic clones for the β subunit have been isolated as yet, there is no indication as to the genomic regulation of this protein although the presence of a glucocorticoid responsive element (GRE) is inferred.

100 μg of membranes prepared from control cells and from the differentiated cells were subjected to immunoblotting with antiserum BN1 [**Fig. 4. 13.**]. Only a

Figure 4. 12. (a).

Immunoblotting of NG108 - 15 membranes with antiserum BN1 to determine optimal loading for detection of modulations in β - subunit.

Various amounts of control NG108 - 15 membranes were subjected to deoxycholate / TCA precipitation followed resolution by SDS - PAGE (10 % (w/v) acrylamide) under denaturing conditions and subjected to immunoblotting as described in **sections 2. 7. 1. , 2. 8. & 2. 12.** Antiserum BN1 (1 : 200 dilution) was used as the primary reagent. Lane A contained 10 μg membrane protein; lane B, 25 μg; lane C, 50 μg; lane D, 100 μg; lane E, 150 μg; lane F, 200 μg; lane G, 250 μg. Similar results were obtained in 3 other experiments.

Figure 4. 12. (a).

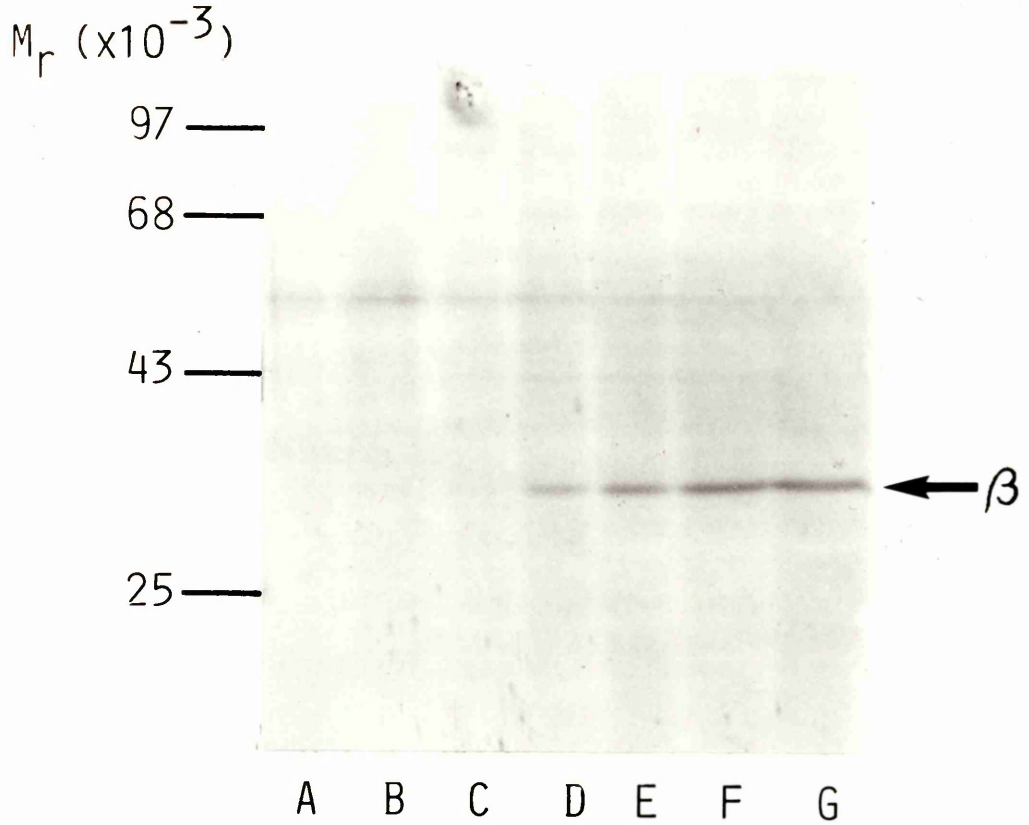


Figure 4. 12. (b).

Quantitative analysis of immunoblot with antiserum BN1 showing optimal loading of NG108 - 15 membranes : Densitometric scanning.

The immunoblot shown previously as **Fig. 4. 12. (a).** was analysed densitometrically. A film positive of the blot was scanned on a Bio - Rad video densitometer as described in **section 2. 12. 4.** The results are presented as changes in immunoreactivity relative to an arbitrary control value.

Inset. Results were expressed graphically as a percentage of the value obtained for the sample corresponding to 100 ug membrane protein and are the mean of three separate experiments ± SEM.

Figure 4. 12. (b).

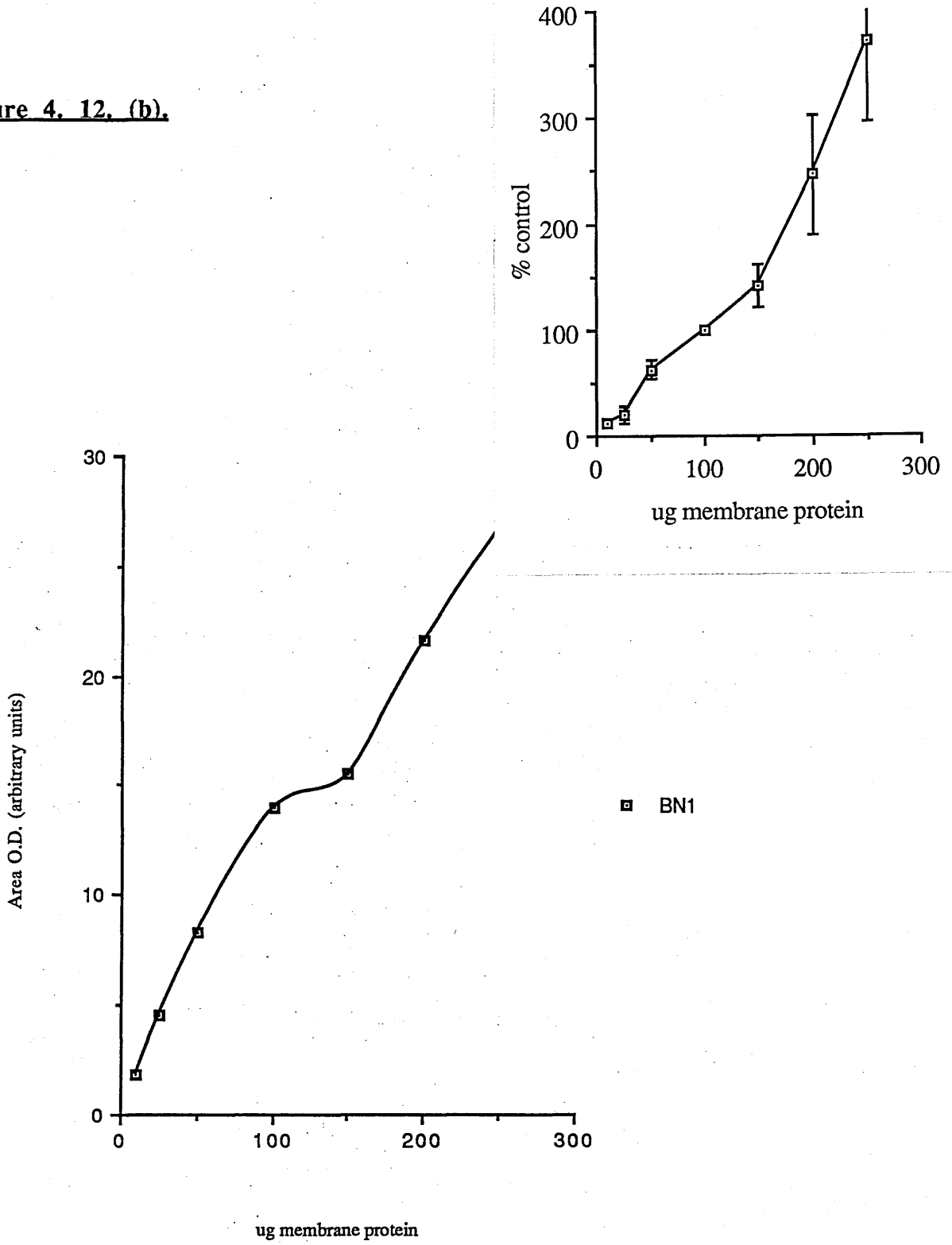

400
300
200
100
0
% control
0
100
200
300
ug membrane protein
30
20
10
0
Area O.D. (arbitrary units)
0
100
200
300
ug membrane protein
BN1

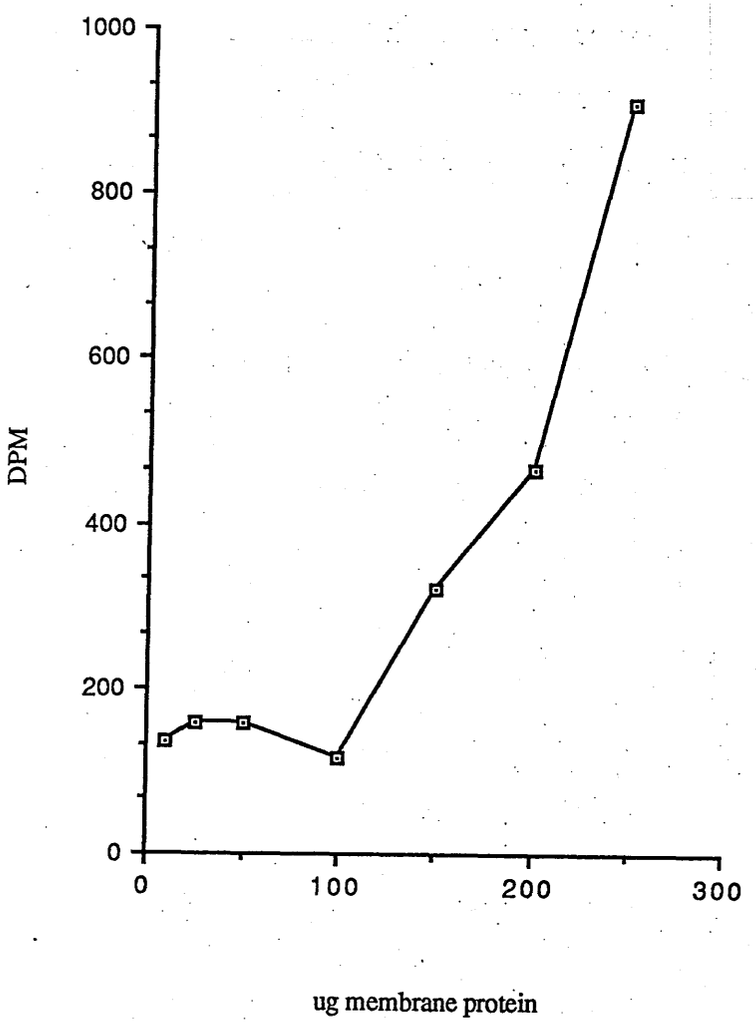
Figure 4. 12. (c).

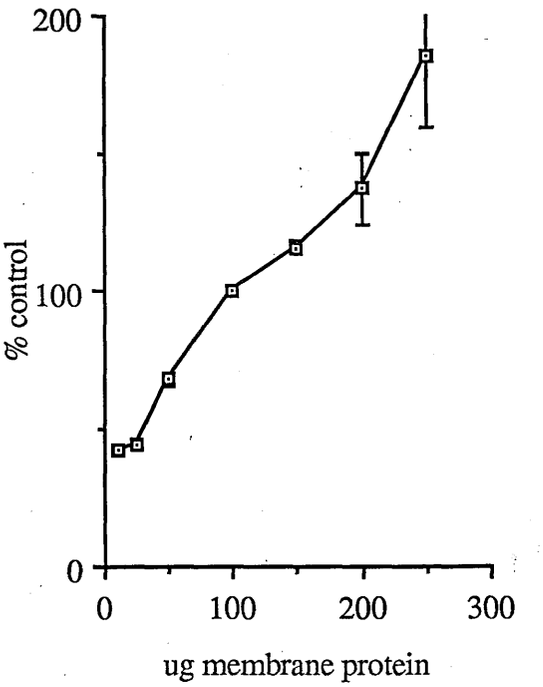
Quantitative analysis of immunoblot with antiserum BN1 showing optimal loading of NG108 - 15 membranes : [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay.

The immunoblot shown previously as **Fig. 4.12. (a).** was analysed using the [^{125}I] - labelled donkey anti - rabbit immunoglobulin overlay technique as described in **section 2. 12. 5.** The immunoreactive bands were excised as counted on a gamma counter. The results are presented graphically as the DPM obtained with each of the different amounts of membrane protein analysed.

Inset. Results were expressed graphically as a percentage of the value obtained for the sample corresponding to 100 ug membrane protein and are the mean of three separate experiments ± SEM.

Figure 4. 12. (c).





BN1

Figure 4. 13. (a).

Immunoblotting of membranes of control and cAMP - differentiated NG108 - 15 cells with antiserum BN1 to detect β-subunit.

Membranes (100 ug) of control (C) and cAMP - differentiated NG108 - 15 (A,B,D,E) cells were resolved by SDS - PAGE (10 % (w/v) acrylamide), transferred to nitrocellulose and immunoblotted as described in **sections 2. 12. 1. & 2. 12. 2.** using antiserum BN1 (1 : 200 dilution) as the primary reagent. A single band of 36 kDa, corresponding to the β subunit which is common to all of the G-proteins was observed in all samples, with only minor variations in intensity of the staining. Cells differentiated with, lane **a**, db cAMP (1 mM), lane **b**, forskolin (10 μM), lane **c**, undifferentiated cells, lane **d**, 8 - bromo cAMP (1 mM), lane **e**, PGE_1 (10 μM). Similar results were obtained in 3 other experiments.

Figure 4. 13. (a).

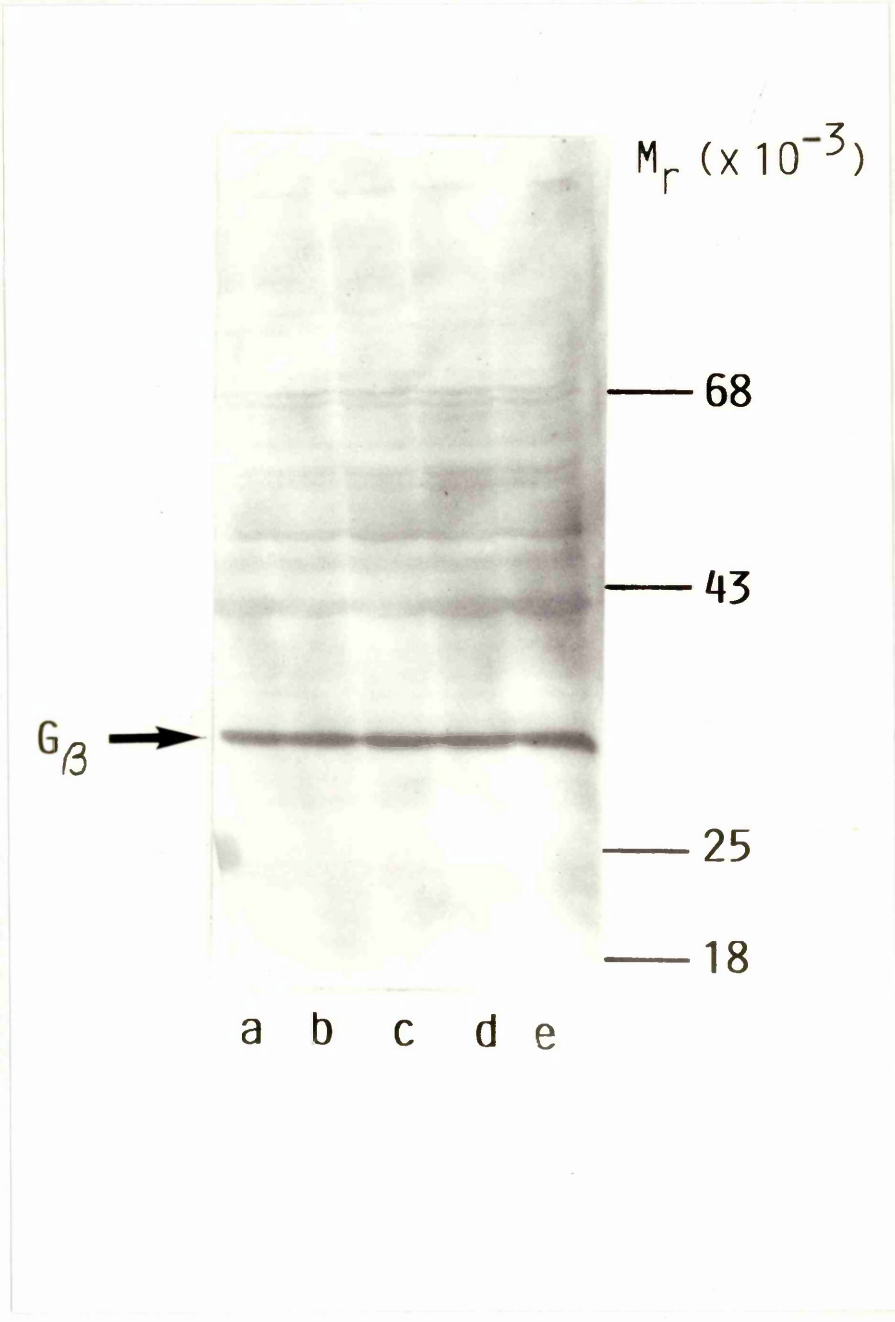


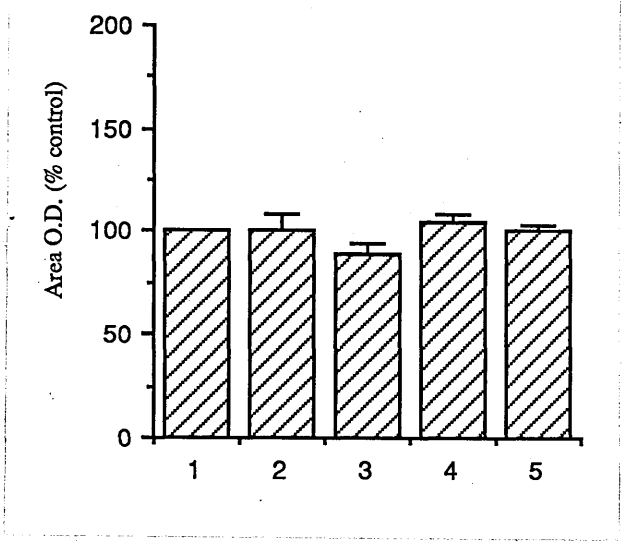
Figure 4. 13. (b).

Quantitative analysis of control and cAMP - differentiated NG108 - 15 cells with antiserum BN1: Densitometric scanning.

The immunoblot shown previously as **Fig. 4. 13. (a).** was analysed densitometrically. A film positive of the blot was scanned on a Bio - Rad video densitometer as described in **section 2. 12. 4.** The results are presented as changes in immunoreactivity relative to an arbitrary control value. Lane C contains membranes from untreated cells. The other lanes represent cells differentiated with db cAMP (1 mM), lane A; forskolin (10 μM); lane B; 8 - bromo cAMP (1 mM), lane D; PGE_1 (10 μM), lane E.

Inset. Densitometric analysis of membranes from each of the variously treated NG108 - 15 cells immunoblotted with antiserum BN1 was performed. Results are expressed as a percentage of an arbitrary control value of 100% given to untreated cells and represent the mean of 4 separate experiments ± S.E.M. Lane 1 contains membranes from untreated cells; lane 2, cells treated with 1mM db cAMP; lane 3, 1 mM 8 - bromo cAMP; lane 4, 10 uM PGE_1; lane 5, 10 uM forskolin.

Figure 4. 13. (b).


Area O.D. (% control)
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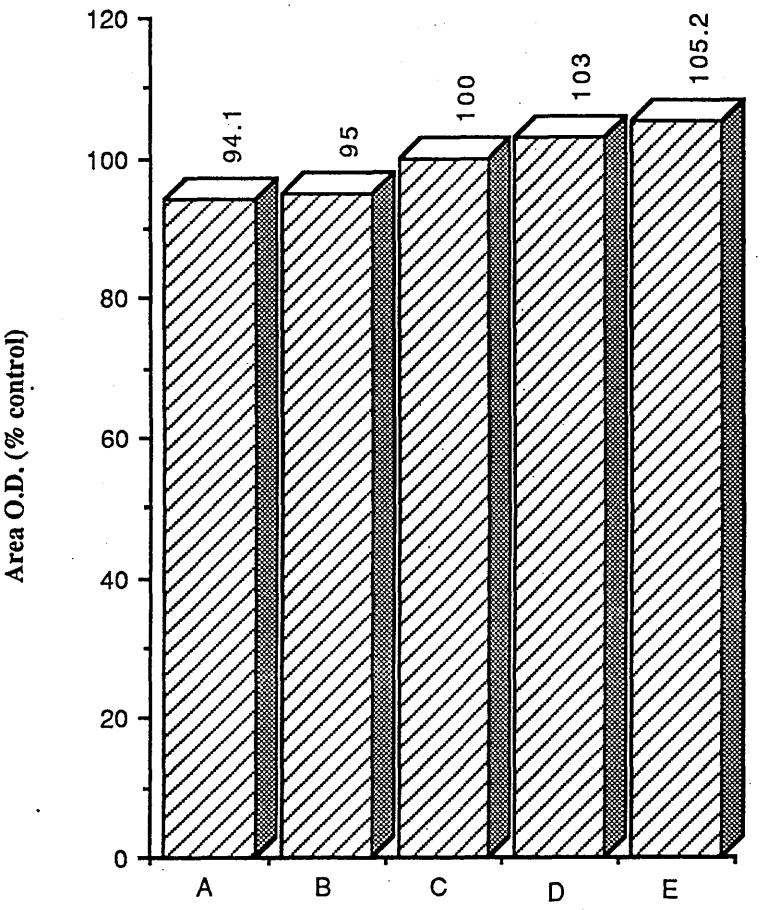
150
100
50
0
1
2
3
4
5


Area O.D. (% control)
120
100
80
60
40
20
0
94.1
95
100
103
105.2
A
B
C
D
E

small change in the levels of the β subunit was observed with any of the treatments when analysed densitometrically [**Fig. 4. 13. (b).**].

A major problem in G - protein quantitation has been the lack of suitable standards with which to compare the levels of the various G - proteins in the membrane. One way round this might be to compare the radioactive incorporation of the pertussis toxin sensitive G - proteins with the densitometric scans obtained with its corresponding immunoblot. This would yield information into the relative amounts of each particular G - protein since pertussis toxin treatment of membranes results in the total pool being ADP - ribosylated and thus radioactively labelled. Comparison of this data with the densitometric scans would enable a rough measure of the comparative levels of the G - protein pool. However, this suggestion has a major drawback; separation of the labelled proteins on an autoradiograph would have to be such that it would be possible to excise the relevant bands accurately. The family of pertussis toxin - sensitive G - proteins have such similar molecular weights that it is not possible at the present time to perform this operation. Further, the existence of at least three pertussis toxin substrates in these cells makes this type of analysis even more complicated.

4. 3. Discussion.

The results in this chapter demonstrate that morphological differentiation of NG108 - 15 cells is accompanied by an elevation in the levels of a pertussis toxin - sensitive G - protein as assessed by mono ADP - ribosylation proceedures. This G - protein was susequently identified as $G_o\alpha$ when immunoblotting with a series of $G_o\alpha$ antisera revealed an increase in specific immunoreactivity. Quantitative analysis of these blots show appreciable increases in the levels of immunoreactive $G_o\alpha$ with each treatment. Antiserum OC1 recording increases of up to seven times the control level [**Fig. 4. 10. (b).**]. No appreciable changes in the levels of the β subunit was detected.

The full complement of pertussis toxin - sensitive G - proteins in NG108 -15 cells has recently been assessed. As well as the α subunits of G_i2 and G_o these cells were shown to contain a further pertussis toxin - sensitive G - protein. This protein, $G_i3\alpha$, runs above $G_i2\alpha$ upon high - resolving SDS - PAGE. $G_i1\alpha$ is either not expressed by these cells or it is present in such low amounts that neither the protein nor the mRNA coding for it can be detected [McKenzie & Milligan, 1990].

It is generally possible to resolve the pertussis toxin substrates using SDS - PAGE since they all appear to have slightly different apparent molecular weights. In such a system the mobility of the various α subunits is such that $G_o > G_i2 > G_i3$. Thus under our resolving conditions one might expect to see a third labelled band corresponding to $G_i3\alpha$ in **Fig. 4. 4.**

One possible reason for its absence in this particular experiment is that perhaps $G_i3\alpha$ has not been fully separated from $G_i2\alpha$ and as such the upper band on the figure represents a mixture of these two proteins. However using exactly these resolving

conditions. McKenzie & Milligan have been able to show a triplet of labelled proteins corresponding to the α subunits of G_i2, G_i3 and G_o in these cells [McKenzie & Milligan, 1990]. Although the triplet was obtained it appears that the $G_i3\alpha$ band is far less intense than the other bands indicating either that $G_i3\alpha$ is a poorer substrate for pertussis toxin or, more likely, that this protein is present in much lower amounts than either $G_i2\alpha$ or $G_o\alpha$ in these cells. However, no reliable information is available about the absolute amounts of the G - proteins in these cells. Since pertussis toxin treatment results in the ADP - ribosylation of the total pool of substrate [see **Fig. 4. 5.**] one must assume that the amount of label incorporated is directly proportional to the amount of protein present in the membrane. This infers that the level of $G_i3\alpha$ in NG108 -15 cells is much lower than the other pertussis toxin substrates and since the exposure time of the autoradiograph resulting in **Fig. 4. 4.** was optimized to observe the separation of $G_i2\alpha$ from $G_o\alpha$, it may not have been sufficient to see any signal resulting from incorporation of label into $G_i3\alpha$. Longer exposure of this particular autoradiograph resulted in loss of resolution, producing a large diffuse band in which it was not possible to see any resolved bands.

One potential drawback in using immunoblotting to investigate changes in the levels of $G_o\alpha$ is in its failure to provide absolute values for the amount of this protein in the membrane. Largely this is a reflection of the lack of availability of the revelant purified proteins for use as standards. It is still a major undertaking to purify homogeneous preparations of individual G - proteins and as such this has led to the hampering of development of quantitative immunoassays. However, recently a number of laboratories have succeeded in producing recombinant protein, derived from cDNA clones, which can be be recognised by the various antisera [Goldsmith *et al.*, 1988b; Rasnas & Insel, 1988] . It is envisaged that with the development of diagnostic assays the present problems involved in quantification of G - proteins in a particular cell or tissue will be solved.

Results to show that increases in the levels of $G_o\alpha$ during neuronal cell differentiation is a common phenomenon have recently been presented. Asano *et al.* were able to demonstrate that differentiation of PC 12 pheochromocytoma cells with nerve growth factor is accompanied by an increase of some 60 - 80 % in $G_o\alpha$ when compared to control cells [Asano *et al.*, 1989] and Brabet has shown that treatment of the murine neuroblastoma NIE 115 with dimethylsulphoxide results in an increase in $G_o\alpha$ immunoreactivity [Brabet *et al.*, 1990].

Despite the virtual identity of reported cDNA sequences for bovine, rat and human $G_o\alpha$, multiple forms of mRNA have been identified [Itoh *et al.*, 1986; Brann *et al.*, 1987; Jones & Reed, 1987; Ovchinnikov *et al.*, 1987; Van Meurs *et al.*, 1987; Price *et al.*, 1989]. Investigation into the molecular basis for this heterogeneity revealed the isolation of a number of cDNA clones which diverge at the 3' - untranslated region and that these differences could, in part, be the basis for the observed multipicity in $G_o\alpha$ mRNAs [Price *et al.*, 1990]. The variations in the relative amounts of the $G_o\alpha$ mRNAs may also reflect differences in the extent of expression of the individual transcripts in specific cell types. Consistent with this idea, *in situ* hybridization [Brann *et al.*, 1987] and immunohistochemical [Worley *et al.*, 1986; Chang *et al.*, 1988] studies have revealed that $G_o\alpha$ expression is limited to specific cell types.

The occurence of $G_o\alpha$ mRNAs with different 3' - untranslated regions could reflect the presence of multiple genes for this G - protein that are nearly identical in the 5' - untranslated and coding regions but differ in the 3' - untranslated regions. The results of Southern blotting of restriction enzyme digests of bovine genomic DNA are, however consistent with the presence of a single $G_o\alpha$ gene and that the multiple mRNAs may result from alternate splicing of transcripts of this gene [Kaziro, 1990].

Differential regulation of expression of mRNA and protein corresponding to $G_s\alpha$ and $G_i\alpha$ in rat brain cortex has been noted following chronic treatment with corticosterone [Saito *et al.*, 1989]. Elevated levels of $G_s\alpha$ have also been noted in a cell line rat pituitary line, GH_3 , following exposure to dexamethasone [Chang & Bourne, 1987] whilst treatment of 3T3 - L1 fibroblasts with dexamethasone, which effectively differentiates these cells into adipocytes, results in an initial increase in $G_o\alpha$ followed by a decrease [Gierschik *et al.*, 1986a]. These results imply that glucocorticoid hormones may be able to regulate expression of G - protein genes.

However there is a precedent for cAMP - induced modulation in the levels of G - proteins. Differentiation of a human promyelocytic leukemia cell line, HL60, with db cAMP or DMSO is accompanied by an increase in the amounts of the α subunit of G_i2 [Murphy *et al.*, 1987] and begs the question at what point is gene expression controlled by cAMP.

Although the primary amino acid sequences of a large number of the G - proteins have been determined using cDNA cloning technology, little is known about their genomic organization and transcriptional control. cDNA cloning analysis has proved most useful in the identification and characterization of the known G - proteins. Since these analyses reveal only the sequence of the final encoded protein and nothing of the upstream non - coded regions, very little is known about the regulation of the various G - protein genes. Isolation of the complete genomic sequence which would include putative promotor regions would be useful.

In a recent paper a number of human genomic clones encompassing the gene for $G_i2\alpha$ were isolated from db cAMP differentiated HL60 cells and characterized. Several transcriptional elements including a possible AP2 binding site were identified [Weinstein *et al.*, 1988]. Analysis of the 5' flanking sequence showed no TATA box

but did reveal the presence of a CCAAT box and at least five GC boxes which are potential DNA binding sites for the transcriptional factor Sp1. In this respect the $G_i2\alpha$ gene appears to be controlled in a similar way to the so - called " housekeeping " genes [Weinstein *et al.*, 1988].

Genomic sequences for $G_o\alpha$ have come from studies on the *dgo* gene from *Drosophila melanogaster*. This gene, which maps to 47A on the second chromosome encodes for two proteins (termed DGo1 and DGo2) which are both 354 amino acids long but differ in seven amino acids in the amino - terminal region [Yoon *et al.*, 1989]. These two polypeptides share 81 % homology with rat $G_o\alpha$ [Jones & Reed, 1987].

Analysis of the genomic clones revealed that there are eight coding exons and that the putative transcripts for the two proteins differ in the 5' - noncoding regions and the first coding exons but share the remaining six coding exons. The difference in the 5' - noncoding regions raises the possibilty of differential transcriptional control through regulation by different promotors. Indeed consensus sequences for a TATA box and a transcription initiation site were found upstream from the translation site for the DGo1 gene product and since the first coding exon for DGo2 is located some 5 kilobases further upstream from this transcription initiation site, it is highly unlikely that these sequences regulate the expression of the DGo2 transcript. Another promotor located further upstream very likely regulates the expression of this transcript. Closer analysis of the 5' - noncoding region of both of the transcripts did not uncover any CRE or GRE consensus sequences.

Chapter 5

CHANGES IN RECEPTOR FUNCTION ACCOMPANY THE DIFFERENTIAL REGULATION OF $G_i2\alpha$ AND $G_o\alpha$ IN db cAMP - TREATED NG108 - 15 CELLS.

Chapter 5

CHANGES IN RECEPTOR FUNCTION ACCOMPANY THE DIFFERENTIAL REGULATION OF $G_i2\alpha$ AND $G_o\alpha$ IN db cAMP - TREATED NG108 - 15 CELLS.

5. 1. Introduction.

Although the subject of much investigation a function for the guanine nucleotide - binding protein G_o has still to be clearly elucidated. A number of immunological and biochemical studies have shown that distribution of $G_o\alpha$ is essentially restricted to neuronal [Gierschik *et al.*, 1986b; Homburger *et al.*, 1987; Asano *et al.*, 1989] and endocrine tissues [Terashima *et al.*, 1987; Journot *et al.*, 1987]. It has been noted that the levels of this protein increase in brain during ontogenic development whereas those of $G_i\alpha$ remained almost constant at all ages. These observations might suggest that G_o and G_i participate in different functions in different cell types within the nervous system [Milligan *et al.*, 1987c; Asano *et al.*, 1988a; Asano *et al.*, 1989].

Reconstitution experiments have been undertaken to clarify the funtion of G_o. It was observed that purified preparations of $G_i\alpha$ and $G_o\alpha$ were equipotent in their ability to interact with partially purified or highly purified muscarinic receptors [Florio & Sternweis, 1985; Haga *et al.*, 1986; Kurose *et al.*, 1986] and with $GABA_B$ receptors [Asano *et al.*, 1985].

However in some reconstitution models $G_i\alpha$ and $G_o\alpha$ were reported to have different efficacies. In membranes of the *cyc-* mutant of S49 lymphoma cells, $G_i\alpha$ activated with GTPγS was found to inhibit adenylyl cyclase, whereas $G_o\alpha$ - activated in a similar manner was inactive [Roof *et al.*, 1985]. Similarly Katada and coworkers

were able to show that activated $G_i\alpha$ inhibited partially purified $G_s\alpha$ - stimulated adenylyl cyclase whereas activated $G_o\alpha$ had no effect [Katada *et al.*, 1986] thus indicating that G_o may not be able to interact with the components of the adenylyl cyclase system.

NG108 - 15 cells have been shown to express opioid receptors of the δ subclass which are able to modulate the inhibition of certain calcium currents. These voltage - sensitive Ca^{2+} channels [VSCC] can only be detected in cells differentiated with agents which maintain an elevated level of intracellular cAMP. Hormonal regulation of the channels appear to be preferentially transduced via G_o [Hescheler *et al.*, 1987].

It has been established however that these same opioid receptors are able to stimulate high - affinity GTPase activity and to inhibit adenylyl cyclase in membranes prepared from NG108 - 15 cells [Koski & Klee, 1981]. Preincubation of the membranes with affinity - purified antibodies directed against the C - terminal region of $G_i2\alpha$, led to an abolition of opioid - peptide stimulation of high - affinity GTPase activity [McKenzie *et al.*, 1988], which led to the conclusion that the opioid receptor interacted selectively with $G_i2\alpha$ in these cells .

Clearly there must be some alteration in the ability of the δ - opioid receptor to interact with the efector systems in these cells which is brought about by differentiation of the cells. It has already been demonstrated in **chapter 4** that morphological differentiation of NG108 - 15 cells following treatment with a variety of agents which act via a common mechanism of raising intracellular cAMP caused an increase in the levels of the α subunit of the guanine nucleotide - binding protein G_o.

The results presented in **chapter 5** looked at potential alterations in the

transducing elements coincident with db cAMP - induced differentiation of NG108 - 15 cells. Firstly, modulation in the levels of the major pertussis toxin - sensitive G - proteins caused by incubation of the cells with db cAMP was more carefully examined and secondly, the effects of these changes on opioid - mediated inhibition of adenylyl cyclase in these cells were assessed.

5. 2. Results.

5. 2. 1. Analysis of membranes from control and db cAMP - differentiated NG108 - 15 cells with pertussis and cholera toxin.

It was decided to examine what effect morphological differentiation has upon a defined signal transducing pathway in NG108 - 15 cells. The pathway examined was the interaction of the δ - opioid receptor with $G_i2\alpha$ which results in an inhibition of adenylyl cyclase. The membranes used in the experiments to be described were produced from a series of incubations with cells cultured and harvested over 5 passages. This ensured that these membranes were as equivalent as possible. Db cAMP was used as the differentiating agent in these experiments simply because it is the most common agent used to differentiate these cells and has the largest literature available for comparison of results obtained with a number of systems.

NG108 - 15 cells in tissue culture were incubated for 6 days with 1mM db cAMP to produce morphological differentiation as previously described [see **section 2. 4. 6.**]. Membranes prepared from these cells were resolved on SDS - PAGE (10 % (w/v) acrylamide). Subsequent staining with Coomassie Brilliant Blue revealed no major changes in the protein profile with differentiation, an observation agreeing with results discussed previously [see **Fig. 4. 2.**].

5. 2. 1. (a). Pertussis toxin.

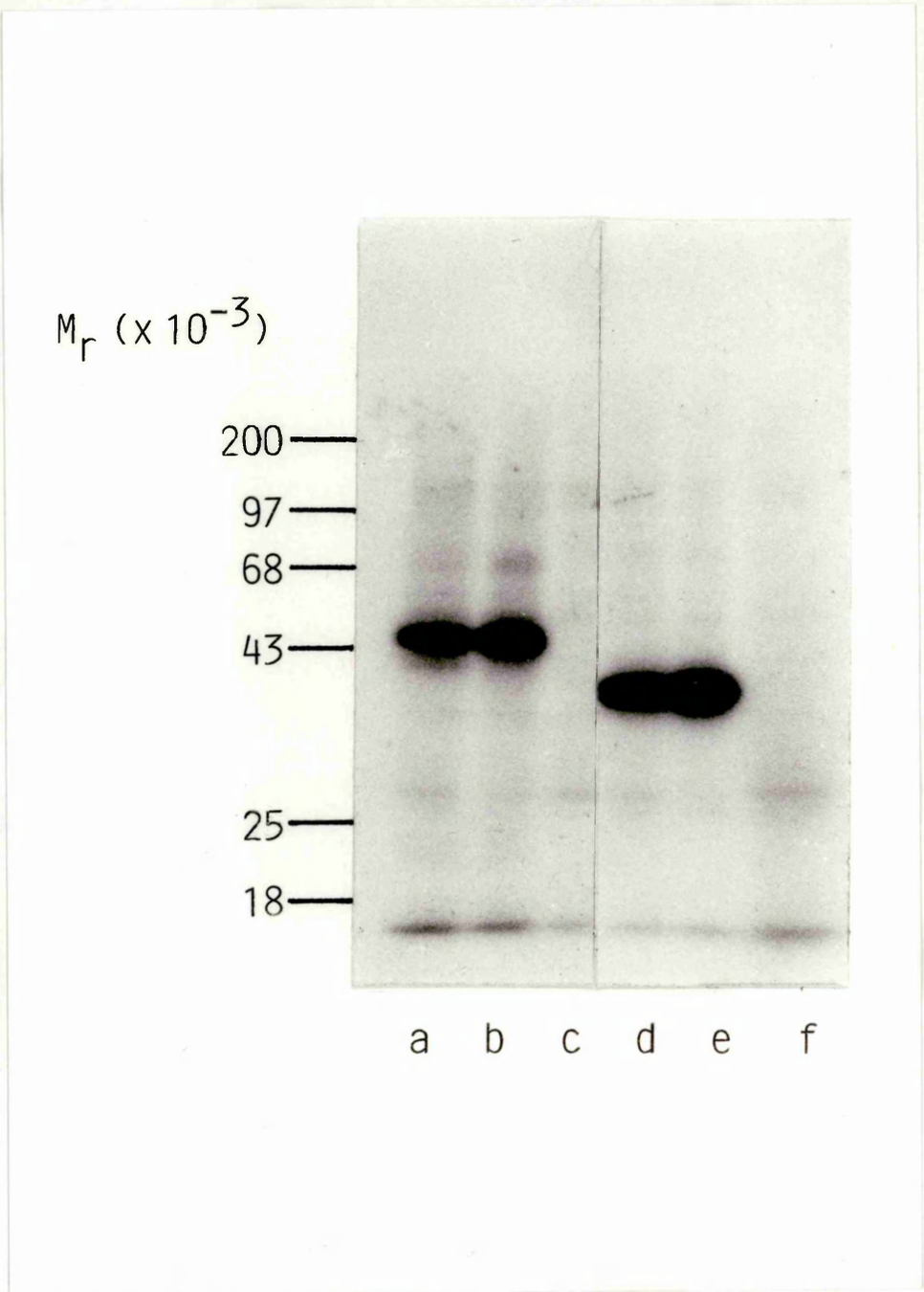
Membranes were treated with [^{32}P] NAD^+ and pertussis toxin and resolved by SDS - PAGE (10 % (w/v) acrylamide). An increase in incorporation of label compared to the control membranes was observed in the differentiated membranes when the gel was subjected to subsequent autoradiography [**Fig. 5. 1.**]. In this

Figure 5. 1.

SDS - PAGE analysis of membranes from control and db cAMP - differentiated NG108 - 15 cells subjected to mono ADP - ribosylation with either cholera or pertussis toxin.

Membranes (25 μg) of control (a,c,d,f) or db cAMP - differentiated (1mM, 6 days) (b,e) NG108 - 15 cells were ADP - ribosylated with [^{32}P] NAD^+ and cholera toxin, (a,b) pertussis toxin, (d,e) or without toxin, (c,f) for 90 min as described in **section 2. 10. 5.** Samples were recovered by deoxycholate / TCA precipitation, resolved using SDS - PAGE (10 % (w/v) acrylamide) and autoradiographed as described in **section 2. 8. 1.** Similar results were obtained in 5 other experiments.

Figure 5. 1.



particular experiment the radioactivity incorporated, upon cutting and counting the appropriate bands, was 753 cpm in the control cells compared to 1233 cpm for the differentiated cells, an increase of some 64 %. This figure agrees well with the increase obtained in previous experiments [see **Table 4. 2.**].

5. 2. 1. (b). Cholera toxin.

Mono ADP - ribosylation of these membranes with cholera toxin also revealed an increase in incorporation of label into membranes of the db cAMP treated cells. Membranes (25 μg) were treated with [^{32}P] NAD^+ and cholera toxin and resolved by SDS - PAGE (10 % (w/v) acrylamide)[**Fig. 5. 1.**]. A broad band was observed at around 45 kDa, the position on the gel where one would expect to find radioactivity incorporated into $G_s\alpha$. In this experiment there were 368 cpm in the control membranes and 500 cpm in the differentiated membranes, an increase of some 36 %. This gave the first indication that modulation of G - proteins other than $G_o\alpha$ may be occuring upon morphological differentiation.

5. 2. 2. Immunological analysis of membranes from control and db cAMP - differentiated NG108 - 15 cells.

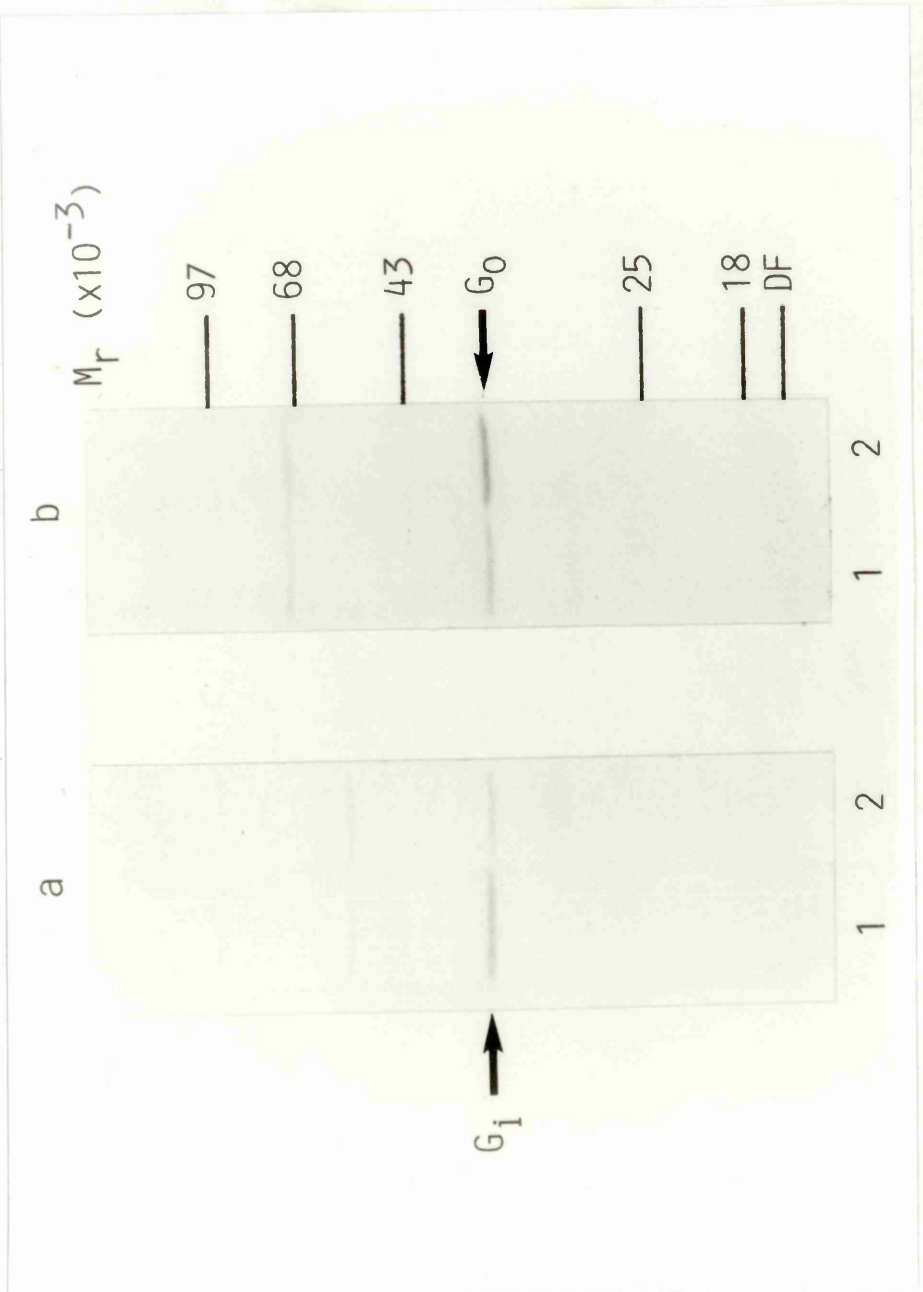
Having previously shown that the associated increase in [^{32}P] ADP - ribosylation by pertussis toxin in membranes from differentiated cells could be accounted for in part by an increase in the levels of $G_o\alpha$. Attempts were made to assess the relevant contributions of the major pertussis toxin substrates, namely the α subunits of G_i2 and G_o, in membranes of control and db cAMP - differentiated cells by using specific antisera which selectively recognize the α subunits of each of these G - proteins.

Figure 5. 2.

Immunoblotting of membranes of control and db cAMP - differentiated NG108 - 15 cells with antisera which discriminate between G_i and G_o.

Membranes (150 μg) from (1) control or (2) db cAMP - differentiated (1 mM, 6 days) NG108 - 15 cells were resolved by SDS - PAGE (10 %(w/v) acrylamide), transferred to nitrocellulose and immunoblotted as described in **sections 2. 12. 1.** and **2. 12. 2.** using either (a) antiserum AS7 (1 : 200 dilution) or (b) antiserum IM1 (1 : 200 dilution). Similar results were obtained in 3 other experiments.

Figure 5. 2.



5. 2. 2. (a). Antiserum IM1.

Membranes from control and db cAMP treated NG108 - 15 cells were resolved by SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum IM1 as the primary reagent. This antiserum, which was raised against a synthetic peptide corresponding to amino acids 22 -35 of the α subunit of G_o, identified a 39 kDa polypeptide which was more abundant in membranes from the differentiated cells [**Fig. 5. 2. (b).**]. This observation confirmed the previous results obtained with the variously treated cells and this antiserum [see **Fig. 4. 9.**]. Densitometric analysis showed that antiserum IM1 immunoreactivity was increased some 4.3 - fold in membranes from differentiated cells, a figure which tallies well with results previously obtained with this antiserum and with the other $G_o\alpha$ antisera [see **Figs. 4. 9. , 4. 10. & 4. 11.**] .

5. 2. 2. (b). Antiserum AS7.

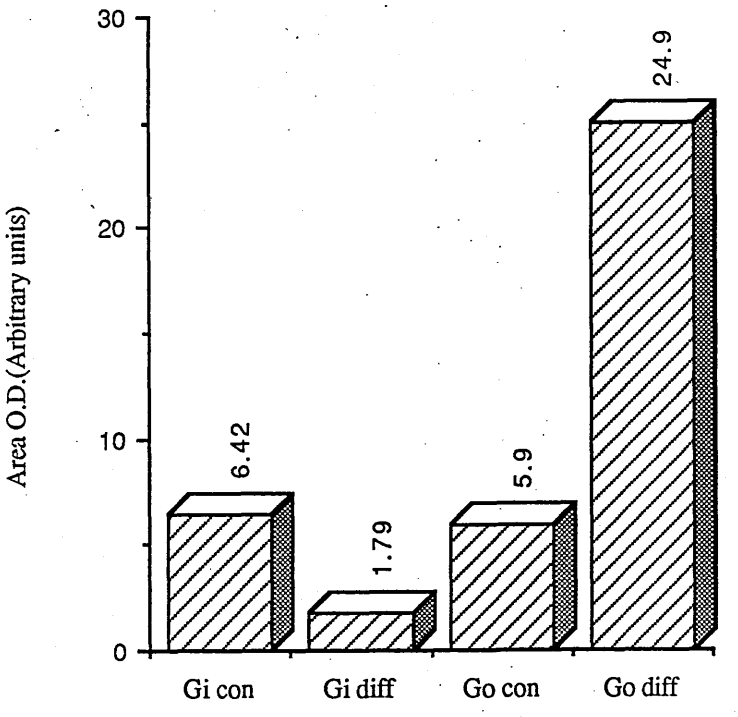
Membranes from control and db cAMP treated NG108 - 15 cells were resolved by SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum AS7 as the primary reagent. This antiserum, which recognizes an epitope within the C - terminal 10 amino acids of the α subunit of G_i1 and G_i2, identified a 40 kDa polypeptide in both control and differentiated membranes. However, staining of this band was more intense in membranes of the control cells, indicating the presence of higher amounts of a form of $G_i\alpha$ in these membranes than in membranes of the db cAMP - differentiated cells [**Fig. 5. 2 (a)**]. Densitometric scanning of **Fig. 5. 2. (a)** indicated that the amount of the α subunit of G_i was some 3 - fold lower in membranes of the differentiated cells in comparison with the control cells [**Fig. 5. 3.**]. In a series of different preparations, the levels of $G_i\alpha$ immunoreactivity with AS7 was decreased by some 2 - 3 fold in membranes from the differentiated cells in comparison with the controls when analysed densitometrically.

Figure 5. 3.

Densitometric analysis of an immunoblot showing membranes from control and dbcAMP - differentiated NG108 - 15 cells with antisera which discriminate between G_i and G_o.

A film positive of the immunoblot shown as **Fig. 5. 2.** was scanned densitometrically as described in **section 2. 12. 4.** on a Bio - Rad 620 video densitometer linked to an Oliveti M24 personal computer. The results are shown graphically with the relevant amounts of each G - protein expressed in arbitrary units in order to give some indication to the extent that differentiation has on the modulation of each particular G - protein.

Figure 5. 3.


Area O.D.(Arbitrary units)
30
20
10
0
6.42
1.79
5.9
24.9
Gi con
Gi diff
Go con
Go diff

5. 2. 2. (c). Antiserum LE2.

Antiserum AS7 was generated against the C - terminal decapeptide of the α subunit of rod transducin [Td1]. This antiserum was able to recognize the predominant form of $G_i\alpha$ in brain, $G_i1\alpha$, because there is only one conservative amino acid change between it and $Td1\alpha$ [Goldsmith *et al.* 1987]. However $G_i2\alpha$ has an identical C- terminal sequence to $G_i1\alpha$ and as such cross - reacts with AS7 to the same extent as $G_i1\alpha$ [Milligan, 1988]. To define the molecular identity of the form of "G_i" it was decided to utilise antiserum LE2 which has been shown to specifically recognize $G_i2\alpha$ but not $G_i1\alpha$. This antiserum was raised against a synthetic peptide sequence which corresponds to amino acids 160 - 169 of the α subunit of a mouse macrophage G_i protein [see **Table 2. 1.**] that has been shown to be identical to $G_i2\alpha$ [Sullivan *et al.*, 1986; Backlund *et al.*, 1988] . Comparison of this sequence with predicted amino acid sequences from cDNA's isolated from rat C6 BU1 glioma cells and human monocytes has shown complete identity in this region [Sullivan *et al.*. 1986; Itoh *et al.*, 1986; Didsbury *et al.*, 1987]. Antiserum LE2 has been shown not to recognize $G_i1\alpha$ from brain but does recognize a pertussis toxin substrate in neutrophils [Goldsmith *et al.*, 1987].

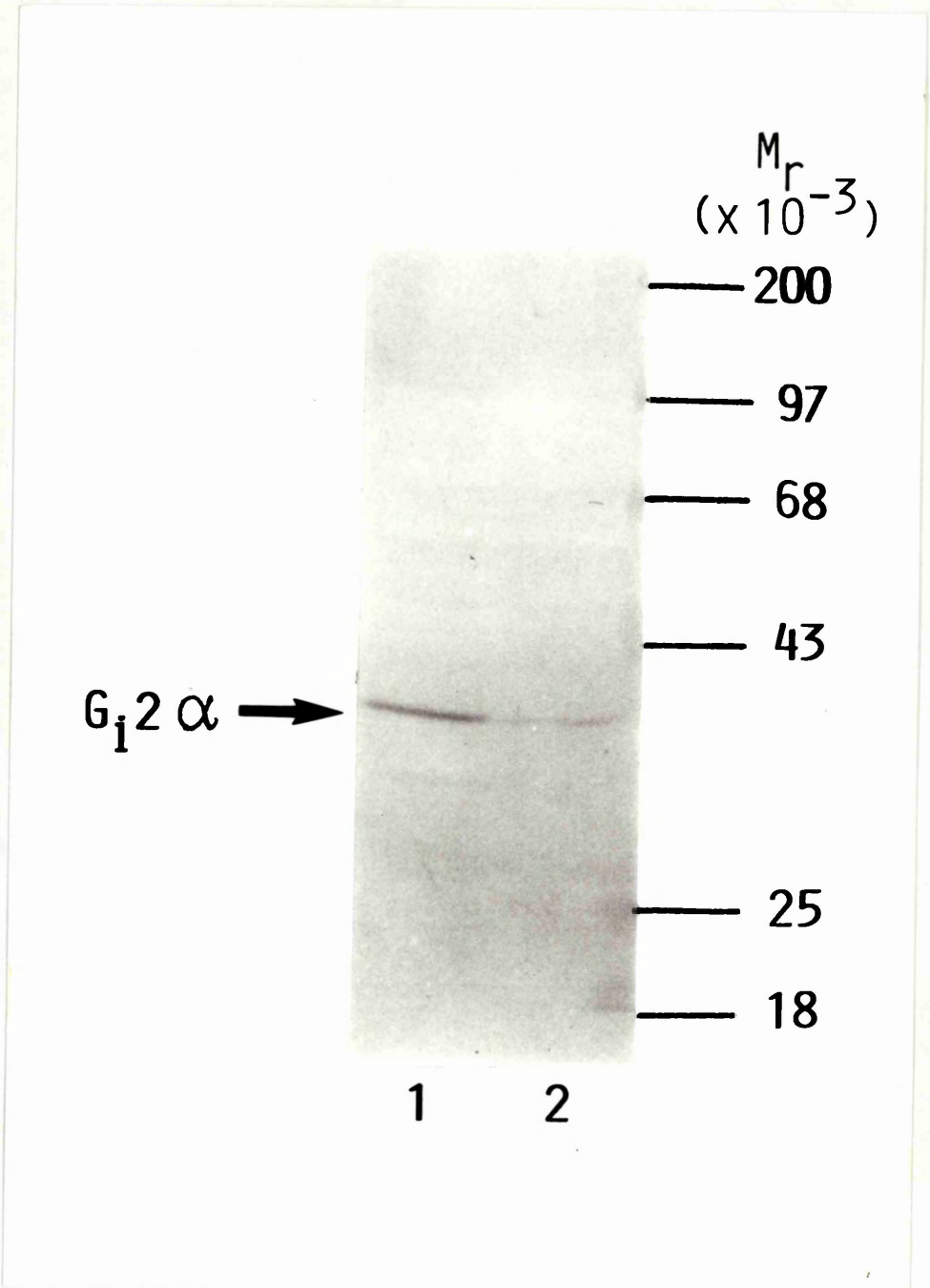
Membranes from control and db cAMP - differentiated cells were resolved on SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted with antiserum LE2 as the primary reagent [**Fig. 5. 4.**]. This antiserum recognized a single polypeptide of some 40 kDa in NG108 - 15 cells. This protein migrated with the same apparent mobility on a 12.5 % (w/v) SDS - PAGE gel as the polypeptide identified by antiserum AS7 in these cells and indeed immunoblotting of these membranes with a mixture of these 2 antisera only revealed a single immunoreactive species [data not shown]. This confirms other reports that $G_i1\alpha$ is either not expressed in these cells or is present in such low abundance that neither the protein nor the mRNA encoding this protein can be detected

Figure 5. 4.

Immunoblotting of membranes of control and db cAMP - differentiated NG108 - 15 cells with antiserum LE2.

Membranes (150 μg) from (1) control or (2) db cAMP - differentiated (1 mM, 6 days) NG108 - 15 cells were resolved by SDS - PAGE (10 % (w/v) acrylamide), transferred to nitrocellulose and immunoblotted as described in **sections 2. 12. 1.** and **2. 12. 2.** using antiserum LE2 (1 : 200 dilution) which specifically recognizes the α subunit of G_i2. Similar results were obtained in 3 other experiments.

Figure 5. 4.



[Milligan, 1988; McKenzie & Milligan, 1990]. Thus antiserum AS7 can be used in these cells as a specific means of detecting $G_i2\alpha$.

When compared to membranes from untreated cells it can be seen that the polypeptide recognized by antiseum LE2 is somewhat less prevalent in db cAMP - differentiated cell membranes [**Fig. 5. 4.**]. Densitometric analysis of this particular immunoblot revealed that the levels of $G_i2\alpha$ decreased to only some 22 % in the membranes of cells differentiated with db cAMP when compared to the controls. This parallels the observations seen in the immunoblots with these membranes using antiserum AS7 [see **Fig. 5. 2.**] and confirms that morphological differentiation of these cells with db cAMP results in differential regulation in the amounts of both the α subunits of G_o and G_i2.

5. 2. 2. (d). Antiserum HPA.

We have already shown, using a specific antipeptide antisera raised against a sequence in the β subunit (antiserum BN1), that differentiation of these cells produces little alteration in the levels of the β subunit [see **Fig. 4. 13.**]. Antiserum HPA was raised by immunization of a rabbit with holomeric G_o purified from bovine brain and as such contains populations of antibodies directed against both the α subunit of G_o and against its associated β subunit. This antiserum was used to confirm those results already obtained with antiserum BN1.

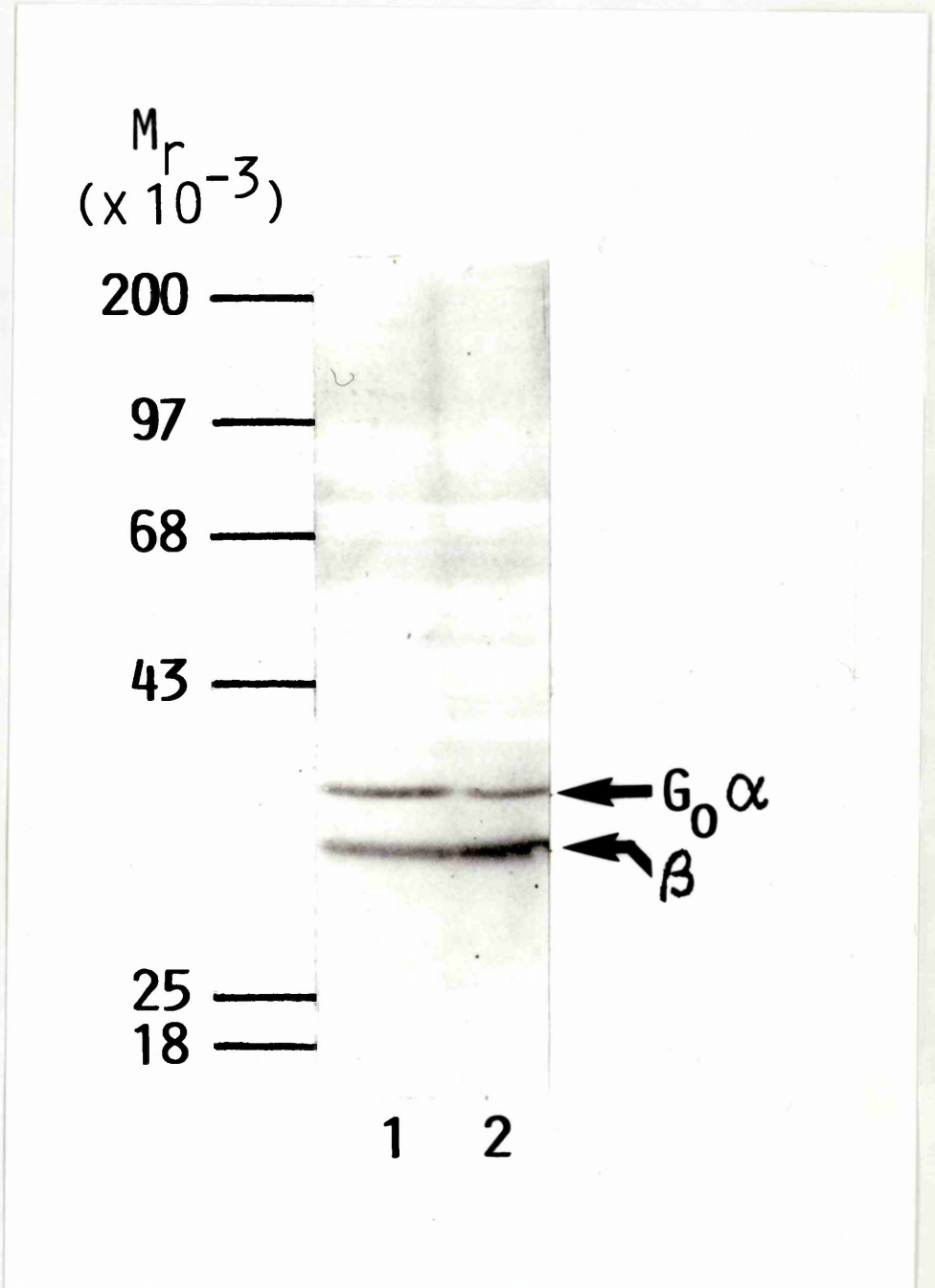
Membranes from control and db cAMP - differentiated cells were resolved by SDS - PAGE (10 % (w/v) acrylamide) immunoblotted with antiserum HPA [**Fig. 5. 5.**]. A single immunoreactive species corresponding to the β - subunit was observed. Little difference in immunoreactivity between the different membranes could be detected, agreeing with the earlier observations with antiserum BN1. As with the

Figure 5. 5.

Immunoblotting of membranes of control and db cAMP - differentiated NG108 - 15 cells with antiserum HPA.

Membranes (150 µg) from (**2**) control or (**1**) db cAMP - differentiated (1 mM, 6 days) NG108 - 15 cells were resolved by SDS - PAGE (10 % (w/v) acrylamide), transferred to nitrocellulose and immunoblotted as described in **sections 2. 12. 1.** and **2. 12. 2.** using antiserum HPA (1 : 200 dilution). Similar results were obtained in 3 other experiments.

Figure 5. 5.



antipeptide antisera against the α subunit of G_o [IM1, OC1, ON1], antiserum HPA demonstrated elevated levels of $G_o\alpha$ in the db cAMP - differentiated cell membranes compared with control cell membranes.

5. 2. 3. Time course of db cAMP - mediated alterations of amounts of $G_o\alpha$ in membranes NG108 - 15 cells.

SDS - PAGE (10 % (w/v) acrylamide) and subsequent immunoblotting with antiserum IM1 of membranes derived from NG108 - 15 cells which had been differentiated with 1mM db cAMP for various times showed that amounts of the α subunit of G_o increased throughout the time period used, with particularly marked increases of $G_o\alpha$ noted between 3 and 4 days after addition of db cAMP [**Fig. 5. 6**]. In contrast, however, amounts of $G_o\alpha$ were not different in untreated cells which were harvested at various times after subculture. This is illustrated in lanes (a) and (b) of **Fig. 5. 6.** where untreated cells harvested at 1 and 6 days after subculture show no obvious change in immunoreactivity with this antiserum.

5. 2. 4. Time course of db cAMP - mediated alterations of amounts of $G_i\alpha$ in membranes NG108 - 15 cells.

Studies on modulation of the levels of $G_i2\alpha$ in response to db cAMP with time demonstrated that amounts of immunoreactive $G_i2\alpha$ fell throughout the period studied [**Fig. 5. 7.**]. As for $G_o\alpha$, alteration in the amounts of $G_i2\alpha$ was strictly dependent on the presence of db cAMP. Growth of NG108 - 15 cells in only 2 % (v/v) foetal bovine serum, a prerequisite for morphological differentiation [Hamprecht *et al.*, 1985], but in the absence of db cAMP did not produce any alterations in the levels of the α subunits of either G_o or G_i2 demonstrating that such alterations were a specific response to the presence of db cAMP [**Figs. 5. 6 & 5. 7.**]. The optimal concentration of db cAMP in order to produce morphological differentiation is 1 mM, at

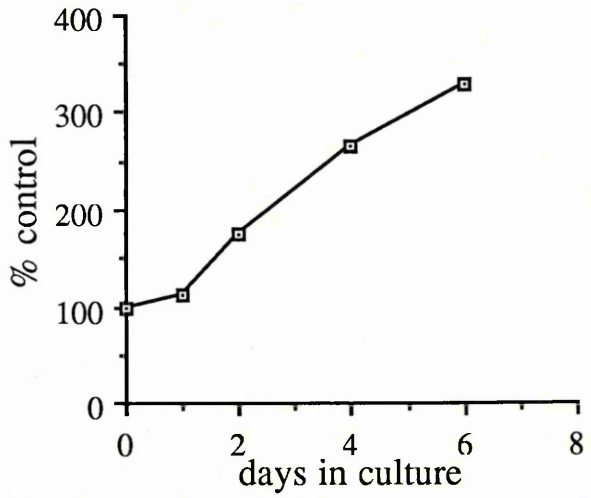
Figure 5. 6.

Time course of db cAMP - mediated alterations of amounts of the α - subunit of G_o in membranes of NG108 - 15 cells.

Antiserum IM1 (1 : 200 dilution) was used as first antibody in an immunoblot following resolution of membranes (150 μg) of NG108 - 15 cells on SDS - PAGE (10 % (w/v) acrylamide). Lane a, untreated cells grown in the presence of 10 % (v/v) bovine foetal serum, 1 day after subculture, lane b; untreated cells 6 days post subculture, lanes c-f, db cAMP-treated cells after 1 (c), 2 (d), 4 (e) and 6 (f) days in the presence of 1mM db cAMP, lane g, untreated cells grown for 6 days in the presence of 2 % (v/v) foetal bovine serum but without db cAMP.

Inset. The results shown as **Fig. 5. 6.** were analysed densitometrically and expressed graphically as a percentage of the control at 0 days.

Figure 5. 6.


400
300
200
100
0
% control
0
2
4
6
8
days in culture

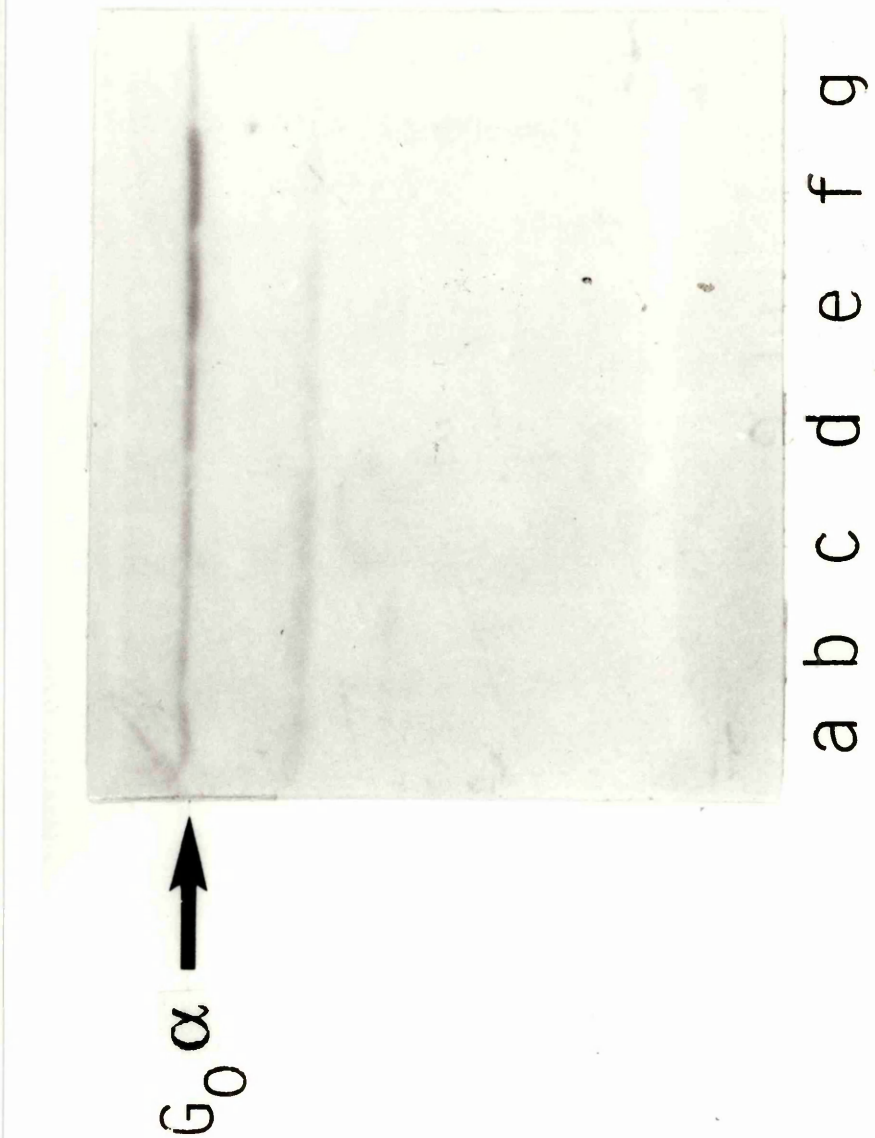

a b c d e f g
$G_O\alpha$

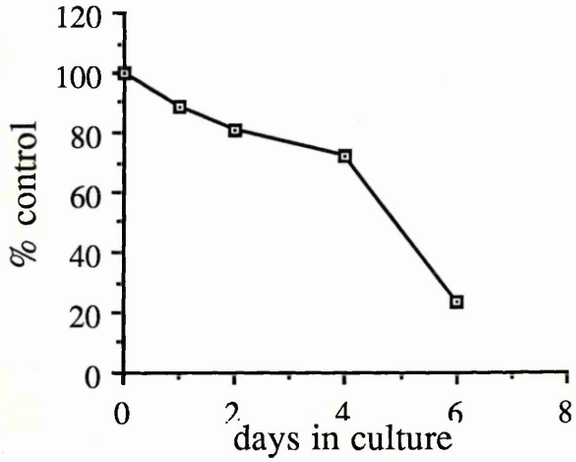
Figure 5. 7.

Time course of db cAMP - mediated alterations in the amounts of the α - subunit of G_i in membranes of NG108 - 15 cells.

Antiserum AS7 (1 : 200 dilution) was used as primary antiserum in an immunoblot following resolution of membranes (150 μg) of NG108 - 15 cells on SDS - PAGE (10 % (w/v) acrylamide) . Lane a, cells grown in the presence of 2 % (v/v) foetal bovine serum but in the absence of db cAMP for 6 days; lanes b-e, cells grown in the presence of 2% (v/v) foetal bovine serum and1mM db cAMP for 6 (b), 4 (c), 2 (d) and 1 (e) days; lane f-g, cells grown in 10 % foetal bovine serum and harvested either 6 (f) or 1 (g) days after subculture..

Inset. The results shown as **Fig. 5. 7.** were analysed densitometrically and expressed graphically as a percentage of the control at 0 days.

Figure 5. 7.



a b c d e f g

0.5 mM it is almost without effect [Hamprecht *et al.*, 1985].

5. 2. 5. The effect of morphological differentiation upon δ - opioid receptor function.

One of the most convenient means to assess the function of a G - protein - linked receptor is to measure changes in the levels of second messenger production caused by activation of the signalling system by ligand. Amongst those readily measured are the production of inositol polyphosphates from receptor - mediated polyphosphoinositide hydrolysis and changes in the concentrations of various ions eg Ca^{2+}, K^{+} brought about by modulation of ion channels [for reviews see Birnbaumer *et al.*, 1987; Litosch, 1990; Dolphin, 1990]. However the most studied is the positive and negative regulation of adenylyl cyclase [Gilman, 1984; Northup, 1985; Gilman, 1987].

Hormonal regulation of adenylyl cyclase manifests itself in the cellular environment as alterations in the levels of intracellular cAMP, changes of which are able to be measured using any of a number of sensitive radioassays.

NG108 -15 cells express a wide variety of cell surface receptors [Hamprecht *et al.*, 1985], a number of which, including those for opioid peptides and α_2 adrenergic agents, are able to mediate the inhibition of adenylyl cyclase [Klee *et al.*, 1985; Sabol & Nirenberg, 1979]. The inhibition by δ opioid agonists requires the presence of both GTP and Na^{+} [Blume *et al.*, 1979] and has been shown to function through a pertussis toxin - sensitive G - protein as prior treatment of the cells with pertussis toxin produces attenuation of the receptor - mediated inhibition [Kurose *et al.*, 1983].

Table 5. 1.

Effects of saturating amounts of the opioid peptide DADLE on forskolin - stimulated adenylyl cyclase activity on membranes from control and db cAMP - differentiated (1 mM, 6 days) NG108 - 15 cells.

Prep	adenylyl cyclase activity			
	Control		db cAMP differentiated	
		+ 10 μM DADLE		+ 10 μM DADLE
1	258	212	328	334
2	190	145	196	210
3	448	382	412	382
4	202	164	290	278
av.	274.5 ± 59	225.8 ± 46.7	306.5± 38.7	301 ±32.1

Membranes (20 μg) of control or db cAMP - differentiated NG108 - 15 cells were incubated for 10 minutes at 30 ^{o}C in the presence or absence of DADLE (10 μM) as described in **section 2. 14.** The inhibition of adenylyl cyclase is expressed as pmol cAMP / min/ mg protein. The data in the table is from individual experiments performed on membranes from 4 separate preparations. Each individual point is expressed as the mean of three replicates and the average value for the 4 preparations represents the mean ± S.E.M.

5. 2. 5. (a). Inhibition of adenylyl cyclase activity by the opioid peptide DADLE is abolished in membranes of db cAMP - differentiated NG108 - 15 cells.

Differences in δ - opioid receptor - mediated inhibition of adenylyl cyclase activity resulting from differentiation were investigated. Measurement of basal activity in membranes was not possible given the sensitivity of the assay used and so in order to measure any inhibition of adenylyl cyclase activity the membranes were stimulated with forskolin, a diterpene compound which directly activates the catalytic domain of the enzyme [Seamon *et al.*, 1981]. The method employed in this study, which measures the amounts of cAMP as an assessment of cyclase activity, was based on the binding - protein assay devised by [Tovey *et al.*, 1974] [see **section 2. 14.**]. This entailed incubation of the membrane with ligand and subsequent measurement of the cAMP levels with a competition assay using a known, fixed amount of the tritium labelled compound and a binding protein which has a high affinity and specificity for cAMP.

Forskolin (100 μM) - stimulated adenylyl cyclase activity in membranes of control (274.5 ± 59.0 pmol / min / mg protein ; mean ± S.E.M., n = 4) and db cAMP - differentiated (306.5 ± 38.7 pmol / min / mg protein ; mean ± S.E.M., n = 4) NG108 - 15 cells was very similar. However this activity was inhibited by a saturating concentration (10 μM) of DADLE by some 21 ± 1 % in the control membranes, whereas opioid - mediated inhibition of adenylyl cyclase was essentially abolished in membranes of differentiated cells (1 ± 1 %)[**Table 5. 1.**]. Statistical analysis by use of student's *t* test for paired samples within each individual experiment indicated that the DADLE inhibition of adenylyl cyclase in control membranes was significant ($P < 0.005$). However, the same analysis of db cAMP - treated membranes revealed no significance ($P > 0.1$). The attenuation of inhibition could be a reflection of the

Table 5. 2.

Maximal stimulation of high affinity GTPase activity in response to the opioid peptide DADLE in membranes of control and db cAMP differentiated (1 mM, 6 days) NG108-15 cells.

	high affinity GTPase activity (pmol/min/mg protein)	
[DADLE]	Control	db cAMP differentiated
0	26.1 ± 3.2	25.2 ± 3.6
1 μM	35.5 ± 3.8	27.2 ± 3.3

Results represent means ± S.E.M. for 6 independent experiments performed with membranes derived from separate cultures.

decrease in the amounts of $G_i2\alpha$, the G - protein demonstrated to be involved in opioid - mediated inhibition of adenylyl cyclase.

5. 2. 5. (b). High affinity GTPase activity stimulated by the opioid peptide DADLE is attenuated in membranes of db cAMP - differentiated NG108 - 15 cells.

NG108 -15 cells have been shown to contain a single subset of opioid receptors of the δ subclass which when activated have been shown to stimulate high - affinity GTPase activity and inhibit adenylyl cyclase [Sharma *et al.*, 1975; Koski & Klee, 1981].

The interaction of agonist with receptor promotes the release of GDP from the relevant G - protein and allows the binding of GTP. This is followed by the subsequent hydrolysis of GTP by the intrinsic GTPase activity of the G - protein. Measurement of this enhanced rate of GTPase activity of a cell in response to agonist thus provides a simple, convenient and direct assessment of the interaction of a G - protein with its particular receptor. Using the assay described by [Cassel & Selinger 1976], alterations in the levels of DADLE - stimulated GTPase activities in membranes from control and db cAMP - treated cells were investigated.

Stimulation of both control and db cAMP differentiated cell membranes with a saturating concentration (1 μM) of the synthetic δ - opioid peptide DADLE produced an elevation over the basal level of membrane high - affinity GTPase activity [**Table 5. 2.**]. There was no change in the basal GTPase activities between control and the db cAMP treated membranes [26.1 ± 3.2 pmol / min / mg protein compared to 25.2 ± 3.6 pmol / min / mg protein]. There was a significant stimulation of GTP ase activity in the control membranes with DADLE [from 26.1 ± 3.2 pmol / min / mg protein to 35.5 ± 3.8 pmol / min / mg protein] However, no significant increase in GTPase activity was

observed in the differentiated membranes upon DADLE stimulation [25.2 ± 3.6 pmol / min / mg protein versus 27.2 ± 3.3pmol / min / mg protein]. Statistical analysis by use of Student's *t* test for unpaired samples indicated that the DADLE stimulation of GTPase activity was significantly greater in the control membranes than in the differentiated membranes ($P = 0.001$). Similar analysis using Student's *t* test for paired samples indicated that within each each individual experiment, DADLE stimulation of membrane high affinity GTPase activity in control cell membranes produced a statistically significant increase ($P < 0.05$). However, response to DADLE in the dbcAMP - differentiated membranes was not significant ($P > 0.1$). Dose response curves of GTPase stimulation in response to DADLE indicated a similar EC_{50} (the concentration of agonist giving 50 % of maximal response) of around 10 nM for the ligand in both the control and the differentiated membranes [**Fig. 5. 8.**].

The loss of ligand - stimulated GTPase activity observed with dbcAMP differentiation of the cells may simply be a reflection of the reduction in the levels of that G - protein which interacts with the receptor, an observation consistant with the attenuation of adenylyl cyclase activity seen in these membranes upon differentiation.

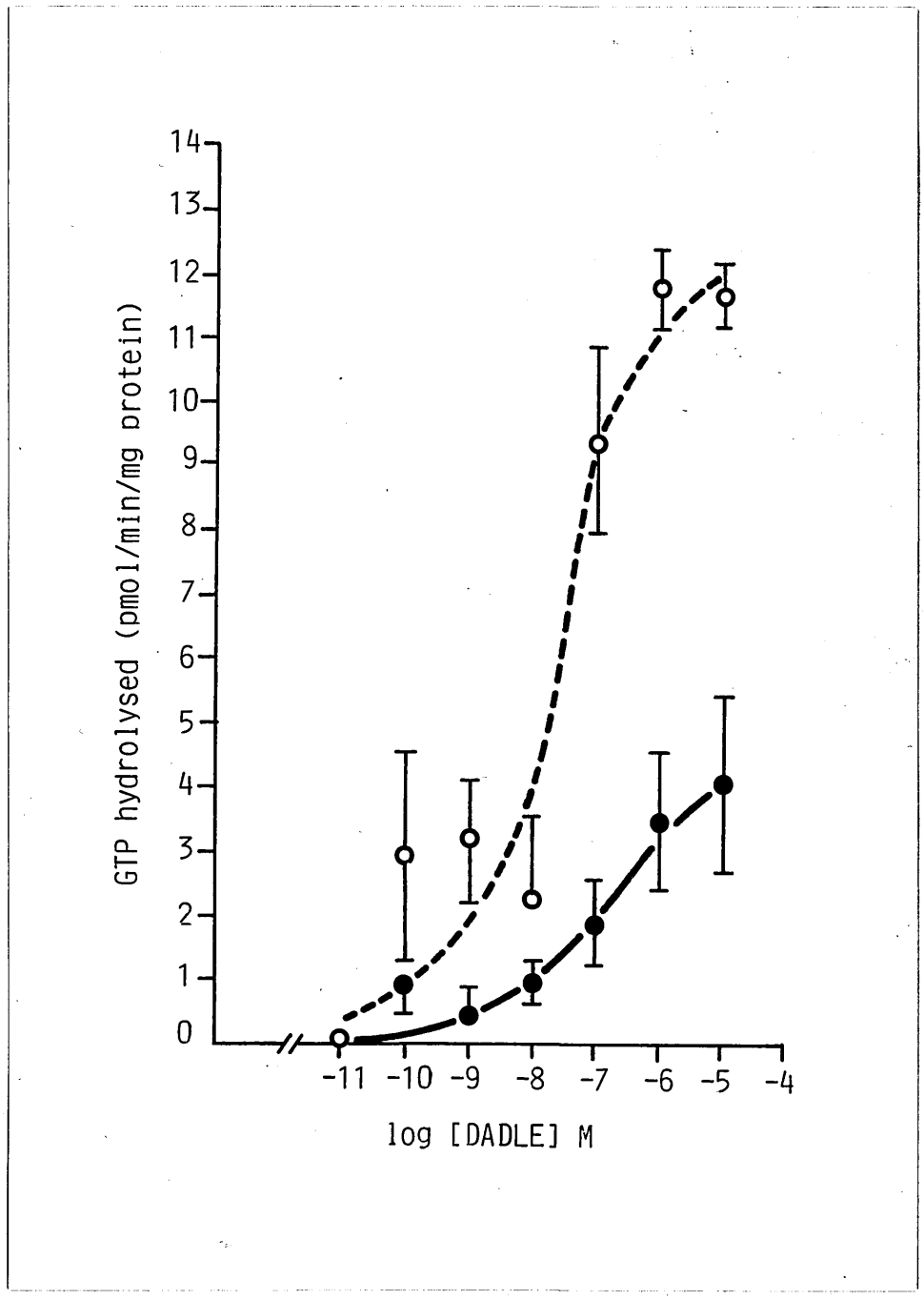
The basal GTPase activity in any membrane system is thought to be a composite of the hydrolysis of GTP by all the G - proteins within that membrane as well as by other enzymic reactions. Thus, the contribution of any particular G - protein to the basal GTPase is dependent on both the amount of that particular G - protein and its rate of GTP hydrolysis. Since there is no difference in the basal GTPase activities between membranes from control and differentiated cells, the contribution to this activity by $G_i2\alpha$ and $G_o\alpha$ is either low, such that alterations in the levels of the G - proteins will have no effect or the fact that these proteins are differentially modulated compensates for any possible changes.

Figure 5. 8.

High affinity GTPase activity stimulated by the opioid peptide DADLE in membranes of control and db cAMP - differentiated NG108 - 15 cells.

The high affinity GTPase activity of membranes (5 μg) from either control cells (o-o) or for those which had been treated for 6 days with db cAMP (1mM) (●-●) was assessed as described in **section 2. 13.** The data is expressed as the stimulation of high affinity GTPase activity by DADLE above that noted for the basal. The data represent means ± S.E.M. derived from 4 separate preparations.

Figure 5. 8.


GTP hydrolysed (pmol/min/mg protein)
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-11 -10 -9 -8 -7 -6 -5 -4
log [DADLE] M

5. 2. 5. (c). Binding of [^{3}H] DADLE to membranes from control and db cAMP - differentiated NG108 - 15 cells.

Although the absolute number of receptors was apt to vary to some degree between different membrane preparations, analysis of saturation - binding studies using [^{3}H] DADLE indicated that membranes from untreated cells contained maximal binding of 173.1± 24.6 fmol bound / mg protein (mean ± S.E.M., n = 4) which decreased to 89.5 ± 13.7 fmol bound / mg protein (mean ± S.E.M., n = 4) upon differentiation [**Table. 5. 3.**]. Thus only some 51.7 % of the number of opioid receptors were present in in membranes of the db cAMP treated cells compared to the control cells in individual sets of membrane preparations

The receptors from both control and differentiated membranes displayed equivalent affinities for [^{3}H] DADLE (control, 2.2 ± 0.3 nM, mean ± S.E.M., n = 5 ; differentiated, 2.8 ± 0.3 nM, mean ± S.E.M., n = 4) [**Fig. 5. 9.**]. No differences were noted in the number of receptors present in individual membrane preparations derived from cells grown in the presence of either 10 % or 2 % (v/v) foetal bovine serum.

Table 5. 3.

The binding of [^{3}H] DADLE to membranes of control and db cAMP differentiated (1 mM, 6 days) NG108 - 15 cells.

Prep	[^{3}H] DALAMID Binding (fmol bound / mg protein)	
	Control	db cAMP differentiated
1	199.5	111.8
2	148	77
3	149	89
4	196	80
av.	173.1 ± 24.6	89.5 ± 13.7

Membranes (150 µg) of control or db cAMP differentiated NG108 - 15 cells were incubated for 20 minutes at 30 ^{o}C with 20 nM [^{3}H] DADLE in the presence or absence of DALAMID (10 µM) as described in **section 2. 15**. The data in the table is from individual experiments performed on membranes from 4 separate preparations and represent the difference between total and non - specific binding and hence are indicative of specific binding. Each individual point is expressed as the mean of two replicates which varied by less than 10 % and the average value for the 4 preparations represents the mean ± S.E.M.

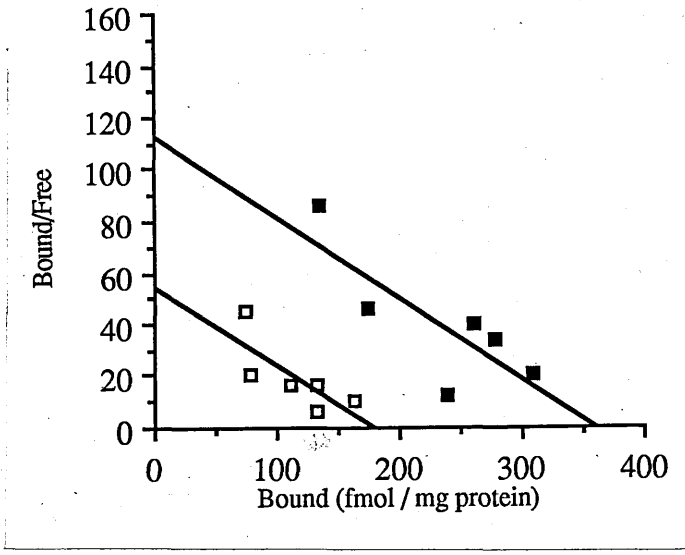
Figure 5. 9.

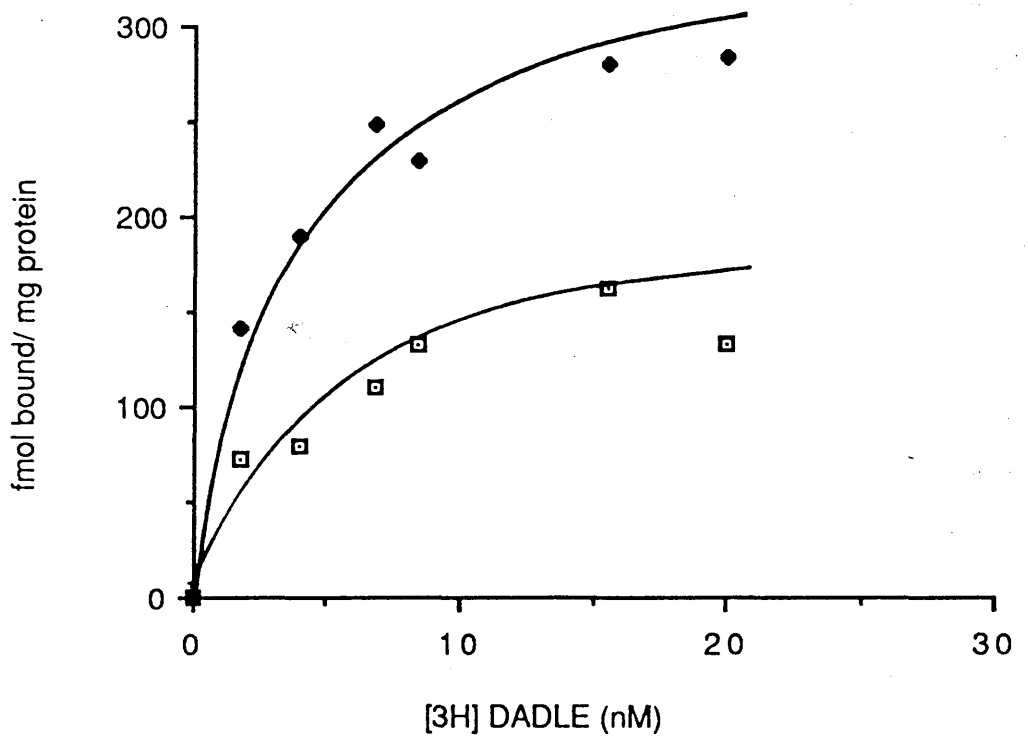
The binding of [^{3}H] DADLE to membranes of control and db cAMP - differentiated NG108 - 15 cells.

Membranes (150 µg), of control (♦-♦) or db cAMP - differentiated (1 mM, 6 days) (◻-◻) NG108-15 cells were incubated with varying concentrations of [^{3}H] DADLE in the presence or absence of DALAMID (10 uM) as detailed in **section 2. 15.** The data in this representative experiment show the difference between total and non-specific binding and hence is indicative of specific binding. Each point is the mean of two replicates which varied by less than 10 %. Similar results were obtained in 3 other experiments.

Inset. The data presented as **Fig. 5. 9.** was subjected to Scatchard analysis. The membranes (150 ug) from control (■) or db cAMP treated (□) NG108 - 15 cells are represented.

Figure 5. 9.


160
140
120
100
80
60
40
20
0
Bound/Free
0
100
200
300
400
Bound (fmol / mg protein)


300
200
100
0
fmol bound/ mg protein
0
10
20
30
[3H] DADLE (nM)

5. 3. Discussion

The results presented in this chapter demonstrate that morphological differentiation of NG108 - 15 cells with db cAMP is accompanied not only by an increase in the levels of $G_o\alpha$ but also by a decrease in the levels of $G_i2\alpha$. These changes were concommitant with alterations in the signal transducing capacity of the δ - opioid receptor as measured by its ability to interact with both its corresponding G - protein and effector.

The responsiveness of both control and db cAMP - treated membranes to opioid peptides as assessed by either interaction with G - protein [GTPase activity] or more distally with the adenylyl cyclase effector system was examined. In both of these cases the maximal effect of the ligand at saturating concentrations was markedly lowered, if not completely abolished, in the treated cell membranes in comparison to those from the untreated cells. There was no marked difference in the concentrations of the agonist required to produce half - maximal effects. However, as the number of opioid receptors present in the differentiated membranes was lower than in the control cells by some 50 %, it is not possible to assess with any certainty whether the alteration in receptors or the decrease in the amounts of $G_i2\alpha$ was primarily responsible for functional changes caused by differentiation. The observed loss of receptor was somewhat suprising since Moses & Snell reported that differentiation of NG108 - 15 cells with db cAMP resulted in a three fold increase in δ- opioid receptor density [Moses & Snell, 1974]. However other studies have indicated that receptor expression in cultured cells can be modulated by agents that induce cellular differentiation [Noronha - Blob *et al.*, 1986; Zhu & Chang, 1988; Monsma *et al.*, 1990].

Cellular responses to many hormones and neurotransmitters decline rapidly despite continuous exposure of cells to these stimuli. This waning of the stimulated response in the face of continuous agonist stimulation has been termed desensitization.

Most investigators have focussed on the β - adrenergic receptor, through which the stimulatory actions of catecholamines on adenylyl cyclase are mediated. A major mechanism for desensitization of adenylyl cyclase appears to be feedback regulation of the receptors via phosphorylation by protein kinase A. Studies with avian erythrocytes have shown a close correlation between catecholamine - induced desensitization and β - adrenergic receptor phosphorylation [Stadel *et al.*, 1983; Stadel *et al.*, 1986; Sibley *et al.*, 1984]. These findings were taken further when it was demonstrated that the purified hamster lung β - adrenergic receptor is stoichiometrically phosphorylated by the cAMP - dependent protein kinase predominantly on two serine residues and that receptor phosphorylation directly inhibited its coupling to $G_s\alpha$ [Benovic *et al.*, 1985].

Protein kinase C is also able to phosphorylate the β - adrenergic receptor *in vitro* , albeit to a lesser stoichiometry than protein kinase A [Bouvier *et al.*, 1987]. Stimulation of certain cells with 12 - *O* - tetradecanoyl 13 - acetate (TPA), a phorbol ester that activates protein kinase C, has been demonstrated to influence hormone - sensitive adenylyl cyclase. In turkey erythrocytes TPA induces desensitization of β - adrenergic receptor - mediated stimulation of adenylyl cyclase. Such treatment had no effect on receptor number but was accompanied by a three - fold increase in phosphorylation of the β - adrenergic receptor [Kelleher *et al.*, 1984].

On the other hand, in wild type S49 lymphoma cells, treatment with TPA resulted in an increase in agonist - stimulated, as well as basal, guanine - nucleotide - and fluoride - stimulated adenylyl cyclase activities [Bell *et al.*, 1985]. These results suggested that phorbol ester facilitated the productive interaction of $G_s\alpha$ with adenylyl cyclase presumably through protein kinase C. Recently Benovic and coworkers have identified a novel cAMP - independent kinase that appears to be involved in desensitization and phosphorylation of the β - adrenergic receptor [Benovic et al., 1986]. This cytosolic enzyme, termed the β - adrenergic receptor kinase (βARK)

appears to be ubiquitous in mammalian tissues so far examined and has the unusual property that it phosphorylates only the agonist - occupied form of the receptor.

The δ - opioid receptor from NG108 - 15 cells has been purified to apparent homogenity as a glycosylated protein with a M_r of around 58 kDa on SDS - PAGE [Simonds *et al.*, 1985]. Preliminary results from cloning experiments of δ - opioid receptors indicate that this receptor and the μ - opioid receptor may be products of the same gene. [Cabon *et al.*, 1988]. However, no complete sequences derived from cDNA sequences have been reported for any of the classes of opioid - receptor and as such little can be stated about any possible covalent modification that they may undergo.

It has been demonstrated that treatment with TPA can produce phosphorylation of the catalytic subunit of adenylyl cyclase in frog erythrocytes and, moreover, purified protein kinase C can directly phosphorylate, *in vitro*, the catalytic subunit of adenylyl cyclase purified from bovine brain suggesting that phosphorylation of adenylyl cyclase by protein kinase C may be involved in the phorbol ester - induced enhancement of adenylyl cyclase activity [Yoshimasa *et al.*, 1987]. A cDNA encoding an adenylyl cylase from bovine brain has recently been cloned [Krupinski *et al.*, 1989]. The cDNA predicts an 1134 amino acid protein with an internally repeated structure and multiple transmembrane spanning regions. The protein also contains a single concensus motif for protein phosphorylation by protein kinase C and another for protein kinase A.

Katada and coworkers were first to demonstrate the phosphorylation of a G - protein α - subunit when they demonstrated that a solubilized purified G_i preparation was a substrate for protein kinase C [Katada *et al.*, 1985]. More recently, both the purified human insulin receptor kinase and protein kinase A have been shown to phosphorylate the α subunit of purified solubilized G_i preparations [O'Brien *et al.*,

1987; Krupinski *et al.*, 1988; Watanabe *et al.*, 1988]. In contrast, Pyne and coworkers were able to demonstrate that $G_i\alpha$ can be phosphorylated in intact hepatocytes treated with either TPA or glucagon but not with insulin [Pyne *et al.*, 1989]. This G - protein was subsequntly identified as the α subunit of G_i2 using immunoprecipitation techniques with specific antisera [Bushfield *et al.*, 1990].

Attenuation of opioid receptor activity can be achieved in NG108 - 15 cells with pharmacological manipulation. Chronic exposure of these cells to opioid agonists results in a loss of the receptor - mediated inhibition of adenylyl cyclase [Law *et al.*, 1983] and incubation of the cells with phorbol ester abolished up to 45 % of the opioid - mediated inhibition of adenylyl cyclase with a loss of up to 40 % of the receptor itself [Louie *et al.*, 1990] thus it would appear that changes in receptor number are in themselves enough to produce functional changes.

A number of previous studies have shown that under certain conditions such as differentiation and in some pathological states, modulations in the levels of some G - proteins can occur [see **chapter four**]. However information about what effects these changes have on the interaction of that G - protein with its receptor and / or effector has not been forthcoming. More information is known about the consequences of these changes upon the complete signalling processes.

Differentiation of 3T3 - L1 cells from fibroblasts to adipocytes is accompanied by increased responsiveness of adenylyl cyclase to lipolytic agents [Rubin *et al.*, 1977]. Gierschik and coworkers were able to demonstrate that this differentiation is associated with alterations in the levels of G_i and G_o [Gierschik *et al.*, 1986a]. Neer and colleagues showed that carbachol, a muscarinic agonist, was able to increasingly inhibit adenylyl cyclase in embryonic chick heart from 2.5 days in ovo until 3 days post hatching, a phenomenon which was primarily a result of the maturation of the muscarinic receptors. However it should be noted that differentiation of these cells was

accompanied with a 2 - 3 fold increase in the levels of $G_o\alpha$ [Liang *et al.*, 1986].

It is well established that activation of opioid receptors of the δ - subtype in NG108 - 15 cells results in an inhibition of adenylyl cyclase activity [Koski & Klee, 1981]. Since this inhibition can be correlated with opioid - stimulation of high - affinity GTPase and because the affinity of binding of opioid ligands to this receptor is reduced in the presence of non - hydrolysable GTP analogues, the effects of the ligand must be transduced via a G - protein. Also, since pretreatment of these cells with pertussis toxin results in the attenuation of both the receptor - mediated stimulation of GTPase activity [Burns *et al.*, 1983] and inhibition of adenylyl cyclase [Kurose *et al.*, 1983], then the G - protein(s) involved must be a substrate for the toxin.

Using a monoclonal antibody which has been epitope mapped to a region close to the C - terminus of rod outer segment Tdα, Hamm and colleagues were able to block the interaction between rhodopsin and transducin [Hamm *et al.*, 1987; Deretic & Hamm, 1987]. This work was extended when it was shown that this same antibody could block G_i - mediated inhibition of adenylyl cyclase [Hamm *et al.*, 1989] thus confirming the prediction from the theoretical model put forward by Bourne and coworkers that the C - terminus of the G - protein is the site of coupling to its receptor [Masters *et al.*, 1986].

NG108 - 15 cells have recently been shown to contain three pertussis toxin - sensitve G - proteins, namely the α subunits of G_i2, G_i3 and G_o [McKenzie & Milligan, 1990]. A recent study has shown that preincubation of NG108 - 15 membranes with antiserum AS7, which specifically recognises the C - terminal decapeptide of $G_i2\alpha$, attenuated the effects of the δ- opioid receptor resulting in an abolition of both DADLE stimulation of GTPase activity and DADLE mediated inhibition of adenylyl cyclase [McKenzie & Milligan, 1990]. Incubation with the other

available C - terminal anti - G - protein antisera had no effect on either of these parameters implying that $G_i2\alpha$ is the transducing element between this receptor and adenylyl cyclase. Further evidence to support this conclusion was obtained as pretreatment of NG108 -15 cell membranes with the IgG fraction of antiserum AS7 reduced the affinity of the [^{3}H] DADLE for the opioid receptor, this reduction being the same as that produced by either GppNHp or by pretreatment with pertussis toxin, both of which prevent interaction of receptors with pertussis - toxin sensitive G - proteins.

In similar studies, Simonds and coworkers were able to show that the $\alpha2$ - adrenergic inhibition of adenylyl cyclase in platelet membranes was blocked by pretreatment with C- terminal antibodies reactive to $G_i2\alpha$ but not with antibodies to $Gi3\alpha$ or $G_{(x)z}\alpha$ [Simonds *et al.*, 1989] and the affinity of binding of agonists to the $\alpha2_B$ adrenergic receptor of NG108 - 15 cells is reduced by antiserum AS7 [McClue & Milligan, 1990].

Unfortunately it was not possible to use these techniques directly to investigate whether differentiation results in a switch in receptor interaction from G_i2 to G_o.The reduction in DADLE - stimulated GTPase noted in membranes from db cAMP - differentiated cells made antibody uncoupling experiments, such as those described by McKenzie & Milligan, impossible to perform, simply because no effect would have been seen in differentiated membranes [McKenzie & Milligan, 1990].The other approach of using the antibodies to reduce the affinity of the receptor for its ligand could not be used since the reduction in receptor number observed upon differentiation made it difficult to see the small shift in the affinity which would result.

Following cAMP - induced differentiation of these cells it is possible to measure an $\alpha2$ - adrenergic receptor - mediated depression of a voltage - dependent calcium current [Docherty & McFadzean, 1989]. This response is abolished by pretreatment of

these cells with pertussis toxin, implying the involvement of a pertussis toxin - sensitive G - protein in transducing this signal. More recently, McFadzean and coworkers have microinjected prostaglandin E_1- differentiated NG108 - 15 cells with the antipeptide antisera raised the C - terminal sequences of $G_i2\alpha$, $G_i3\alpha$ and $G_o\alpha$, namely AS7, I3B and OC1. The effects of each antiserum on adrenaline - induced inhibition of the current was assessed. Adrenaline, at maximally effective concentrations, was able to depress the Ca^{2+} current by some 26 %. Intracellular injection of antiserum OC1 essentially abolished the $\alpha 2$ - adrenergic inhibition whereas the other antisera had no effect thus providing strong evidence for the involvement of $G_o\alpha$ in the regulation of Ca^{2+} channel function [McFadzean *et al.*, 1989]. Similar conclusions had been reached by Hescheler *et al.* who were able to reconstitute the α subunits of G_i and G_o, which had been purified from bovine brain, into differentiated NG108 - 15 cells which had been pretreated with pertussis toxin. They were able to demonstrate a reconstitution of opioid - mediated depression of Ca^{2+} current and that the $G_o\alpha$ fraction was 10 times as effective as $G_i\alpha$ [Hescheler *et al.*, 1987]. It should be noted that since the G - proteins were purified from brain then the G_i was probably largely G_i1 which still has its function to be clearly defined, also, since the purification of G - proteins is extremely difficult the possibility of other contaminating G - protein α subunits cannot be discounted.

Upon differentiation of NG108 - 15 cells there would appear to be a change in δ - opioid function from inhibition of adenylyl cyclase in the undifferentiated cell to modulation of Ca^{2+} currents in cells in the differentiated state. However this could simply be a reflection of receptor loss and not primarily due to modulation of the G - proteins. It is tempting to speculate however, that it is the respective amounts of a particular G - protein which would dictate the defined transducing pathway for that receptor. NG108 -15 cells appear to only contain one subset of opioid receptors, namely the δ - receptor. The other opioid subsets can themselves be further subdivided into subgroups. The μ - opioid receptor consists of two such subtypes, μ_1 and μ_2, that

can be distinguished pharmacologically [Pasternak, 1986] and the κ_1 - opioid receptor subtype differs from κ_2 in its ability to selectively bind benzeneacetamidine [Zukin *et al.*, 1988]. Although diversity of the δ - opioid receptor has yet to be established the possibility of the existance of multiple forms cannot be discounted. Thus, two distinct opioid receptors with pharmacological characteristics of the δ - subtype, each interacting via different G - proteins, would be able to produce distinct effects within a particular cell. This could certainly be the case with the altered effects of the $\alpha 2$ - adrenergic ligands noted with differentiation in the NG108 -15 cells. Two distinct $\alpha 2$ - adrenoreceptors, $\alpha 2_A$ and $\alpha 2_B$, have been pharmacologically characterized on the basis of ligand binding [Bylund, 1985]. Purification of the isoforms from human platelet and neonatal rat lung revealed that they differed both in apparent molecular weight and in the number or type of their associated oligosaccharide moieties suggesting distinct gene products [Lanier *et al.*,1988]. Molecular characterization of these two receptors has recently been performed, these studies show that although the receptor isoforms display some heterogeneity in primary structure there is some 77 % agreement in what are the putative transmembrane domains [Kobilka *et al.*, 1987; Zeng *et al.*, 1990].

The effects of dopamine on pituitary D_2 receptors were first assumed to be mediated solely by the inhibition of adenylyl cyclase, thus subsequently inhibiting the release of prolactin [Cote *et al.*, 1984]. However recent evidence has questioned the role of cAMP in these dopamine - mediated effects since they appear to be associated with Ca^{2+} mobilization, [Delbeke & Dannies, 1985] a phenomenon that is probably mediated by dopaminergic inhibition of phosphatidylinositol turnover in pituitary tissues [Baudry *et al.*, 1986; Enjalbert *et al.*, 1986]. Moreover D_2 receptors have been show to activate K+ channels in rat corpus striatum [Freedman & Weight, 1988] and rat pituitary [Israel *et al.*, 1985] and dopamine has been shown to inhibit a Ca^{2+} current in snail neurons [Harris - Warrick *et al.*, 1988]. Thus D_2 receptors appear to

be functionally coupled to the inhibition of adenylyl cyclase, to the attenuation of phospholipase C and to the regulation of various ion channels. Senogles *et al.* were able to show that partial purification of the D_2 receptor by affinity chromatography resulted in the co - purification of two associated approximately 40 kDa pertussis toxin substrates [Senogles *et al.*, 1987] and more recently that the α - subunit of G_i2 appeared to couple with the D_2 receptor with a 10 fold higher affinity than any of the other G - protein α subunits tested [Senogles *et al.*, 1990]. Two forms of the D_2 receptor have been cloned from a number of cDNA sources including rat brain [Bunzow *et al.*, 1988; Giros *et al.*, 1989; Monsma *et al.*, 1989], rat pituitary [Eidne *et al.*, 1989] and human pituitary [Dal Toso *et al.*, 1989] cDNA libraries. The longer form of the cDNA includes an additional 87 base pair insertion in the coding region and hence encodes an additional 29 amino acids in the functionally important third cytoplasmic loop. It is this loop which is one of the important sites for the coupling of a G - protein to its receptor. In the case of the β - adrenergic receptor, deletions or substitutions in the corresponding loop area tend to diminish or abolish G - protein activation [O'Dowd *et al.*, 1988]. Mutational analyses of this kind will prove invaluable in the determination of G - protein specificity with the dopamine D_2 receptor and indeed with all other G - protein - linked receptors.

Chapter 6.

IDENTIFICATION OF 2 DISTINCT ISOFORMS OF THE GUANINE NUCLEOTIDE - BINDING PROTEIN $G_o\alpha$ IN NG108 - 15 CELLS AND THEIR INDEPENDENT REGULATION DURING cAMP - INDUCED DIFFERENTIATION.

Chapter 6.

IDENTIFICATION OF 2 DISTINCT ISOFORMS OF THE GUANINE NUCLEOTIDE - BINDING PROTEIN $G_o\alpha$ IN NG108 - 15 CELLS AND THEIR INDEPENDENT REGULATION DURING cAMP - INDUCED DIFFERENTIATION.

6. 1. Introduction.

It was shown in **chapter 4.** that morphological differentiation of NG108 - 15 cells with any of a number of pharmacological agents,which act by a common mechanism of raising intracellular cAMP, is accompanied by an increase in the levels of $G_o\alpha$ as assessed by ADP - ribosylation by pertussis toxin and by immunoblotting with a series of specific antipeptide antisera raised against various regions of $G_o\alpha$.

Four pertussis toxin - substrates have recently been purified from bovine brain using high resolution Mono - Q anion - exchange chromatography [Goldsmith *et al.*, 1988a]. Comparison of these polypeptides with cloned cDNAs encoding putative α subunits using either specific anti - peptide antisera or their migrations on two - dimensional SDS - PAGE identifed the α subunits of G_o, G_i1 and G_i2. The fourth protein was a novel G -protein of slightly above 39 kDa which had a more basic pI (6.0 against 5.6) and was indistinguishable from $G_o\alpha$ on immunoblots with a number of specific antisera. It was concluded that this protein, termed $G_o\alpha$*, could represent a novel $G_o\alpha$, although it is unclear at the present time if these proteins are the products of distinct genes, stem from differential splicing of nuclear RNA transcribed from a single gene or represent covalently modified forms of the same peptide.

One - dimensional SDS - PAGE analysis revealed only a single immunoreactive

species of $G_o\alpha$ in NG108 - 15 cells. The experiments described in **chapter 6** investigate the possibility of multiple forms of immunoreactive G_o in these cells using two - dimensional gel analysis.

6. 2. Results.

6. 2. 1. Analysis of $G_o\alpha$ in membranes of NG108 - 15 cells by two - dimensional immunoblotting reveals two isoforms which are differentially modulated upon morphological differentiation.

Membranes from control NG108 - 15 cells and from cells differentiated by treatment for 6 days with each of dibutyryl cAMP (1 mM), 8-bromo cAMP (1 mM), prostaglandin E_1 (10 μM) or forskolin (10 μM) were subjected to 2 - D SDS - PAGE analysis. The membranes were first solubilized for 1 hr at 4 ^oC with a buffer containing 1 % (w/v) nonidet P - 40 in order to extract the G - proteins from the membranes. The solubilized fraction was then subjected to an isoelectric focussing first dimension followed by conventional SDS - PAGE (10 % (w/v) acrylamide) and subsequent immunoblotting [**sections 2. 11. & 2. 12.**].

Resolution of membranes on such a system indicated that there was no gross alteration in the relative amounts of the most prevalent polypeptides brought about by differentiation. **Fig. 6. 1.** shows a silver stained gel from control (**a**) and db cAMP - differentiated (**b**) membranes resolved using such a 2 - D strategy. All of the other differentiating treatments produced similar silver staining profiles (results not shown).

Fig. 6. 2. shows membranes from control and db cAMP - differentiated which had been subjected to resolution with 2 - D SDS - PAGE (10 % (w/v) acrylamide) and subsequent immunoblot analysis with antiserum OC1. In contrast to the 1 - D analysis of these membranes [see **chapter 4**], which revealed only one immunoreactive band, immunoblotting of 2 - D gels using antiserum OC1 identified distinct immunoreactive species of some 39 kDa with isoelectric points of 5.5 and 5.8.

Figure 6. 1.

Two - Dimensional SDS - PAGE analysis of membranes from control and db cAMP - differentiated NG108 - 15 cells.

Membranes (150 μg) from control (a) and db cAMP - differentiated cells (b) were solubilized for 1 hour at 4 ^{o}C and resolved by 2 - D SDS - PAGE (10 % (w/v) acrylamide) as described in **section 2. 11.** The resultant gels were silver stained as in **section 2. 9. 2.** and the positions of marker proteins with known molecular weights are indicated

Figure 6. 1.

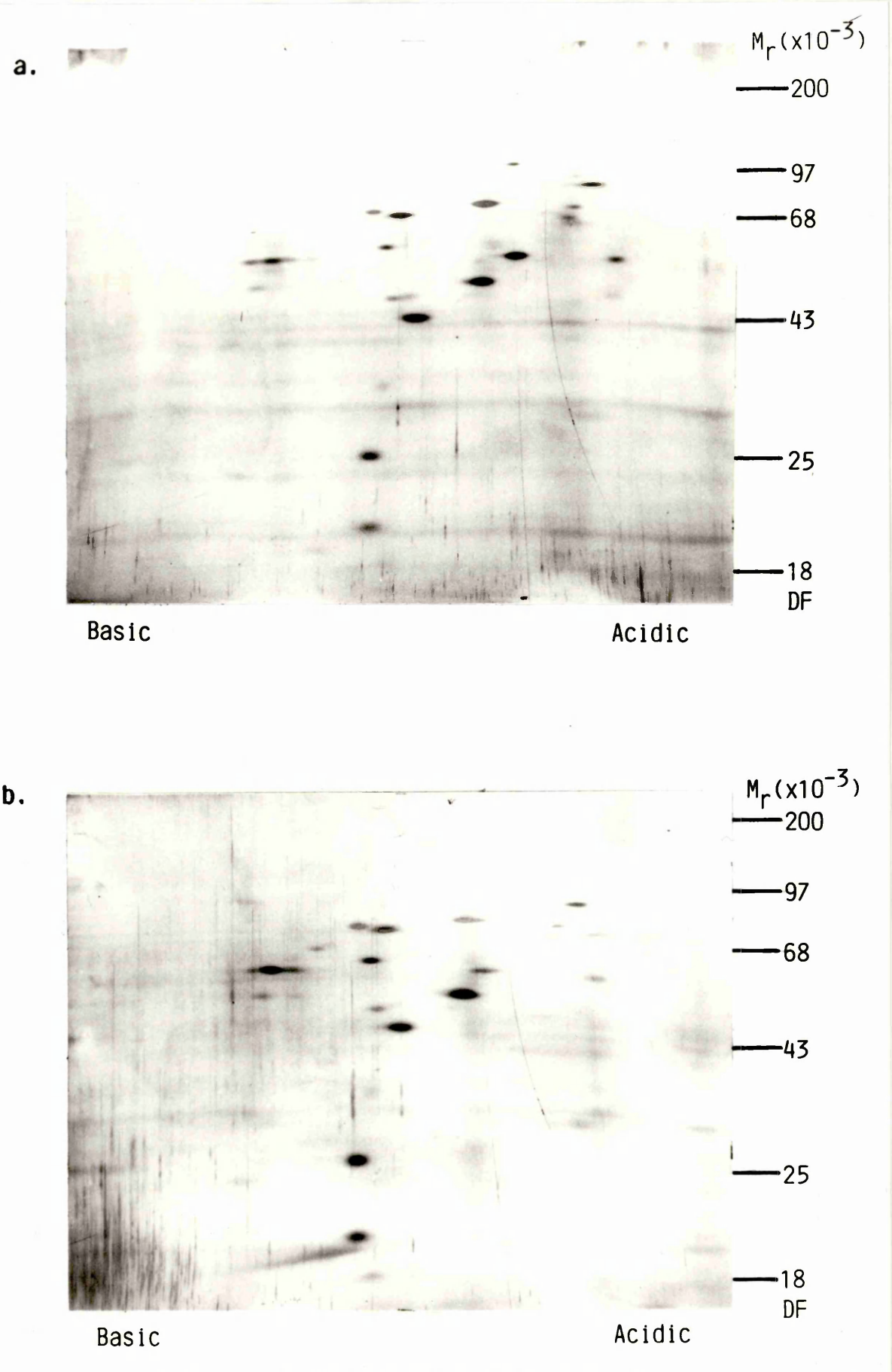
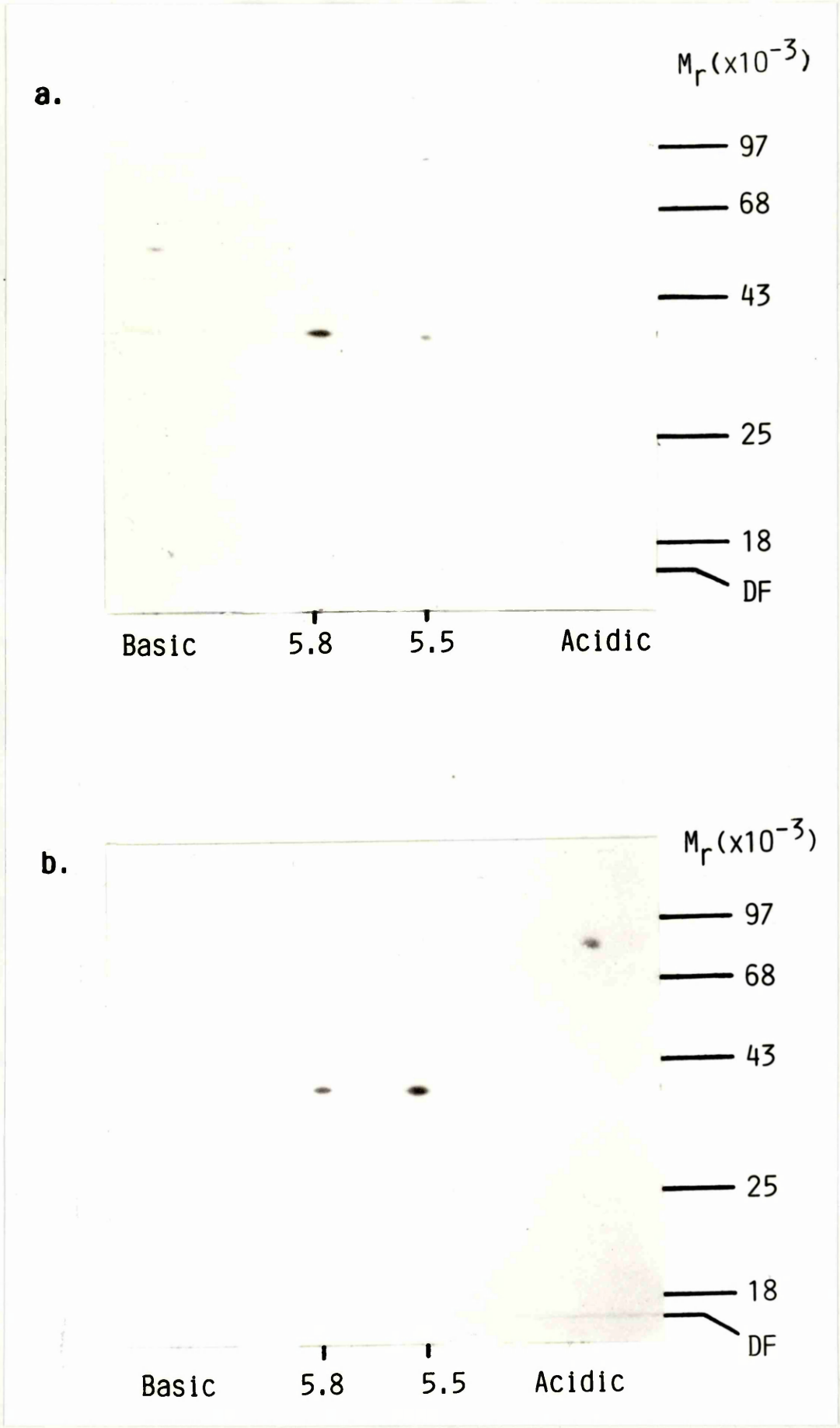

a.
M_r (x10^{-3})
200
97
68
43
25
18
DF
Basic
Acidic
b.
M_r (x10^{-3})
200
97
68
43
25
18
DF
Basic
Acidic

Figure 6. 2.

Two - Dimensional immunoblot analysis of membranes from control and db cAMP - differentiated NG108 - 15 cells with antiserum OC1 to detect multiple isoforms of $G_o\alpha$.

Membranes (150 µg) from control (a) and db cAMP - differentiated cells (b) were solubilized for 1 hour at 4 ^oC, resolved by 2 - D SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum OC1 (1 : 200 dilution) as described in **section 2. 11. & 2. 12.** Similar results were obtained in 3 other experiments.

Figure 6. 2.


a.
$M_r(x10^{-3})$
97
68
43
25
18
DF
Basic
5.8
5.5
Acidic
b.
$M_r(x10^{-3})$
97
68
43
25
18
DF
Basic
5.8
5.5
Acidic

Greater immunoreactivity was observed routinely for the more basic form of $G_o\alpha$ in membranes from control cells [**Fig. 6. 2. (a)**] and, assuming that the antiserum identifies these two polypeptides equally, this suggested that the basic isoform was present in higher amounts in these cells. Immunoblots of db cAMP - differentiated cells also shows the presence of two specific immunoreactive 39 kDa polypeptides [**Fig. 6. 2. (b)**]. Whilst the levels of the basic form of $G_o\alpha$ were almost unchanged from those of the control cells, the levels of the acidic isoform were markedly increased. Indeed it can be seen that the acidic isoform was now the predominant species in differentiated cells.

Treatment of NG108 - 15 cells with the other differentiating agents produced qualitatively similar results. Membranes from control cells (**a**) and membranes from cells differentiated for 6 days with 1 mM 8 - bromo cAMP (**b**), 10 μM forskolin (**c**) and 10 μM PGE_1 (**d**) (see **section 2. 4. 6.**) were subjected to 2 - D SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted with antiserum OC1 [**Fig. 6. 3.**]. In all cases there was an increase in immunoreactivity of the acidic isoform caused by each of the treatments suggesting that the observed increase in $G_o\alpha$ imunoreactivity on 1 - D SDS - PAGE elicited by morphological differentiation was due to an increase in the acidic isoform of two observed species of $G_o\alpha$ in the NG108 - 15 cell.

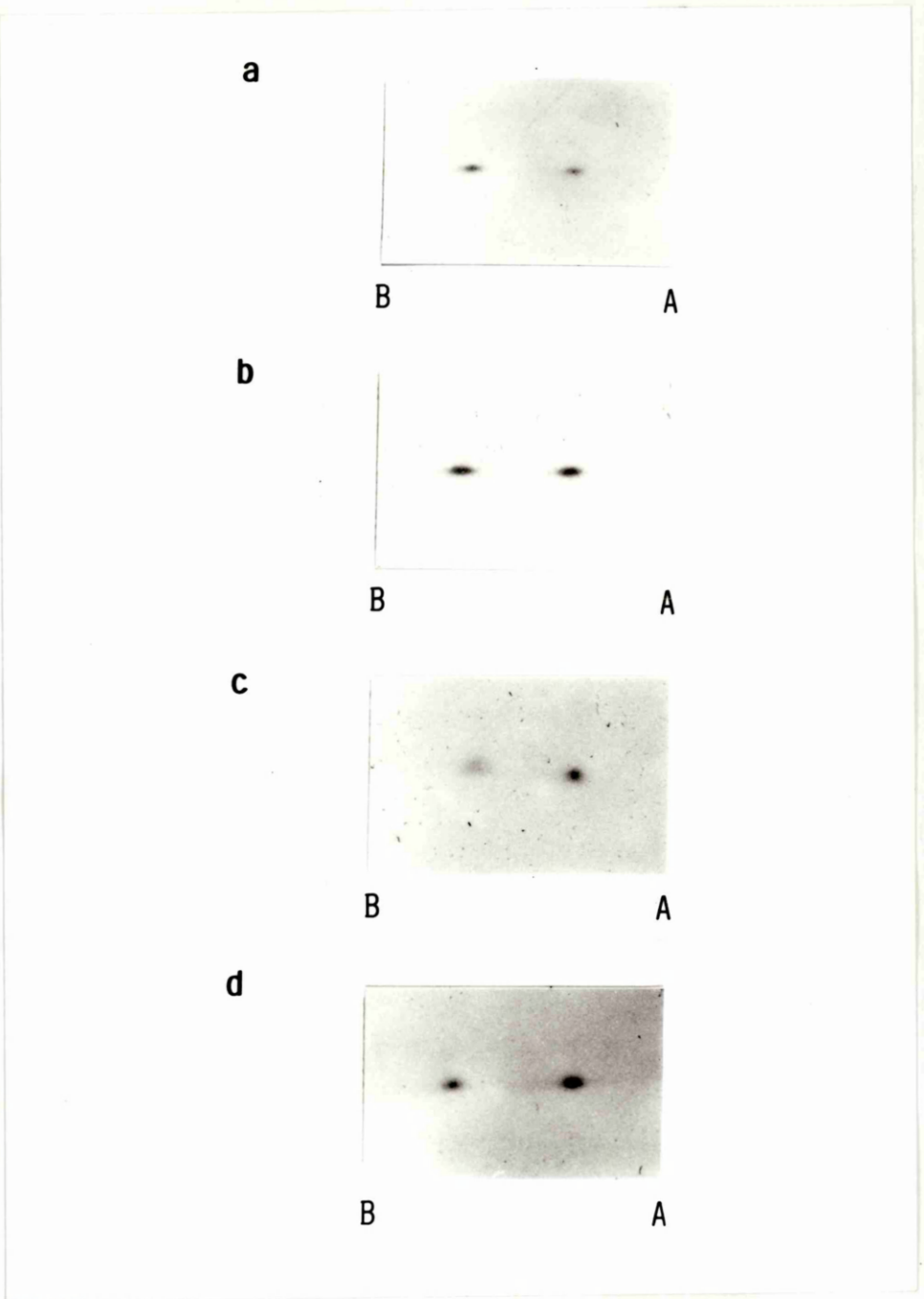
To confirm that each of the two proteins identified with antiserum OC1 did indeed represent forms of $G_o\alpha$, identical studies on the variously - treated membranes were performed using antiserum ON1which was raised against a different region of $G_o\alpha$ from antiserum OC1 [see **Table 2. 1.**]. This antiserum also specifically identified two 39 kDa polypeptide species with isoelectric points of 5.5 and 5.8 which were equivalent to those observed with antiserum OC1. In control cells the basic isoform was the most prevalent and there was a marked increase in immunoreactivity in the acidic isoform upon differentiation with each of the treatments [**Figs. 6. 4. & 6.**

Figure 6. 3.

Two - Dimensional immunoblot analysis of membranes from control and cAMP - differentiated NG108 - 15 cells with antiserum OC1 to detect multiple isoforms of $G_o\alpha$.

Membranes (150 µg) from control (a) and cAMP - differentiated cells (b - d) were solubilized for 1 hour at 4 ^oC, resolved by 2 - D SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum OC1 (1 : 200 dilution) as described in **section 2. 11. & 2. 12.** Two immunoreactive $G_o\alpha$ species with pIs of 5.8 and 5.5 were observed in all immunoblots. Panel (b) shows cells differentiated with 8 - bromo cAMP (1 mM), panel (c) with forskolin (10 µM) and panel (d) with PGE_1 (10 µM). Similar results were obtained in 3 other experiments. **B** is the basic side and **A** is the acidic side from the isoelectric focussing first dimension.

Figure 6. 3.



5.].

The possibilty that the two $G_o\alpha$ isoforms found in NG108 - 15 differed in some way from each other by some form of covalent modification was investigated.

6. 2. 2. The two forms of $G_o\alpha$ found in NG108 - 15 cells are substrates for ADP - ribosylation by pertussis toxin.

The first possibility considered was that the two forms of $G_o\alpha$ might represent ADP - ribosylated and non - modified forms of the same protein. To assess this, NG108 - 15 cells were treated in culture with pertussis toxin (25 ng / ml, 16 hours) as described in **section 2. 4. 7.** and membranes prepared. Incubation of cells *in vivo* with pertussis toxin catalyses the transfer of a ADP - ribose group from the donor NAD^+ onto a conserved cysteine which is four residues from the carboxy terminus of those G - proteins, including $G_o\alpha$, which are pertussis toxin substrates [Milligan 1988].

Membranes from control and pertussis toxin - treated NG108 -15 cells were subjected to 2 - D SDS - PAGE (10 % (w/v) acrylamide), immunoblotted with antiserum OC1 and the mobility of the forms of $G_o\alpha$ were compared. Following toxin treatment, two polypeptides of some 39 kDa were still identified by antiserum OC1, but both migrated as more acidic forms in comparison to the polypeptides identified in membranes from the untreated cells [**Fig. 6. 6.**]. The presence of a polypeptide of some 64 kDa and pI 5.7, which was identified by antiserum OC1 but which was not a substrate for pertussis toxin - catalysed ADP - ribosylation and hence did not alter in mobility following pertussis toxin treatment, provided, in a serendipitous manner, an internal control for pertussis toxin - catalysed shifts in mobility of the two $G_o\alpha$ isoforms. The 64 kDa polypeptide was not identified by antiserum ON1 and hence is

Figure 6. 4.

Two - Dimensional immunoblot analysis of membranes from control and db cAMP - differentiated NG108 - 15 cells with antiserum ON1 to detect multiple isoforms of $G_o\alpha$.

Membranes (150 μg) from control (a) and db cAMP - differentiated cells (b) were solubilized for 1 hour at 4 ^oC, resolved by 2 - D SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum ON1 (1 : 200 dilution) as described in **sections 2. 11. & 2. 12.** Similar results were obtained in 3 other experiments.

Figure 6. 4.

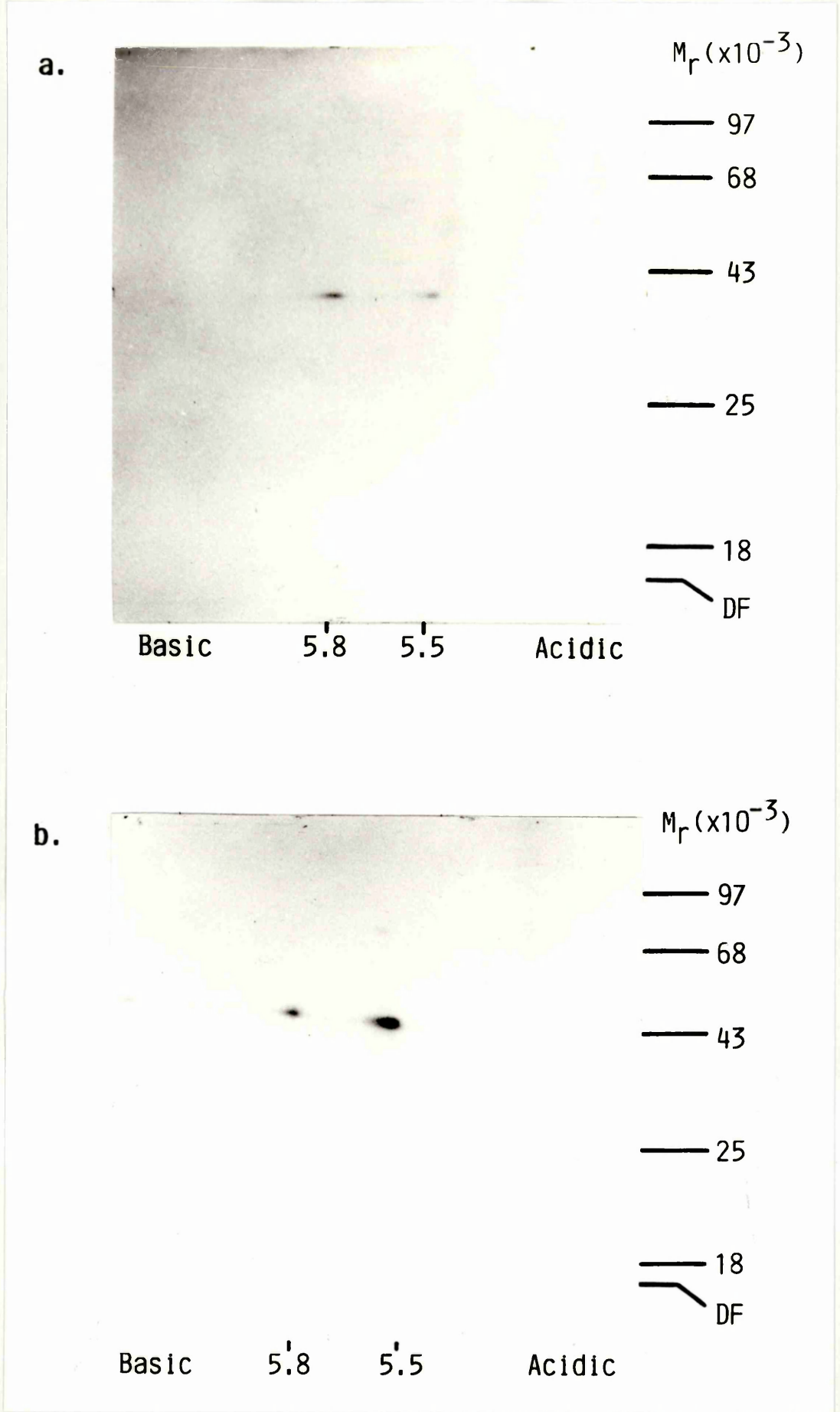

a.
$M_r (x10^{-3})$
97
68
43
25
18
DF
Basic
5.8
5.5
Acidic
b.
$M_r (x10^{-3})$
97
68
43
25
18
DF
Basic
5.8
5.5
Acidic

Figure 6. 5.

Two - Dimensional immunoblot analysis of membranes from control and cAMP - differentiated NG108 - 15 cells with antiserum ON1 to detect multiple isoforms of $G_o\alpha$.

Membranes (150 µg) from control (a) and cAMP - differentiated cells (b - d) were solubilized for 1 hour at 4 ^oC, resolved by 2 - D SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum ON1 (1 : 200 dilution) as described in **section 2. 11. & 2. 12**. Two immunoreactive $G_o\alpha$ species with pIs of 5.8 and 5.5 were observed in all immunoblots. Panel (b) shows cells differentiated with 8 - bromo cAMP (1 mM), panel (c) with forskolin (10 µM) and panel (d) with PGE_1 (10 µM).**B** is the basic side and **A** is the acidic side from the isoelectric focussing first dimension. Similar results were obtained in 3 other experiments.

Figure 6. 5.

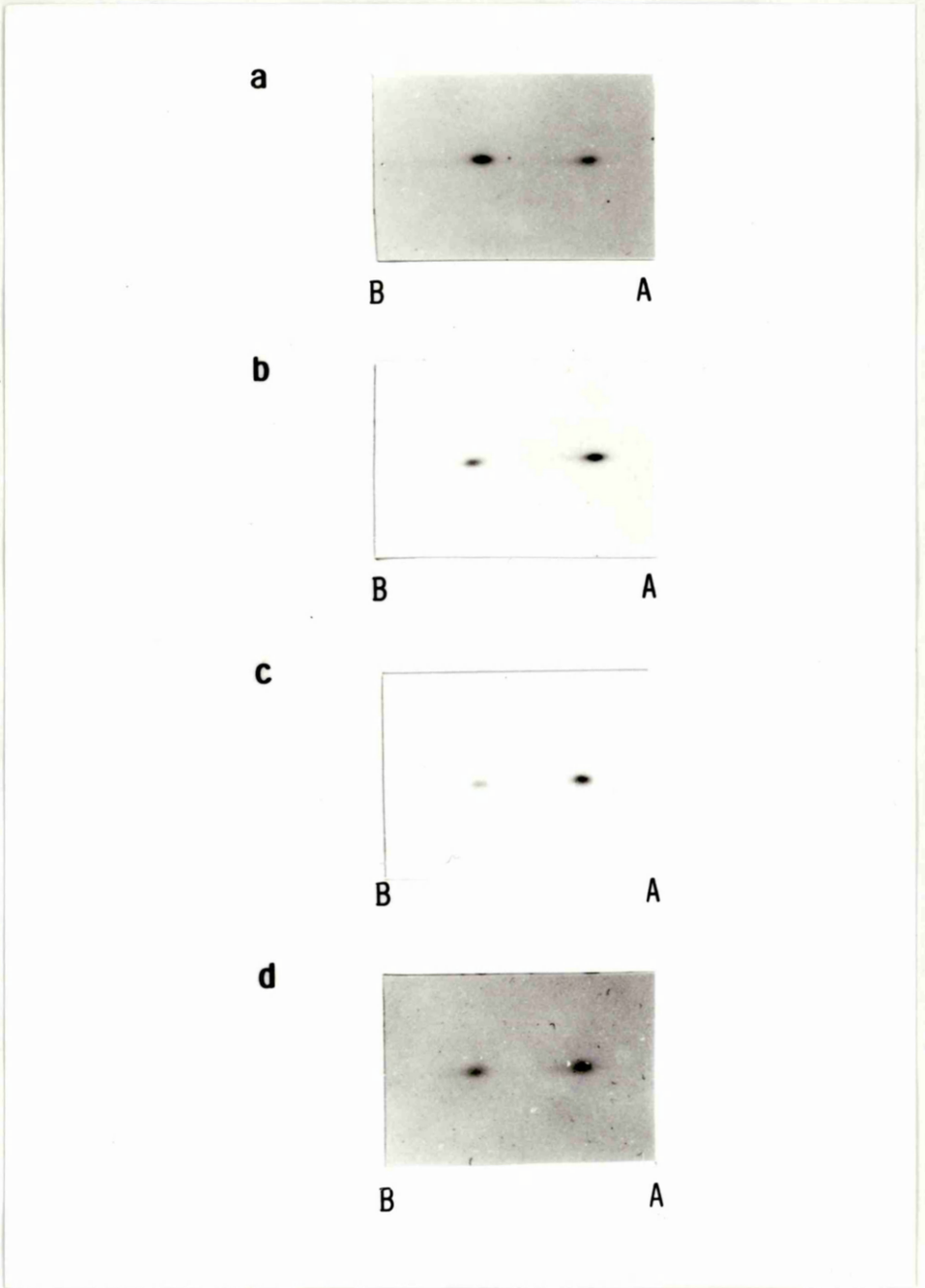
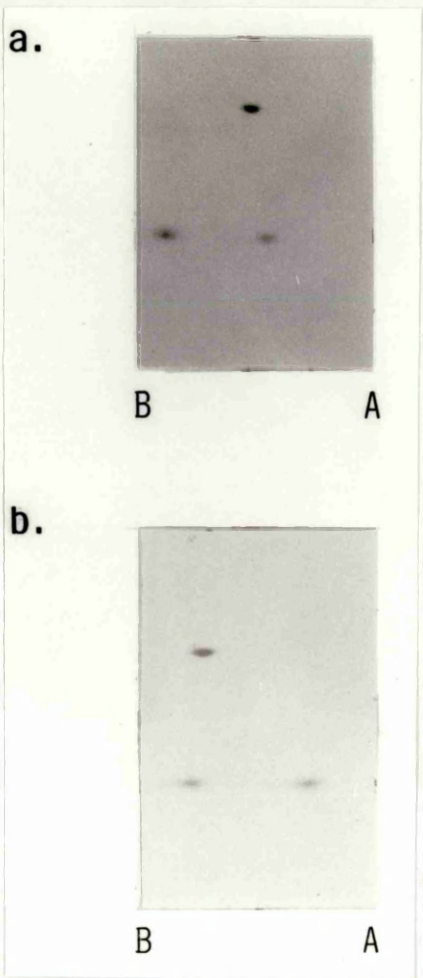


Figure 6. 6.

Both acidic and basic forms of $G_o\alpha$ are substrates for pertussis toxin - catalysed ADP-ribosylation.

Membranes (150 μg) of untreated (a) and pertussis toxin-pretreated (25 ng/ml, 24 h.) (b) NG108-15 cells were resolved in 2-dimensional SDS-PAGE and immunoblotted with antiserum OC1 (1 : 1000 dilution) as primary reagent. Two immunoreactive $G_o\alpha$ species with pIs of 5.8 and 5.5 were observed in all immunoblots. A 64 kDa polypeptide which was identified by the antiserum but which was not a substrate for pertussis toxin-catalysed ADP-ribosylation acted as an internal control to demonstrate that both isoforms of $G_o\alpha$ migrated as more acidic species following pertussis toxin treatment of the cells.**B** is the basic side and **A** is the acidic side from the isoelectric focussing first dimension.A second experiment produced similar results.

Figure 6. 6.



unlikely to be closely related to $G_o\alpha$. Attempts to incorporate radioactivity, using [^{32}P]NAD^+ and thiol-activated pertussis toxin, into polypeptides in membranes of the pertussis toxin-treated NG108-15 cells were unsuccessful, demonstrating that the entire pool of pertussis toxin-sensitive G-proteins had been ADP-ribosylated by the pretreatment. This being so it would appear that if the differences between the two isoforms of $G_o\alpha$ in NG108 - 15 are indeed due to one of them being ADP - ribosylated, presumably the acidic form, then such a modification cannot be at the cysteine residue which is the target for pertussis toxin.

6. 2. 3. Differences in the $G_o\alpha$ isoforms are not due to phosphorylation of one of the polypeptides.

It has been demonstrated recently that the pertussis toxin - sensitive G - protein, $G_i2\alpha$, can act as a substrate for phosphorylation by protein kinase C in hepatocytes [Bushfield *et al.*, 1990]. Further, this protein, which appears to be the true G_i of the adenylyl cyclase cascade in hepatocytes [Bushfield *et al.*, 1990] and in NG108 - 15 cells [McKenzie & Milligan, 1990], is inactivated by such phosphorylation. Guanine nucleotide regulation of the inhibition of adenylyl cyclase can be restored by treatment of hepatocyte membranes with alkaline phosphatase. Phosphorylation of $G_o\alpha$ would result in the protein migrating as a more acidic entity under the conditions used in the 2 - D immunoblot system and may account for the presence of the two $G_o\alpha$ isoforms.

To assess if the two isoforms of $G_o\alpha$ differed only in their phosphate content, membranes of db cAMP - differentiated NG108-15 cells were treated with alkaline phosphatase, under conditions which can remove phosphate from the α subunit of G_i2.

Membranes from db cAMP - treated NG108 -15 cells were incubated with alkaline phosphatase (800 units / ml) for 30 minutes at 37 ^oC as described in **section 2. 16.** After this incubation the membranes were subjected to analysis by 2 - D SDS -

PAGE (10 % (w/v) acrylamide) and immunoblotted with antiserum ON1 [**Fig. 6. 7.**]. No difference was observed between the untreated membranes (**panel a**) and those treated with alkaline phosphatase (**panel b**).

6. 2. 4. Analysis of the isoforms of $G_o\alpha$ using SDS - urea - PAGE immunoblotting.

Whilst 2 - dimensional electrophoresis allowed the resolution of the two forms of $G_o\alpha$, it was noted that in some experiments a distinct fraction of the total $G_o\alpha$ immunoreactivity appeared not to penetrate adequately into the isoelectic focussing tube gel and following the 2nd dimension separation, was thus detected at the extreme basic edge of the immunoblot. Resolution of the forms of $G_o\alpha$ in a 1 - D gel system in which the total sample has been accepted into the gel would have distinct advantages over the 2 - D system. Resolution of the two isoforms on a 1 - D system is possible if 4 M deionized urea is included in the SDS - PAGE resolving gel [**section 2. 8. 3.**].

When analysed on a 2 - D immunoblot system the isoforms of $G_o\alpha$ migrate as 39 kDa polypeptides. **Fig. 6. 8.** shows membranes from db cAMP - differentiated NG108 - 15 cells which were subjected to an IEF first dimension followed by SDS - PAGE (12.5 % (w/v) acrylamide) analysis and subsequent immunoblotting with antiserum ON1 [**panel a**] and those resolved on a similar SDS - PAGE gel containing 4 M deionized urea [**panel b**]. Whilst there appears to be no difference in mobility between the isoforms on the conventional immunoblot, a distinct shift was observed on the proteins separated in the presence of urea. The acidic isoform, termed $G_o1\alpha$, was seen to migrate slightly more slowly on the blot when compared to the basic form, $G_o2\alpha$.

Figure 6. 7.

Two - dimensional immunoblot analysis of db cAMP - differentiated (1 mM, 6 days) NG108 - 15 membranes. Effect of alkaline phosphatase treatment.

Membranes (150 μg) from cAMP - differentiated cells were incubated for 30 minutes at 37 ^{o}C in the absence (a) or presence (b) of alkaline phosphatase (800 units / ml) as described in **section 2. 16. 1.** The membranes were then solubilized for 1 hour at 4 ^{o}C, resolved by 2 - D SDS - PAGE (10 % (w/v) acrylamide) and immunoblotted using antiserum ON1 (1 : 200 dilution) as described in **section 2. 11. & 2. 12.**Two immunoreactive $G_o\alpha$ species with pIs of 5.8 and 5.5 were observed in all immunoblots.**B** is the basic side and **A** is the acidic side from the isoelectric focussing first dimension. Similar results were obtained in 3 other experiments.

Figure 6. 7.

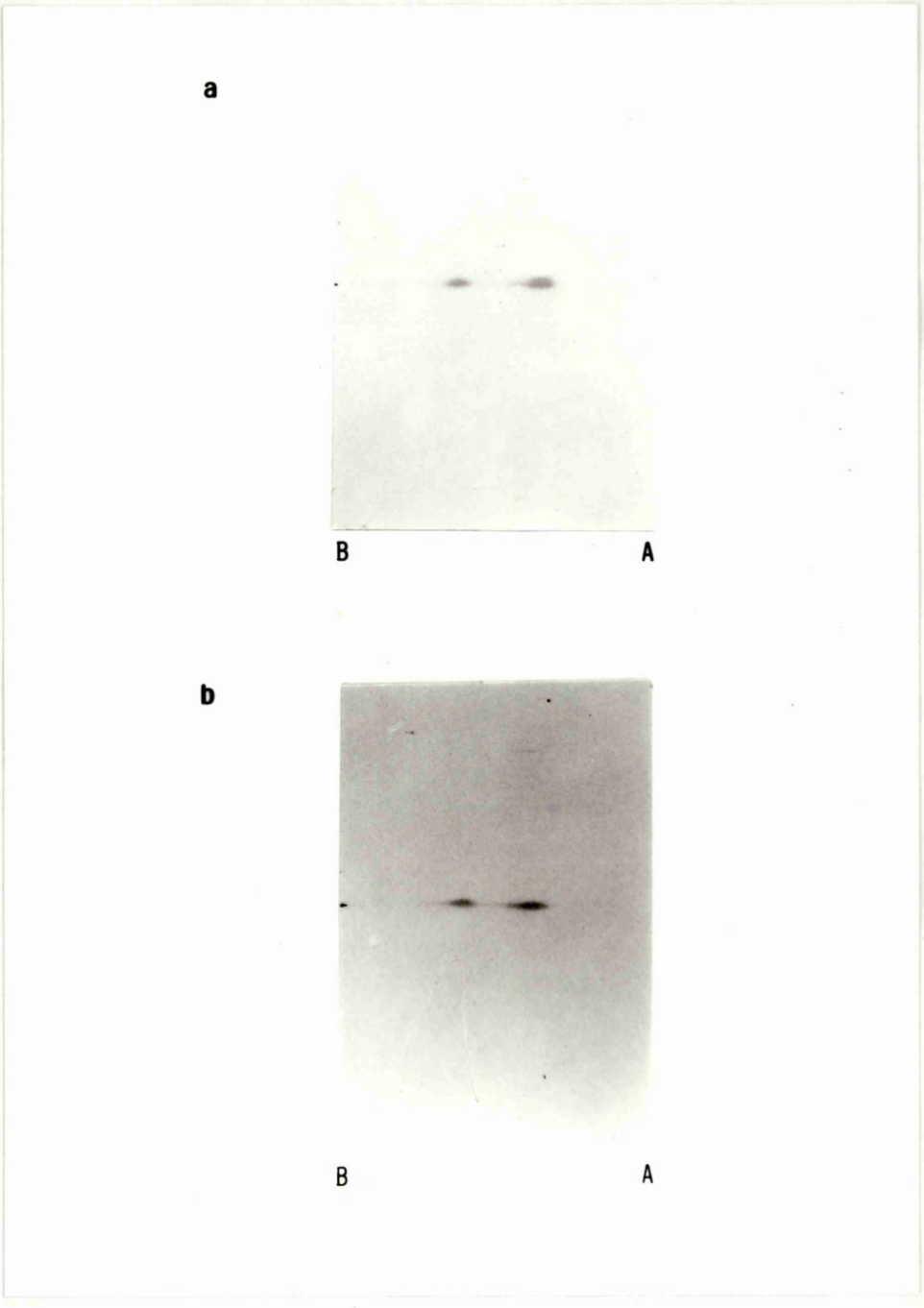
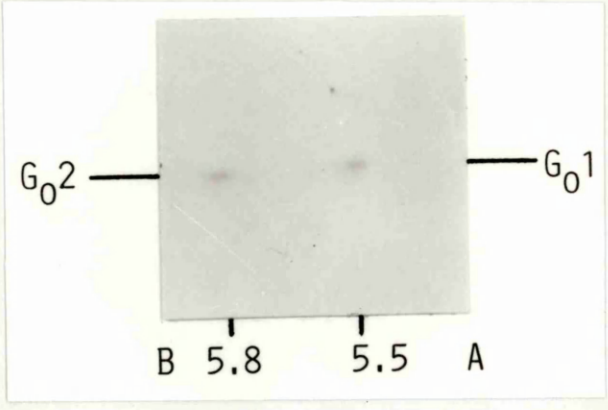


Figure 6. 8.

Two - dimensional SDS-urea PAGE analysis of membranes of NG108-15 cells. The acidic form (G_o1) of $G_o\alpha$ migrates more slowly than the basic form (G_o2) in this gel system.

Membranes (150 µg) from db cAMP - differentiated cells were solubilized for 1 hour at 4 ^{o}C. The membranes were then resolved by 2 - D SDS - PAGE (12.5 % (w/v) acrylamide) gel (panel a) or on the same gel system containing 4 M deionized urea (panel b) as described in **section 2. 8. 3.** Immunoblotting of protein following transfer to nitrocellulose was performed using antiserum ON1 (1 : 1000 dilution) as primary reagent. Similar results were obtained in 3 other experiments.

Figure 6. 8.



The possibility of separating $G_o1\alpha$ and $G_o2\alpha$ on a 1 - D gel was investigated. Membranes from rat brain and control NG108 - 15 cells were resolved on 1 - D SDS - urea - PAGE (12.5 % (w/v) acrylamide) and immunoblotted with antiserum ON1 [**Fig. 6. 9.**]. Antiserum ON1, under these conditions, now recognized a second polypeptide in both brain (**lane 1**) and in NG108 - 15 membranes (**lane 2**). However, the second polypeptide identified in brain (Go*α in the terminology of [Goldsmith *et al.*, 1988]) migrated considerably more slowly in the 1-dimensional SDS-urea-PAGE system than did either of the $G_o\alpha$ forms in NG108 - 15 cells suggesting that G_o*α is a different protein from those in NG108 - 15 cells and indeed that G_o*α is not found in these cells. This is not entirely unexpected since the isoelectric points measured for $G_o\alpha$ and G_o*α were 5.6 and 6.0 respectively [Goldsmith *et al.*, 1988a] compared to 5.5 and 5.8 for $G_o1\alpha$ and $G_o2\alpha$ suggesting more fundamental differences in polypeptide identity.

From **Fig. 6. 9.** it can be seen that $G_o\alpha$ and G_o*α from rat brain were well resolved on the 1 - D system (**lane 1**) whereas $G_o1\alpha$ and $G_o2\alpha$, being much closer together, made it impossible to determine whether $G_o\alpha$ from brain has a mobility identical to either of the NG108 - 15 isoforms.

To circumvent this problem a smaller amount of rat brain (5 µg as opposed to 10 µg) was resolved as before and compared this time to membranes of control and db cAMP - differentiated NG108 -15 cells [**Fig. 6. 10.**]. These were resolved on a 1 - D SDS - urea - PAGE (12. 5% (w/v) acrylamide) and immunoblotted with either antiserum IM1(**panel a**) or OC1 (**panel c**). The form of $G_o\alpha$ with lower mobility through the gel ($G_o1\alpha$) was present in greatly increased levels in membranes of db cAMP - differentiated cells compared to the undifferentiated cells, whilst the form with greater mobility ($G_o2\alpha$) was unaltered in amount (compare **lanes 2** and **3**). The more slowly migrating form of $G_o\alpha$ in NG108 - 15 cells ($G_o1\alpha$) appears to have

Figure 6. 9.

Neither $G_o1\alpha$ or $G_o2\alpha$ correspond to $G_o*\alpha$.

Membranes from (1) rat brain (10 μg) and (2) untreated NG108-15 cells (50 μg) were resolved by 1 - D SDS - PAGE (12.5 % (w/v) acrylamide) gel and immunoblotted with antiserum ON1 (1:1000 dilution) as primary reagent. Similar results were obtained in 3 other experiments.

Figure 6. 10.

One - dimensional SDS - urea - PAGE analysis of $G_o1\alpha$ and $G_o2\alpha$.

Membranes of (1) rat brain (5 μg), (2) untreated NG108-15 cells (50 μg), (3) db cAMP - treated (1 mM, 6 days) NG108-15 cells (50 μg) and (4) mouse brain (5 μg) were resolved by 1-dimensional SDS-urea-PAGE and immunoblotted with antisera (a) IM1 (1 : 200 dilution), (b) I3B (1 : 200 dilution) and OC1 (1 : 1000 dilution) as primary reagents. Equivalent results were produced in 4 further experiments in which the membranes were derived from separate cultures.

Figure 6. 9.

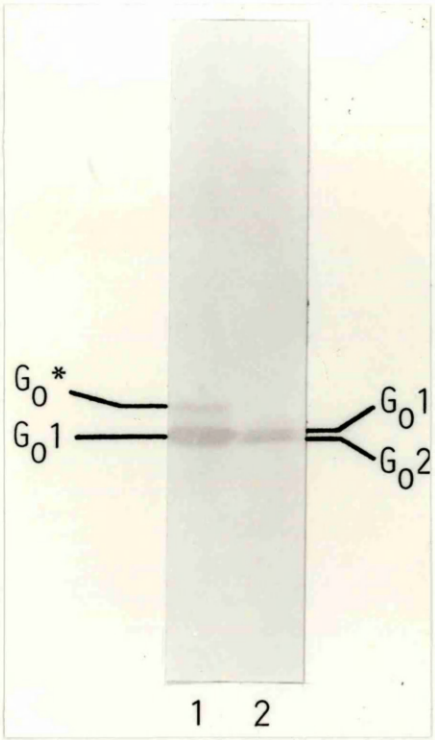
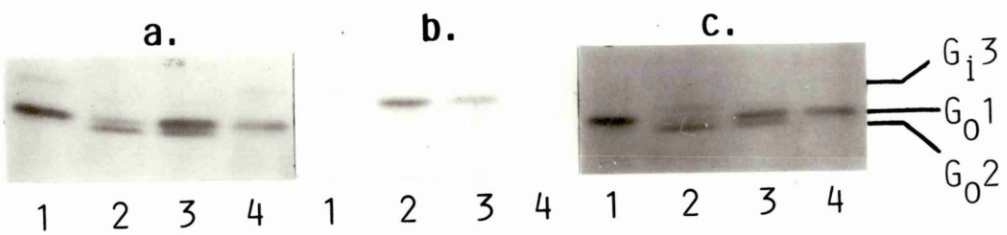


Figure 6. 10.



migrated identically with $G_o\alpha$ from rat brain. However no polypeptide corresponding to the more rapidly migrating form of $G_o\alpha$ in NG108-15 cells ($G_o2\alpha$) was detected in rat brain (**panels (a)** and **(c)**).

It should be noted that NG108 - 15 cells were generated by fusion of rat glioma C6 BU1 cells and mouse neuroblastoma N18TG2 cells and as such the possibility exists that the two $G_o\alpha$ -like polypeptides present in these cells were rat and mouse variants of $G_o\alpha$ produced by distinct chromosomes derived from the parental cells of the hybrid.

This was examined and it was demonstrated that mouse brain $G_o\alpha$ also migrated identically with the more slowly migrating polypeptide of NG108-15 cells in the SDS-urea-PAGE system [**Fig. 6. 10.**] . Membranes prepared from mouse brain cortex were immunoblotted with antiserum IM1 (**lane 4, panel a**) and antiserum OC1 (**lane 4, panel c**). Two isoforms of G_o were detected which corresponded to $G_o\alpha$ and $G_o{}^*\alpha$ when their mobilities were compared to the isoforms found in rat brain. As with rat brain, no immunoreactive polypeptide was detected in mouse brain which corresponded to the more rapidly migrating form of $G_o\alpha$ of NG108 - 15 cells.

Since there was the possibilty of a cross - reactivity of antiserum OC1 with $G_i3\alpha$ [see **chapter 3**] the possibility that $G_o2\alpha$ was actually the $G_i3\alpha$ gene product was assessed.

Membranes from rat brain, mouse brain and from control and db cAMP - differentiated NG108 - 15 cells were resolved upon 1 - dimensional SDS - urea - PAGE (12.5 % (w/v) acrylamide) and immunoblotted with antiserum I3B which was generated against the C - terminal decapeptide of $G_i3\alpha$ [**Fig. 6. 10. ; panel (b), lanes 1 - 4.**]. $G_i3\alpha$, which is expressed to a considerable degree in NG108 - 15 cells [McKenzie & Milligan, 1990], was detected by antiserum I3B but this polypeptide

migrated considerably more slowly through the gel [**Fig. 6. 10.** ; **panel (b)**] than did either form of G_o α as identified by antisera IM1 or OC1 [**Fig. 6. 10.**; **panels (a), (c)**]. From this figure it seen that treatment of NG108 - 15 cells with db cAMP resulted in a considerable decrease in the immunoreactive $G_i3\alpha$ detected with antiserum I3B.

6. 3. Discussion.

The results presented in this chapter demonstrate the presence of two isoforms of the α subunit of the guanine nucleotide - binding protein G_o in NG108 - 15 cells. Morphological differentiation of these cells results in an increase in the more acidic isoform as assessed using 2 - dimensional immunoblotting with specific $G_o\alpha$ antisera. These results are consistent with observations in the neuroblastoma cell line NIE 115 where differentiation with dimethylsulphoxide resulted in the appearance of a novel pertussis toxin - sensitive polypeptide with immunological characteristics of $G_o\alpha$ which had an isoelectric point similar to that of the acidic isoform of $G_o\alpha$ in NG108 - 15 cells [Homburger *et al.*, 1988.; Brabet *et al.*, 1990]. As with the isoforms in NG108 - 15 cells, the biochemical basis of the differences between these polypeptides is unknown at this time.

The possibility that these proteins differed by a covalent modification was investigated. Although these isoforms were not separable under normal 1- dimensional SDS - PAGE conditions inclusion of 4 M deionized urea to the gel resulted in the observation of an immunoreactive doublet. Comparison with the brain $G_o\alpha$ isoforms revealed that one of the proteins ($G_o1\alpha$) had identical mobility on the gel with brain $G_o\alpha$; the other ($G_o2\alpha$) differed from both brain $G_o\alpha$ and $G_o{*}\alpha$.

The α subunit of G_o was first identified as a polypeptide of 39 kDa which co-purified through a number of chromatographic steps with the major form of $G_i\alpha$ ($G_i1\alpha$) from bovine brain [Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Milligan & Klee, 1985] where it may represent as much as 1% of brain membrane protein [Sternweis & Robishaw, 1984; Gierschik *et al.*, 1986b].

However, detailed examination of fractions containing pertussis toxin-sensitive

G-proteins during purifications from bovine brain have indicated the presence of two distinct populations of $G_o\alpha$ [Goldsmith *et al.*, 1988a]. These two forms differed in isoelectric point and as such could be resolved on high performance ion exchange resins [Goldsmith *et al.*, 1988a]. These proteins termed, $G_o\alpha$ and $G_o{}^*\alpha$, were identified with two - dimensional analysis consisting of an isoelectric focussing first dimension followed by conventional immunoblotting with a wide selection of antisera and although their molecular identities were not defined the authors reasoned that $G_o{}^*\alpha$ may be a post translationally modified form of $G_o\alpha$.

Since different anti - $G_o\alpha$ - antisera were unable to discriminate between the two polypeptides found in the NG108 - 15 cells, it is unclear if the two forms were derived from distinct but highly homologous genes or if they were generated by either the differential splicing of pre-mRNA derived from a single gene or by alterations in covalent modification subsequent to synthesis. Both of the potential explanations which would be consistent with the two forms being derived from a single gene have parallels in other G-proteins. Firstly, it is well established that up to 4 forms of $G_s\alpha$ can be generated via differential splicing mechanisms [Bray *et al.*, 1986; Robishaw *et al.*, 1986a; Robishaw *et al.*, 1986b] and that multiple forms of G_s α so generated can be detected in a single cell. Furthermore, the recent demonstration of the production of alternatively spliced forms of $G_o\alpha$ in *drosophila* [de Sousa *et al.*, 1989; Thambi *et al.*, 1989; Yoon *et al.*, 1989] offers a further potential explanation for the identification of distinct isoforms of $G_o\alpha$ in NG108-15 cells as demonstrated herein. Similar production of isoforms of $G_o\alpha$ produced by differential splicing of mRNA has recently been observed in mammalian systems in terms of isolation of corresponding cDNA species. Screening of a hamster insulin - secreting tumor cell cDNA library for $G_o\alpha$ revealed the presence of two inserts each coding for a distinct $G_o\alpha$. One of the $G_o\alpha$ cDNAs encoded for a protein identical to that cloned previously from rat and bovine tissues whilst the other encoded for a protein that appeared to be derived from an alternatively spliced mRNA. This novel cDNA coded for a protein that is identical to

$G_o\alpha$ in its first two - thirds and differed in the remaining carboxyl third of the polypeptide [Hsu *et al.*, 1990].

Multiple transcripts have been identified in northern blots using a mammalian $G_o\alpha$ cDNA as probe [Jones & Reed, 1987]. Secondly, members of the "G_i-like" gene products have been demonstrated to be substrates for each of protein kinase C [Katada *et al.*, 1985; Pyne *et al.*, 1989; Bushfield *et al.*, 1990], protein kinase A [Watanabe *et al.*, 1988] and the insulin receptor tyrosyl kinase activity [O'Brien *et al.*, 1987; Krupinski *et al.*, 1988] under various conditions .

In a recent paper, Lang was able to isolate five pertussis toxin - sensitive G - protein α – subunits from bovine brain includng two subforms of $G_o\alpha$, presumably $G_o\alpha$ and $G_o{}^*\alpha$, which were not discriminated against with a number of specific antisera. *In situ* peptide mapping using SDS - PAGE revealed different cleavage products with different proteases on each of these proteins [Lang, 1989] suggesting that these proteins were different gene products each with a discrete amino acid primary sequence. It should be noted that in the same study only one isoform of $G_o\alpha$ was detected in NG108 - 15 cells, but this is probably as a consequence of using gel conditions under which the two isoforms would not be separated.

Heterogeneity of $G_o\alpha$ is not confined to only two subtypes; Katada and coworkers have identified and characterized the α - subunits of four immunologically G_o - like proteins, termed 1 - 4, which were distinguishable from one another on elution profiles from high - resolution Mono Q column chromatography [Kobayashi *et al.*, 1989; Inanobe *et al.*, 1990]. When subjected to digestion with lysylendopeptidase or V8 protease and then analysed by immunoblot with a polyclonal antiserum raised against purified $G_o\alpha$ from rat brain, there were significant differences in the fragments which were immunoreactive with the antibody among the four α - subunits. The

peptide fragments tended to fall into two groups, one group consisting of subunits 1 and 2 and the other consisting of 3 and 4 and no apparent differences were observed in their digested patterns between 1 and 2 or 3 and 4. All four proteins served as substrates for pertussis toxin - catalysed ADP - ribosylation and had a measurable GTPase activity suggesting that they were existent in the native membranes and not artificial products due to proteolysis or denaturation during the purification process [Inanobe *et al.*, 1990].

An NAD^+ : arginine ADP - ribosyltranferase that catalyses a reaction similar to that of cholera toxin has been purified from turkey erythrocytes [Moss & Vaughan, 1978; Moss *et al.*, 1979]. This enzyme has been shown to ADP - ribosylate a number of polypeptides in both crude mixtures and purified protein preparations including both the α and β - subunits of purified transducin [Watkins *et al.*, 1987]. This modification is not restricted to retinal G - proteins, Jacquemin was able to show that $G_s\alpha$ was subject to ADP - ribosylation by an endogenous ADP - ribosyltransferase activity in membranes prepared from adipocytes [Jacquemin *et al.*, 1986].

One possibility for differential post-translational modification might have been that the more acidic form had become ADP - ribosylated by this endogenous ADP - ribosyltransferase activity. Both isoforms of $G_o\alpha$ in NG108 -15 cells are substrates for pertussis toxin - catalysed ADP - ribosylation, as following pretreatment of the cells with pertussis toxin and subsequent 2 - dimensional electrophoresis both isoforms now migrated as more acidic proteins [**Fig. 6. 6.**]. However since neither an ADP - ribosyltranferase inhibitor nor any means of stripping ADP - ribose groups from the protein was available it cannot be demonstrated definitively that the more acidic isoform had not been ADP - ribosylated by an endogenous ADP - ribosyltransferase activity at a site other than the cysteine residue which acts as the ADP-ribose acceptor during pertussis toxin catalysed ADP-ribosylation. If this is true however, then given that the

addition of ADP-ribose would cause the polypeptide to migrate as a more acidic species and that the acidic isoform of $G_o\alpha$ from NG108-15 cells appears to be identical with the predominant form of $G_o\alpha$ from both rat and mouse brain, it would suggest that virtually all of the $G_o\alpha$ in brain contains endogenously added ADP - ribose. Moreover, ADP - ribosylation of transducin by an endogenous NAD^+: arginine ADP-ribosyltransferase from turkey erythrocytes [Watkins *et al.*, 1987] produced a large alteration (4 kDa) in the apparent size of transducin α subunit as judged by its ability to move through an SDS-PAGE gel. ADP - ribosylation of G -protein α subunits by either pertussis or cholera toxin also produce marked mobility shifts in SDS - PAGE [see **Fig. 4. 5.** for example). Given that the mobility of $G_o\alpha$ in 1 -dimensional SDS - PAGE is entirely consistent with the size of the polypeptide as assessed from isolation of a corresponding cDNA [Jones & Reed, 1987] and that the two isoforms show extremely similar mobility in 1-dimensional SDS-PAGE, endogenous ADP-ribosylation of the more acidic polypeptide is unlikely.

In an attempt to assess whether the two forms of $G_o\alpha$ were identical save for the post-translational addition of a phosphate group, membranes from db cAMP - differentiated NG108 - 15 cells, which contain high levels of the acidic form of $G_o\alpha$ ($G_o1\alpha$), were incubated with alkaline phosphatase, the membrane proteins resolved by 2-dimensional electrophoresis and then immunoblotted using antiserum ON1. No alterations in the relative amounts or mobilities of the two forms were noted. This is despite the fact that treatment of hepatocyte membranes under these conditions causes the dephosphorylation of phosphorylated $G_i2\alpha$ and the restoration of guanine nucleotide mediated inhibtion of adenylyl cyclase which had been ablated by treatment of the cells with agents which produce an activation of protein kinase C (M. Bushfield, G.Murphy, G. Milligan and M.D. Houslay, unpublished).

As the α - subunits of forms of G_i and G_o are myristoylated at their N - terminus [Buss *et al.*, 1987; Schultz *et al.*, 1987], then it might be suggested that the

two $G_o\alpha$ isoforms differ in that one isoform does not contain covalently linked myristic acid and that this could account for the differences in isoelectric mobility. Such an argument is not consistent with the fact that myristoylation is essential for membrane attachment of such G - proteins [Jones *et al.* 1990] and that both of the $G_o\alpha$ isoforms are membrane associated polypeptides as membrane preparations were the starting material for these studies. Furthermore, Homburger and coworkers have provided evidence that both isoforms of $G_o\alpha$ in NIE 115 cells contain covalently linked myristic acid [Homburger *et al.*, 1988; Brabet *et al.*, 1990].

The possibility that one of the isoforms of $G_o\alpha$ represents a proteolytic product of the other must be considered unlikely by the fact that the antisera used were directed against both the extreme N - terminal (antiserum ON1) and C - terminal (antiserum OC1). Further evidence to suggest the integrity of the C-terminal region of each polypeptide is that, as noted above, both isoforms are substrates for pertussis toxin - catalysed ADP -ribosylation. As this modification occurs only 4 amino acids from the C - terminus then any modification at the C - terminus would have to be extremely minor to be consistent with the immunological detection of this polypeptide. Indeed, detailed examinations of the cross - reactivity or otherwise between antisera raised against the C-terminal deapeptides of the α subunits of G_i2, G_i3 and G_o indicate a key role for the C - terminal tyrosine residue in recognition of $G_o\alpha$ by antiserum OC1 [**Fig. 3. 8.**].

Resolution of the forms of $G_o\alpha$ in a 1 - dimensional system was achieved by the inclusion of 4 M deionized urea in the resolving phase of SDS - PAGE. The results obtained by this approach were entirely consistent with those obtained using the 2 - dimensional system. Each of three distinct anti-$G_o\alpha$ antisera identified each of the two polypeptides and each antiserum demonstrated that cAMP - induced differentiation of NG108 -15 cells produced elevated levels of only one of these isoforms ($G_o1\alpha$). Because the polypeptide whose expression was increased by the differentiation

procedure was the acidic form and because this polypeptide migrated identically with rat brain $G_o\alpha$ in the SDS - urea - PAGE system then this can be identified as $G_o\alpha$ rather than $G_o*\alpha$ [Goldsmith *et al.*, 1988a]. The more basic $G_o\alpha$ - like polypeptide of NG108 - 15 cells ($G_o2\alpha$) is not, however, identical with $G_o*\alpha$. This conclusion is based on the differences in mobility of $G_o*\alpha$ and $G_o2\alpha$ from NG108 - 15 cells in 1 - dimensional SDS - urea - PAGE [**Fig. 6. 10.**]. $G_o*\alpha$ migrates in this system considerably more slowly than $G_o1\alpha$ whilst $G_o2\alpha$ actually migrated slightly more rapidly than $G_o1\alpha$.

As none of the available antisera provide a selective probe to discriminate between the two isoforms of $G_o\alpha$ in NG108 - 15 cells then the assessment of which of these polypeptides plays the key role in receptor regulation of voltage-sensitive Ca^{2+} channels in NG108 - 15 cells is unclear [Hescheler *et al.*, 1987, McFadzean *et al.*, 1989]. As cAMP - induced differentiation is necessary to record neurotransmitter-mediated inhibition of such currents, it is tempting to speculate that the acidic isoform ($G_o1\alpha$) is of primary importance, as this is the form which is increased markedly in levels during the differentiation process.

FUTURE PERSPECTIVES.

The work described in this thesis consists of two main parts. Firstly a series of antipeptide antisera were generated against a number of G - protein subunits, primarily the α subunit of G_o, and these tools were used to assess changes in the levels of the various G - protein components in NG108 - 15 cells which had been morphologically differentiated. One of the major disadvantages in trying to quantitate the levels of G - protein α subunits, and any subsequent modulations, is the current lack of available purified native protein for use as standards. More recently, with the advent of genetic engineering, it has become possible to obtain pure preparations of recombinant G - protein α subunits. This would enable the development of quantitative assays calibrated with recombinant protein giving mass, rather than relative, amounts of G - proteins in cell preparations.

The second part of this thesis investigated the function of $G_o\alpha$ in these cells and identified two immunoreactive species of $G_o\alpha$ in membranes prepared from NG108 - 15 cells. This raises two questions which will have to be addressed in the near future. Firstly, determination as to whether these are two distinct proteins or covalently modified forms of the same protein and secondly, what consequences this has in ascribing functionality to each of these polypeptides.

Although alkaline phosphatase treatment of membranes had no effect on the immunoreactivity of these two polypeptides, the possibilty that one of the proteins differs by addition of a phosphate group cannot be discounted. A number of kinases including protein kinase A, protein kinase C and the tyrosyl kinase activity of the insulin receptor are able to catalyse the phosphorylation of certain purified G - proteins [see **chapter 6**]. Phosphorylation of a particular G - protein may inactivate it, thus allowing for the expression of a number of such proteins and the possibilty of their

interaction with a single species of receptor. Evaluation of the role of cAMP - dependent protein kinase and protein kinase C - mediated phosphorylation in the control of expression of $G_o\alpha$ should be investigated..

$G_o\alpha$, as well as being myristoylated, has recently been reported to contain thioester - linked palmitate [M. Parenti, G. Milligan & A.I. Magee, personal communication], therefore the possibilty that the $G_o\alpha$ isoforms differ by additional fatty acid moeities arises. Metabolic labelling studies using various radiolabelled fatty acids could be used to answer this problem. Indeed such studies have already indicated that the half life of $G_o\alpha$ differs from tissue to tissue. Silbert and coworkers have demonstrated that $G_o\alpha$ is degraded more quickly in GH_4 pituitary cells when compared to cardiocytes and, since the steady - state protein levels were similar, suggested that the rate of $G_o\alpha$ synthesis was different between the cell types [Silbert *et al.*, 1990]. They also noted that the levels of mRNA encoding $G_o\alpha$ were approximately equal in both cardiocytes and GH_4 cells implying that protein translation controls could be important determinants of G - protein α subunits concentrations in biological membranes. Indeed our laboratory could not detect any changes in $G_o\alpha$ mRNA levels elicited by differentiation of NG108 - 15 cells [C. Loney, personal communication], thus the observed increase in the levels of immunoreactive $G_o\alpha$ upon differentiation in these cells could be a reflection of changes in degradation rates of the protein as well as changes in the rate of synthesis. Metabolic labelling studies using [^{35}S] methionine and subsequent immunoprecipitation with specific antisera could be used to address this problem.

One major drawback in investigating the molecular nature of the two immunoreactive species of $G_o\alpha$ has been the lack of discriminating antisera. The isolation, by Hsu and collaborators, of cDNAs encoding two $G_o\alpha$ proteins generated by alternate splicing of a single gene enables for the production of novel specific

antisera generated against distinct peptide regions of the predicted amino acid sequences of these proteins [Hsu *et al.*, 1990]. These antisera may prove successful in the defining the molecular identity of these polypeptides. Also generation of distinct oligonucleotide probes from these sequences could be used to screen a cDNA library from NG108 - 15 cells. Such work is already in progress in our laboratory.

If antisera ,which were able to discriminate between the two forms of $G_o\alpha$, were generated, these could be used to investigate possible functions of each polypeptide. It should be possible be to look at the effect of these antibodies on the noradrenaline - induced calcium current inhibition in NG108 - 15 cells in experiments similar to those reported in McFadzean *et al.*, [1989]. It may be that only one the antisera would have the effect that the general $G_o\alpha$ antiserum had. It is also possible, however, that the two species of $G_o\alpha$ isolated by Hsu and coworkers do not correspond to those identified in NG108 - 15 cells. Probing of a cDNA library from these cells with a specific $G_o\alpha$ probe would allow isolation of any cDNA copies specifically for this (these) protein(s) and subsequent predicted primary amino acid sequences derived from this analysis could be used to develop specific antisera. Assessment as to whether one or both forms of $G_o\alpha$ co - localise with GAP 43 may also provide an indication to differential functions for these isoforms.

Publications.

The following publications have resulted in work arising from this thesis:

1. GTP-binding proteins in brain and neutrophil are tethered to the plasma membrane via their amino termini.
Eide, B., Gierschik, P., Milligan, G., Mullaney, I., Unson, C,.Goldsmith, M, & Spiegel, A. (1987)
Biochem. Biophys. Res. Commun. 148 1398-1405.

2.The use of antipeptide antisera to probe interactions between receptors and guanine nucleotide-binding proteins.
McKenzie, F.R., Mullaney, I., Unson, C.G., Spiegel, A.M. & Milligan, G. (1988)
Biochem. Soc. Trans. 16 434-437.

3. GTP analogues promote release of the alpha subunit of the guanine binding-protein protein, G_i2, from membranes of rat glioma C6 BU1 cells.
Milligan, G., Mullaney, I., Unson, C.G., Marshall, L., Spiegel, A.M. & McArdle, H. (1988)
Biochem. J. 254 391-396

4. GTP analogues cause release of the alpha subunit of the GTP binding protein, G_o from the plasma membrane of NG108 - 15 cells.
McArdle, H., Mullaney, I., Spiegel, A., Magee, A., Unson, C. & Milligan, G. (1988)
Biochem. Biophys. Res. Commun. 152 243-251.

5. Differential expression of the guanine nucleotide-binding proteins G_i and G_o elicited by dibutyryl cyclic AMP in neuroblastoma x glioma hybrid cells.
Mullaney, I., Magee, A.I., Unson, C.G., & Milligan, G. (1988)
Biochem. J. 256 649-656.

6. Elevated levels of guanine nucleotide-binding protein G_o are associated with cyclic AMP-mediated differentiation of neuroblastoma x glioma hybrid cells.
Mullaney, I.& Milligan, G. (1989) FEBS Lett. 244 113-118.

7. Antibodies to the GTP-binding protein, G_o, antagonise noradrenaline-induced calcium current inhbition in NG108 - 15 hybrid cells.
McFadzean, I., Mullaney, I., Brown, D.A. & Milligan, G. (1989)
Neuron 3 177-182

8. Identification of two distinct isoforms of the guanine nucleotide-binding protein, G_o, in neuroblastoma x glioma hybrid cells. Independent regulation during cyclicAMP induced differentiation.
Mullaney, I. & Milligan, G. (1990) J. Neurochem. (in press)

9. Identification and analysis of two isoforms of the guanine nucleotide binding protein G_o in NG108 - 15 cells.
Mullaney I. & Milligan, G. (1990) Biochem. Soc. Trans. 18 396-399

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