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LEVELS AND REGULATION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR IN HUMAN BREAST CANCER

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE FACULTY OF SCIENCE

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DEPARTMENT OF BIOCHEMISTRY

UNIVERSITY OF GLASGOW

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DEDICATION

I had originally dedicated this thesis to my wife, Angela, without her love and encouragement I would not have got this far.

During the rewrite of thesis my father, Gerry, died (19 Sept., 1991), it is to his memory that I dedicate this thesis. He taught me many lessons in his life and death.

"It is not the molecules that make up life that are important, it is life itself."

I would also like to include my mother, Winnie, in this dedication. The way she loved and nursed my father through a long debilitating illness, is worthy of a saint.

So long Gerry, I will never forget you.....

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I would not have been able to do this project without the permission of Tom Mathieson, Dept. of Biochemistry, whose progressive attitude towards the technical staff made this thesis possible.

On a more personal note there are a lot of people who have helped make my 13 years in this department enjoyable.

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CONTENTS

Tables

FIGURES

ABBREVIATIONS

The standard abbreviations recommended in the Biochemical Journal "Policy of the Journal and Instructions to Authors" (Biochem J **241**: 11-21(1987)) are used throughout this thesis with the following additions or exceptions

BSA Bovine serum albumin

cpm Counts per minute

DCC Dextran Coated Charcoal

DES Diethylstilboestrol

DMEM Dulbecco's modification of Eagle's medium

DMSO Dimethyl sulphoxide

DNA Deoxyribonucleic acid

EDTA Ethylene diamine tetra-acetic acid

EGF Epidermal growth factor

EGFR EGF receptor

E_2 17β Oestradiol

ER Oestrogen receptor

ETN EDTA-Tris-NaCl buffer

FCS Foetal calf serum

FITC Fluorescein isothiocyanate

[^{3}H] Tritiated label

HED HEPES-EDTA-Dithiothreitol

HEPES 4-(2)-hydroxyethyl-1-piperazine-ethanesulphonic acid

HIDCCFCS Heat-inactivated dextran coated charcoal stripped FCS

[^{125}I] - Iodinated Label

IgG Immunoglobulin G

IP Inositol phosphates

IP_3 Inositol Triphosphate

IP_4 Inositol Tetraphosphate

kDa. KiloDaltons

mol. wt. Molecular Weight

NP-40 Nonidet P-40

OD Optical Density

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate buffered saline

PIP Phophatidyl Inositol Pathway

hPRL Human Prolactin

oPRL Ovine Prolactin

PRLR Prolactin receptor

RNAase Ribonuclease

[^{35}S] Sulphonated

SDS Sodium dodecyl sulphate

TBS Tris buffered saline

TBST Tris buffered saline tween

TEMED N,N,N',N'-Tetramethylene-diamine

(v/v)Volume per volume

(v/w) Volume per weight

All other abbreviations (e.g. chemical names, buffer solutions) are explained in the text.

Abstract

Recent data suggest that, in addition to hormones, growth factors and their receptors play a role in human breast cancer. The epidermal growth factor receptor (EGFR) is proposed to play a significant part in breast tumour cell mitogenesis and has been used as a marker of poor patient prognosis. Utilising a ligand binding assay system it was demonstrated that EGFR was present in 38% (75/199) of primary breast tumours analysed. Concentrations of EGFR found ranged from 69.6 - 28201.0 fmoles. mg^{-1} tumour cell DNA for the high affinity , low capacity binding site, and 163.0 - 9680.0 fmoles mg^{-1}. DNA for the low affinity binding site. Mean values were 1581 ± 3647.4 and 5223.8 ± 6233.5 fmoles. mg^{-1} DNA respectively, median values were 686.5 and 3557.4 fmoles. mg^{-1} DNA respectively. Within the EGFR positive group only 14 (7%) contained oestrogen receptors (ER). This contrasts with the EGFR negative group in which 99/124 (79%) were ER positive. These results help to confirm the hypothesis that there is a negative correlation between ER and EGFR in human breast cancer, and that there are two subclasses of primary breast tumours in terms of their receptor biochemistry. The ER positive subclass is assumed to be under endocrine control and the EGFR subclass is assumed to be under more local paracrine/autocrine control.

Whilst it is accepted that the majority of EGFR is located within the cytoplasmic portion of cells, there has been some suggestion within the literature that there is an EGF binding component located within the nuclear fraction of mammalian cells in tissue culture and in placental tissue. Studies contained within this thesis indicate that there may be a single high affinity, low capacity EGF binding component within the nuclear fraction of a "bulk" breast tumour preparation (82.4 fmoles. mg^{-1} DNA; Kd: 0.013 nmoles. $litre^{-1}$). High salt extraction of the nuclear fraction with 2M Nacl failed to dislodge this binding component.

Western blotting with a polyclonal antibody to EGFR (BG 48) revealed the presence of the 170 kDa. native receptor in both the membrane fraction and in the nuclear fraction both before and after salt extraction. To confirm that there was no contamination of the nuclear fraction by membrane proteins, the nuclear fraction was assayed for the presence of two known plasma membrane enzymes, 5' Nucleotidase and Alkaline phosphatase. No activity was found in the detergent washed nuclear fraction for either enzyme. These results imply that there is an EGF binding component in the nuclear fraction of breast tumours which is immunologically recognised as the same molecular weight as the membrane bound form.

The regulation of EGFR was studied in an ER positive breast cancer cell line (ZR-75-1) . Studies within this cell line revealed that ovine prolactin (oPRL) at a pharmacological concentration of 1 $\mu g.ml^{-1}$ was able to enhance EGF stimulated mitogenesis. This was demonstrated to be through its ability to increase EGFR availability. Peak levels of EGFR were observed after four days exposure to the hormone in media containing phenol red (1600 fmoles EGFR. mg^{-1} DNA in hormone treated cell membrane fraction compared to 400 fmoles EGFR. mg^{-1}.DNA in control cell membrane fraction). Treatment with the protein synthesis inhibitor, cycloheximide (40 nmoles. $litre^{-1}$) completely inhibited this stimulation.

Whole cell binding was also increased by human PRL (hPRL) at a physiological concentration of 40 ng. ml^{-1} (153% stimulation) after 48 hours incubation in phenol red free media. However the greatest increases were observed with hPRL and oestradiol (E_2) at a concentration of $10^{-9}M$ (251% stimulation).

Subsequent Scatchard analysis revealed that the increase in EGFR binding was due to an increase in receptor concentration with no apparent effects on binding affinity.

Studies using other parameters to assess increases in EGFR levels such as indirect immunofluorescent and immunoprecipitation using the EGFR 1 monoclonal antibody, reveal increases in specific fluorescence and immunoprecipitated receptor respectively. Studies as to the possible mechanism of receptor induction point to a paracrine/autocrine approach. It is possible that the induction of EGFR is due to a heat labile polypeptide.

Chapter 1. INTRODUCTION

1.1. Historical Perspective

The first recorded instances of breast cancer can be seen in the Edwin Smith Surgical papyrus which dates around 3000 - 3500 B.C. In it there are descriptions of eight cases of tumours or ulcers of the breast . Tumours that were hard, were cool to touch, and contained fluid were distinguished from inflammations and abscesses ; for the former the author admits " there is no treatment" . Reference is made however to one case treated by cauterisation with a fire stick.

Other instances have been recorded throughout early civilisations. One of the most famous being that of Hippocrates (460-370 B.C.). When he described what must have been a common course of the disease. "... and hard tumours appear in the breast, some larger and some smaller, these do not suppurate, but continually grow harder and harder. From these grow hidden cancers..... and everything (the patients) eat tastes bitter, and if you give them more to eat, they refuse it, and shut their mouths. They become delirious, their eyes are hard, and they do not see clearly and pains dart from the breast to the neck and beneath the shoulder blades, thirst seizes upon them, the nipples are dry, and the whole body becomes emaciated ..... When they have gone as far as this, they do not recover, but die of this disease ! ". In his own experience he counselled, " It is better not to apply any treatment of occult cancer, for if treated, the patients die quickly, but if not treated they hold for a long time " .

The first real attempts at treatment of the disease where performed in the second century by Galen (131-203 A.D.). He concluded that breast cancer was brought about by an excess of black bile in the body, caused by a local manifestation of the constitutional disturbance, Melancholia. He likened the tumour growth to a crab with the large veins extending from all sides giving

the appearance of legs. Where possible he advised " Make accurate incisions surrounding the whole tumour, so as not leave a single root " .

This advice has been carried out in one form or another according to the prevailing medical dictates right up to the twentieth century.

1.2 Epidemiology

Cancer of the breast frequently occurs in mice, rats, dogs, and humans, and almost exclusively affects the female of the species. Low rates are found in Japan, Asia and African countries, intermediate rates in Southern European countries, and high rates in North America and Northern European countries. (Seidman & Mushinski, 1983). The incidence rate for England and Wales has risen steadily during the last twenty years and is around 21,000 registrations per year, a similar rise in the number of deaths recorded has also been observed (Brinkley *et al..*, 1984). The distribution pattern of occurrence in terms of age differs from that of most other common epithelial malignancies. A steep rise through to the menopausal age group with a slight plateau between 45-55 and then a slow but steady rise thereafter (Williams & Buchanan, 1987). The age adjusted mortality rate for the United Kingdom as a whole is around 34 per 100,000 women. The disease is the predominant cause of death in women between the ages of 15-74 with 40-54 being the years during which it exceeds all other causes of death.

An American study (Silverberg, 1987) estimated that the lifetime probability of developing breast carcinoma among girls born in 1987, was 10 per cent and eventually dying of breast cancer 3 per cent.

1.3. Prognostic Factors

A thorough review of this field is outwith the scope of this thesis. Many factors have been suggested to play a role. The identification of prognostic factors is critical for two reasons in that it (a) provides invaluable insights into

the biology of breast cancer and, (b) allows first line of therapy to be selected on the basis of expected short or long term survival.

1.3.1 Histological Classification

Several classification systems have been proposed. The most commonly used system is that of the World Health organisation (1981). In essence, this relies on the identification of the degree of tumour cell differentiation compared to normal cell type histology and that of the original germinal cell tissue type. As can be seen from table **1.1**, malignant epithelial tumours comprise the majority of cancers of the breast and can be divided into three parts.

(1) Non-invasive tumours
(2) Invasive tumours of various types
(3) Paget's disease of the nipple

The most common histological subtype is invasive ductal carcinoma followed by invasive lobular carcinoma.

1.3.2. Tumour Grade

The hisological grade of a tumour is normally classified by the Bloom & Richardson technique (Bloom & Richardson, 1957). This involves a subjective assessment of the degree of tubule formation, nuclear pleomorphism, elastosis, and the frequency of hyperchromatic and mitotic figures.

1.3.3. Blood vessel and Lymphatic invasion

The frequency of blood vessel invasion varies greatly (5-45%) (Stewart & Rubens, 1984); prognostic outcome appears to negatively correlate with degree of vascular invasion (Sampat *et al.*, 1977). A similar pattern is also

Table 1.1 Histologcal classification of breast tumours

1. Epithelial Tumours

A. Benign

1. Intraductal papilloma'
2. Adenoma of the nipple
3. Adenoma a. Tubular
   b. Lactating

.

B. Malignant

1. Non-invasive
a. Intraductal carcinoma
b. Lobular carcinoma in situ

2. Invasive
a. Invasive ductal carcinoma
b. Invasive ductal carcinoma with a predominant intraductal component
c. Invasive lobular carcinoma
d. Mucinous carcinoma
e. Medullary carcinoma
f. Pappillary carcinoma
g. Tubular carcinoma
h. Adenoid cystic Carcinoma
i. Secretory (juvenile) carcinoma
k. Carcinoma with metaplasia
i Squamous type
ii Spindle-cell type
iii Cartiliginous and osseeous type
iv Mixed type
l. Others

3. Paget's disease of the nipple

II Mixed connective tissue and epithelial tumours

A. Fibroadenoma
B. Phyllodes tumour (cytosarcoma)
C. Carcinosarcoma

III Miscellaneous tumours

A. Soft tissue tumours
B. Skin Tumours
C. Tumours of hematopoeitic and lymphoid tissues.

IV Unclassified tumours

V Mammary dysplasia/fibrocystic disease

VI Tumour-like lesions
A. Duct ectasia
B. Inflammatory pseudotumours
C. Hamartoma
d. Gynecomastia
e. Others

From WHO (1981) International Histological Classification of Tumours, No2.

seen with the degree of lymphatic invasion (Fisher *et al.*, 1975).

1.3.4. Elastosis

It has been observed that some tumours contain bundles of elastic fibres in the stromal compartment. The significance of their presence is at best controversial. Shivas & Douglas (1972) found improved survival with increased elastosis. This contrasts with the findings of Fisher *et al.* (1980) who found no such correlation. Masters *et al.* (1978;1979) suggested that there may be a correlation between elastosis, menopausal status and the presence of oestrogen receptors (another index of long term survival).

1.3.5. Tumour Necrosis

Tumour necrosis is most commonly seen in high grade (grade III) tumours. Several studies (Canter *et al.*, 1978; Fisher *et al.*, 1978) have shown that the presence of necrosis correlates with poor patient survival.

1.3.6. Staging (TNM)

The characteristics of the primary tumour and the presence or absence of axillary lymph node involvement are strongly correlated with survival. They account for two elements of the TNM system, the third being metastatic disease (see table **1.2**). Developed over thirty five years ago it has been updated by subsequent UICC committees. The rules governing the use of the system are set out in the TNM classification of malignant tumours handbook. They have been reviewed extensively (Israel & Chaniman, 1975; Salvadori, 1984), patients can be grouped into stages (see table **1.3**). With a relatively short follow up period there is a clear correlation between the risk of recurrent disease and increasing stage. (Paterson *et al.*, 1984). Nodal involvement is generally agreed to be the best single prognostic single index.

Table 1.2 TNM staging classification for breast cancer (UICC manual for staging of cancer, 1978)

3.1 Primary Tumour (T)
Clinical-diagnostic classification

Tx Tumour cannot be assessed

TIS Paget's disease of the nipple with no demonstratable tumour(n.b. Paget's disease with a demonstrable tumour is classified according to size of the tumour)

T1* Tumour 2cm or less in greatest dimension
TIa No fixation to underlying pectorial fascia and/or muscle
T1b Fixation to underlying pectorial fascia and/or muscle

T2* Tumour more than 2cm but less than 5cm in greatest dimension
T2a No fixation to underlying pectorial fascia and/or muscle
T2b Fixation to underlying pectorial fascia and/or muscle

T3* Tumour more than 5cm in its greatest dimension
T3a No fixation to underlying pectorial fascia and/or muscle
T3b Fixation to underlying pectorial fascia and/or muscle

T4 Tumour of any size with direct extension to chest wall or skin (n.b. chest wall includes ribs, intercostal muscles, and serratus anterior muscle, but not pectoral muscle)
T4a Fixation to chest wall
T4b Odema (including peau d'orange), ulceration of the skin of the breast, or satellite skin nudules confined to the same breast
T4c Both of the above
T4d Inflammatory carcinoma

Nodal Involvment (N)
Clinical-Diagnostic Classification

Nx Regional lymph nodes cannot be assessed clinically

N0 No palpable homolateral axillary nodes

N1 Movable homolateral nodes
N1a Nodes not considered to contain growth
N1b Nodes consdered to contain growth

N2 Homolateral axillary nodes containing growth and fixed to one or to other structures

N3 Homolateral supraclavicular or infraclavicular nodes containing growth or odema of the arm (n.b. Oedema of the arm may be caused by lymphatic obstruction and lymph nodes may not be palpable)

3.3 Distant Metatases (M)

Mx Not assessed

M0 No (known) distant metastasis
M1 Distant metastasis present

Table 1.3

Stage grouping for breast cancer (UICC Manual for Staging of Cancer, 1978)

In situ cancer (in situ lobular, pure intraductal and paget's disease of the nipple)

TIS

Invasive Cancer

Stage I			
	T1a or T1b	N0 or N1a	M0
Stage II			
	T0	N1b	M0
	T1a orT1b	N1b	M0
	T2a or T2b	N0 or N1a or N1b	M0
Stage III			
	T1 or T1b	N2	M0
	T2a or T2b	N1b	M0
	T3a or T3b	N0 or N1 or N2	MO
Stage IV			
	Any T4	Any N	Any M
	Any T	N3	Any M
	Any T	Any N	M1

1.3.7. Tumour Growth Rate

The favoured method of assessing growth rate is that of mammography. In a study (Galante *et al.*, 1984) of 196 women comparing mammograms at the time of tumour detection to those immediately after surgery (mean interval 30 days), it was found that 16% of the patients had a "fast" doubling time defined as less than 20 days. Almost equal numbers were reported as having " intermediate" (31-90 days) or "slow" (more than 90 days) doubling times. The median doubling time was 60 days, which suggests that breast cancer takes an average of five years to reach 1 cm in diameter. This is relatively slow when compared to other malignancies.

1.4. The Biochemistry of Breast Cancer Growth

Whilst the physical manifestations of breast cancer can yield information on patient survival, it is obvious that this is a reflection of the biochemical events controlling the tumour cell growth, and must therefore depend on those factors which influence these events. From a biochemical viewpoint carcinogenesis relies on the interactions between chemical carcinogens, radiations, oncogenic viruses, inherited genetic factors, mitogenic hormones, and other promotional agents (Bishop, 1987).

One of the first observations that mitogenic hormones may play a role in tumour promotion was that of Beatson (1896), when he noted that some of his patients obtained significant breast disease regression following oophorectomy. Further clinical observations (Huggins & Bergestal, 1952; Luft & Olivecrona, 1955; Dao, 1972; Kennedy, 1974) have indicated that 25 to 40 per cent of breast cancers respond to the surgical removal of endocrine producing glands.

1.4.1. Oestrogens in normal breast tissue

Oestrogens are of major importance in the development of normal breast epithelium. They are essential for the ductal phases of mammary gland

development (Nandi, 1958; Kratochwil, 1987) and administered systematically to castrated female mice cause quiescent ductal end buds to synthesise DNA and effect ductal elongation (Bresciani, 1968). However this effect is negated by the prior removal of the pituitary glands in addition to the ovaries (Lyons, 1958; Lieberman *et al.*, 1978)

These results have been used to postulate that oestrogen does not act directly on mammary tissue, but possibly via an endocrine agent such as growth hormone or prolactin. *In vitro* studies using normal mammary epithelial cell cultures would seem to support this hypothesis (Yang *et al.*, 1980; Stampfer & Bartley, 1987). Oestrogen induced epithelial proliferation has been observed only when epithelial cells are cocultured with mammary stromal cells (MacGrath, 1983; Haslem & Levely, 1985). Supporting the view that oestrogen action is an indirect local mechanism involving close communication between stromal and epithelial components. *In vivo* studies involving the topical application of oestradiol to one nipple area of a mammary gland in monkeys, rabbits and equine pigs promotes lobuloalveolar growth only in the treated gland (Lyons & Sulco, 1940; Chamberlin *et al.*, 1941; Nelson, 1941). Daniel *et al.* (1987) using implanted oestrogen containing pellets, demonstrated local but not distant oestrogen induced alveolar development in mice. Further, the autoradiographic localisation of oestrogen receptors was observed in the ductal, epithelial and luminal cells of the end bud, but not in the proliferation cap cells of the end bud.

The postulation that oestrogen effects target tissue by local indirect mediators draws support from both *in vivo* and *in vitro* studies. If adolescent female mice are "primed" with oestrogen and progesterone, their glands are capable of lobuloalveolar development when transferred to primary tissue culture in response to a combination of hormones and growth factors. Insulin, prolactin, aldosterone, and hydrocortisone, can act along with an extract of "primed" gland to induce *in vitro* development in organ culture. The gland

extract contains epidermal growth factor binding activity. Either epidermal growth factor (EGF) or transforming growth factor alpha (TGFα) can substitute for the activity supplied by the gland extract. EGF and TGFα are both able to promote lobuloalveolar development *in vitro* in isolated ductal end buds from mice (Richards *et al.*, 1982; Vonderhaar *et al.*, 1988). Oestradiol stimulation of uterine growth may depend on both the induction of the EGF receptor and an EGF related growth factor (DiAugustin *et al.*, 1988; Lingham *et al.*, 1988). Cell lines derived from reduction mammoplasties display low levels of oestrogen receptor be they of epithelial or myoepithelial origin, contain high levels of EGF receptor and TGFα. (Hacket *et al.*, 1977; Stampfer, 1985; Le Vay-Young *et al.*, 1987; Rudland, 1987).

In summary, the interaction of multiple hormones including oestrogen regulates mammary gland development. While there is some evidence for oestrogen receptors in the epithelium, it is not clear if growth response of the normal epithelium is in direct response to oestrogen. It is likely that stromal epithelial interactions and growth factor mediators such as TGFα appear to be involved.

1.4.2. Oestrogen role in Breast Neoplasia

Oestrogenic regulation of neoplastic growth is a modified remnant of the normal regulatory mechanisms involved in mammary epithelial proliferation and differentiation. It has been demonstrated that pharmacological amounts of oestrogens can be carcinogenic (Dickson *et al.*, 1980; Fishman, 1980; Fishman *et al.*, 1983; Degan & Metzler, 1987; Metzler, 1987). This resulted from the observations showing increased risk of vaginal cancer in daughters of women who took diethylstilbestrol (DES) to prevent spontaneous abortions.

Oestrogens are mitogenic for both normal and malignant breast epithelia. The hypothalamic pituitary axis is indirectly involved in the control of ovarian oestrogen secretion by virtue of gonadotrophin releasing hormone (GnRH) and

gonadotropin (Ross *et al.*, 1980). In addition the pituitary gland (or other organs) may also secrete other direct or indirect acting mitogens (Eide *et al.*, 1985; Wilding *et al.*, 1987) such as insulin like growth factor II (IGF II), basic and acidic fibroblast growth factor (FGF) and lutenising hormone releasing hormone.

Murine model systems have shown that oestrogen can control breast tumour growth by inducing pituitary hyperplasia and prolactin secretion. Sirbasku has employed the term "estromedin" for other analogous but still hypothetical oestrogen induced endocrine acting mitogens (Peres *et al.*, 1987). Oestrogen might also act by allowing breast cancers to overcome growth inhibitory agents in their environment by synergy with other stimulatory agents (Lykkesfeldt & Briand, 1986; Devleeschower *et al.*, 1987, Soto & Sonneschein, 1987). These interacting components could be serum derived or produced by the cancer itself or nearby tissue.

Studies into the hormonal control of breast cancer have been facilitated by the availability of cell lines usually derived from pleural or ascites fluid of patients. Several oestrogen responsive cell lines exist including MCF-7, T47-D, MDA-MB-134, ZR-75-1, PMC42 and CAMA-1. MCF-7 (Brooks *et al.*, 1973) display an absolute requirement for oestrogen for tumour formation when introduced into athymic(nude) mice (Soule & MacGrath, 1980). *In vitro* hormone sensitivity of cell lines, in particular that of the most intensively studied, MCF-7 have produced conflicting results with regard to oestrogen sensitivity .

Lippman *et al.* (1976) have demonstrated oestrogen receptors and direct responses to physiological doses of 17-β-Oestradiol *in vitro*, this has been confirmed by others (Chablose *et al.*, 1982; Darbre *et al.*, 1983; Katzenellenbogen *et al.*, 1983; Page *et al.*, 1983; Berthois *et al.*, 1986; Nelson *et al.*, 1987; Osborne *et al.*, 1987). Initially a number of other groups have failed to note this response (Edward *et al.*, 1980 ; Butler *et al.*, 1981; Lykkesfeldt & Briand, 1986; Devleeschower *et al.*, 1987; Soto & Sonneschein, 1987). This may be due to studies with an

incorrectly identified or characterised cell line (Osborne *et al.*, 1987). Another problem is a more complete understanding of the relevant variables in culture conditions. Serum is a rich source of oestrogenic compounds including sulphate conjugates, and it has been shown that phenol red may act as oestrogen (Nelson *et al.*, 1987).

Numerous oestrogen independent cell lines have been used as models for hormone unresponsive breast carcinoma such as the adenocarcinoma MDA-MB-231 or the carcinosarcoma HS5787 (Butcher *et al.*, 1981). Whilst these models can be categorised according to their sensitivity to oestrogen. It is worth noting that all are derived from metastatic sites and are fully malignant in that sense. Therefore it is difficult to assess the biological significance when related to premetastatic tumour, or the influence that the metastatic process has had on the original tumour population.

1.4.3. Biochemical effects of Oestrogen

Oestrogen has been shown to induce a large number of enzymes and other proteins involved in nucleic acid synthesis in breast cancer cell lines, including DNA polymerase, C-myc protooncogene (Dubik *et al.*, 1987), thymidine and uridine kinases, thymidylate synthetase, carbamyl-phoshate synthetase, aspartate transcarbamylase, dihydroorotase, glucose-6-phosphate dehydrogenase and dihydrofolate reductase (Edwards *et al.*, 1980; Aitken & Lippman, 1983, 1985; Dickson *et al.*, 1987). Physiological concentrations stimulate DNA synthesis by both scavenger and *de novo* biosynthetic pathways. Oestrogen regulates thymidine kinase and dihydrofolate reductase at the mRNA level (Cowan *et al.*, 1982; Kasid *et al.*, 1986). Oestrogen appears to induce progesterone receptor protein (Horwitz & MacGuire, 1978, Chablos & Rochefort, 1981; Lippman, 1985) and mRNA levels (Read *et al.*, 1988). The progesterone receptor is generally coupled to functional growth regulation by oestrogens *in vivo* and *in vitro* . Progesterone receptor content in breast tumours has been used as a

marker of oestrogen and antioestrogen responsiveness (Nardulli *et al.*, 1988).

Oestrogen has been shown to alter cellular synthesis and/or secretion of several other proteins, whose roles in growth control is unclear. These include various proteases which could contribute to tumour growth and progress by allowing the tumour to digest and transverse the encapsulating basement membrane (Huff & Lippman, 1984; Liotta *et al.*, 1986; Terranova *et al.*, 1986; Butler *et al.*, 1987). Another possibility is that proteases may facilitate the release of active growth factors from carrier proteins, processing inactive precursor growth factors and other proteases to active species (Kaufman *et al.*, 1977). It has been noted that oestrogen induces the secretion of proteins of various molecular weights of which little is known, 24 kilodaton (kDa.) (Cioca *et al.*, 1983), 52 kDa. and 160 kDa. (Westley and Rochefort, 1980; Westley and May, 1987), 37-39 kDa., 32 kDa. (Bronzert *et al.*, 1987; Sheen & Katzenellenbogen, 1987) and 7 kDa. (Jakolew *et al.*, 1984, Nunez et al., 1987). The function of the proteins are still open to conjecture. The 52 kDa. glycoprotein is one of the major secreted proteins and it has beeen indentified as cathepsin D, which has been demonstrated to be mitogenic to MCF-7 cells *in vitro* (Vignon *et al.*, 1986; Caponay *et al.*, 1987).

1.4.4. The Oestrogen Receptor

The actions associated with oestrogen are presumed to be mediated via its specific receptor. Gorski *et al.* (1968) and Jensen *et al.* (1968) from results gained in retention studies using tritiated oestradiol in rat uteri, proposed the now classical "two step" model for oestrogen. In this model binding of oestradiol to its soluble "cytosol" receptor results in translocation to the nuclear compartment, where it was assumed that the hormone receptor complex bound to the chromatin and initiated the responses attributed to oestrogen action. Both the unoccupied and occupied receptor are now believed to reside in the nuclear fraction. Receptor is produced in the cytoplasm by the ribosomal assembly

proteins. The mechanism of transfer to the nuclear matrix is unknown but a sequence conferring karyophilic properties has recently been indentified by Milgrom *et al.* (1990). Welshons *et al.* (1984 & 1985) using cell separation and ligand binding techniques, have demonstrated that "empty" receptors are loosely attached to the nucleus and that tight nuclear binding occurs once hormone binds to the receptor. This has been confirmed immunohistochemically by King & Greene (1984) using a number of monoclonal antibodies (recognising different epitopes), which have shown exclusively nuclear staining in fixed sections of human breast tumours, human uterus, rabbit uterus, and in MCF-7 cells.

Toft and colleagues (Shuh *et al.*, 1985) have shown that receptors for oestrogen and other steroids associate (at least *in vitro*) with a 90 kDa. heat shock protein (HSP-90), which may play a role in stabilisation of the unbound receptor. Loss of HSP-90 binding to the oestrogen receptor is thought to greatly facilitate steroid binding. Work within this laboratory by Chalmers (1990) has demonstrated nuclear migration of HSP-90 upon heat shock. Under these conditions total loss of oestrogen binding was observed, although almost two thirds of total immuno-recognisable oestrogen receptor levels were present. Another protein, a 29 kDa. phosphoprotein may also associate with the oestrogen receptor . Its function is unknown, but its presence in breast cancer appears to correlate with hormone responsiveness (Cano *et al.*, 1986).

cDNA clones for the oestrogen receptor have been obtained from MCF-7 cells (Walter *et al.*, 1985). The mRNA codes for a 66 kDa. protein which contains a long 3'-untranslatable region similar to the glucocorticiod receptor (Gigume *et al.*, 1986). The DNA binding domains of the receptors for oestrogen, glucocorticoid, mineralocorticoid, vitamin D_3, progesterone, and retinoic acid share a strong sequence homology with one of the transforming proteins of avian erythroblastosis virus v-erb-A (Greene *et al.*, 1986; Green *et al.*, 1986; Arriza *et al.*, 1987; deThe *et al.*, 1987; Giguere *et al.*, 1987,1988; MacDonnell *et al.*,

1987; Thomson *et al.*, 1987, Evans, 1988;). The cellular homolog of v-erb-A is known as c-erb-A and is a receptor for thyroid hormones. (Sampat *et al.*, 1986; Weinberger *et al.*, 1986). The DNA binding domains of each receptor is highly conserved (Lumar *et al.*, 1986).

1.4.5. The Oestrogen Receptor in Breast Cancer

Oestrogen receptors are found by ligand binding assay in 50-70% of breast carcinomas. Nuclear receptors are found in 26-46% of cases depending on menopausal status of the patients (Pregoraro *et al.*, 1980; Leake *et al.*, 1984). Nuclear receptors are usually found in tumours with cytoplasmic receptors, but can occur without cytosolic receptors (Leake *et al.*, 1981a).

In terms of patient prognosis, cytosolic oestrogen receptor positive patients have a better prognosis, better disease free interval and overall survival (Osborne & MacGuire, 1978; Stewart & Rubens, 1984). This can be further enhanced by the the addition of data on nuclear receptor presence (Leake *et al.*, 1981b). The inclusion of progesterone receptor presence appears to further enhance predictive response. (Bertuzzi *et al.*, 1981). The significance of steroid receptors in other gynaecological malignancies is still unclear, though progesterone receptors indentify the majority of hormone sensitive tumours in Endometrial (for a review see Soutter & Leake, 1987) and ovarian cancer (Harding *et al.*, 1990)

1.5. Growth Factors Activities in Normal and Malignant Epithelium

It is now becoming clear that the action of oestrogen and other hormones is mediated at least in part by the regulation of local growth control mechanisms. These may be autocrine and/or paracrine in their mode of action. These local mechanisms have been termed growth factors. Experimental evidence suggests that growth factors play extensive roles in the growth of both the epithelial and stromal compartments in the breast.

1.5.1. Insulin and Insulin-like Growth Factors and their Receptors

The insulin family of growth factors is a complex group of cross reacting ligands and receptors. Alternative splicing of the mRNA for insulin-like growth factors, particularly IGF-I (Rotwein, 1986) further adds to the complexity. Insulin is a two chain disulphide linked growth factor, processed from a single gene product whose primary site of synthesis is the pancreas. In contrast, the single chain of (uncleaved 7.5 kDa. size) IGF-I and IGF-II (somatomedins) are synthesised by many body tissues (including the liver) and are under different hormonal regulation particularly that of growth hormone (Daughaday *et al.*, 1987; Hymes *et al.*, 1987). Several other growth factors, such as relaxin and lentropin (which controls lens fibre formation) are members of an even larger insulin related family (Beebe *et al.*, 1987).

Somatomedins are required both for anchorage dependent and independent proliferation of fibroblasts. IGF-I is mitogenic in some breast cancer cells *in vitro* (Furlanetto & DiCarlo, 1984). An IGF-I related species has been found to be secreted by all breast cancer cell lines examined by radioimmunoassay (Baxter *et al.*, 1983; Huff *et al.*, 1986). Oestrogen induction of IGF-I like growth factor has been noted, along with insulin, TGF α and EGF (Huff *et al.*, 1988). Secretion of IGF-I related factors are inhibited by antioestrogen, transforming growth factor beta (2) and glucocorticoids. Growth hormone stimulation of IGF-I in liver, fibroblasts, and other normal tissue is not seen in the MCF-7 cell line (D'ercole *et al.*, 1980; Clemmons *et al.*, 1983; Jansen *et al.*, 1983).

It is unknown whether IGF-I produced by breast cancer acts primarily on breast cancer itself in an autostimularory mode or on the surrounding stroma to promote chemotaxis and growth. Alternatively it may be true that breast cancers induce the surrounding mesenchyme to produce IGF activities which may stimulate the mesenchyme or breast epithelium. Antibody blockade of the IGF-I receptor (Flier *et al.*, 1986) is capable of inhibiting *in vitro* cloning of the

MDA-MB-231 cell line (Rohlick *et al.*, 1981), and tumour growth *in vivo* (Arteaga *et al.*, 1981) suggesting the importance of an autocrine role.

1.5.2. Transforming Growth Factor Beta

TGF β is a 25 kDa. polypeptide initially purified from platelets and various normal tissues. It is required, along with other growth factors, for the full induction of the transformed phenotype in fibroblasts. It is also produced autonomously in fibroblasts transformed by oncogenes (Sporn *et al.*, 1987). TGF β is a member of a multigene family which includes mullerian inhibiting substance, inhibins, and activins (Carter *et al.*, 1986). Breast cancer cells *in vitro*, like normal breast epithelium is inhibited by TGF β, which has been proposed to be an autocrine inhibiting substance in breast cancer (Wang & Hsu, 1986).

TGF β like activity has been noted in breast cancer cells (Knabbe *et al.*, 1988; Derynck *et al.*, 1985) and has been shown to transform AKR-2B and NRK fibroblasts. Comigration on gel exclusion columns with TGF β and immunoprecipitation with TGF β antiserum has been documented (Knabbe *et al.*, 1987). All Breast cancer cell lines analysed express the 2.5 kb mRNA species (Derynck *et al.*, 1987). Secretion is inhibited in the MCF-7 cell line by oestrogen and insulin. Knabbe *et al.* (1987) have shown growth inhibitory antioestrogens and glucocorticoids strongly stimulate its secretion. TGF β also inhibits the growth of an oestrogen receptor negative cell line (MDA-MB-231). Alleviation of inhibition is achieved by the addition of a polyclonal antibody to native TGF β.

The TGF β receptor is a high molecular weight complex of 615 kDa., consisting of two 280 kDa. receptor subunits, together with two other binding subunits of 65 kDa. and 85 kDa. (Cheifetz *et al.*, 1986). The receptor does not appear to contain tyrosine kinase activity (Goustin *et al.*, 1986). Whilst the receptor has not been purified, cloned or sequenced, an alternate gene for TGF β exists (Ikeda *et al.*, 1987). The gene product forms either a homodimeric or a

heterodimeric complex with TGF β, and appears to bind the 280 kDa. receptor species as does TGF β but has a lower affinity for the 65 and 85 kDa. forms of the receptor (Cheifetz *et al.*, 1987; Segarini *et al.*, 1987). It is equipotent in inhibiting epithelial cell proliferation (Ohta *et al.*, 1987). High affinity receptors appear to be present in greater numbers in oestrogen receptor negative breast cancer cell lines. However both oestrogen receptor positive and negative cell lines have been reported to be growth inhibited by both TGF β and TGF β_2 (Zugmaier *et al.*, 1988). The mechanism(s) of action of TGF β is largely unknown.

1.5.3. Platelet Derived Growth Factor

Found in high concentrations in platelets, PDGF is a heterodimeric protein of approximately 30 kDa.. The v-sis oncogene is related to the PDGF B chain homodimer and can transform PDGF receptor containing cell types. In tumours derived from cell types lacking receptor, v-sis is not transforming. PDGF is presumed to act in a paracrine manner, stimulating angiogenesis (desmoplasia), chemotaxis, stromal proliferation and degranulation of monocytes and neutrophils (Ross *et al.*, 1986).

Many breast cancer cell lines secrete PDGF related activity as detected by anchorage independent growth stimulation of mouse 3T3 fibroblasts in the presence of platelet poor plasma (Rosengurt, 1986); 25 kDa. and 16 kDa. species of PDGF have been observed by immunoprecipitation of metabolically labelled MCF-7, MDA-MB-231 and other cell lines. Upon examination of Poly-(A)-selected mRNA from either cell line, transcripts of both PDGF A and B chains were observed (Rosengurt *et al.*, 1985; Betscholtz *et al.*, 1986; Bronzert *et al.*, 1987; Peres *et al.*, 1987)

It is likely that in breast cancer, oestrogen induced PDGF (along with other growth factors) act in a paracrine manner on fibroblasts and other surrounding tissue. This could result in the proliferation of fibroblasts, and further enhance tumour growth by released mediators such as IGF-I. It is

possible that fibroblast derived IGF-I might be one of the stromal factors required *in vivo* to initiate all the effects associated with oestrogen action on epithelial proliferation. Ro *et al.* (1987) have reported that there is a correlation between PDGF mRNA expression and the degree of stromal desmoplasia in primary breast cancer.

1.5.4. Fibroblast Growth Factor

Members of this family include acidic and basic fibroblast growth factor (aFGF and bFGF) (Esch *et al.*, 1985), Kaposi's FGF (Ks FGF), and Int 2 (Delli Bovi et al., 1987; Dickson & Peters, 1987), a more distant homology exists with interleukin 1 (IL-1) (Thomas *et al.*, 1985). Both aFGF and bFGF bind to a 140-210 kDa. receptor and stimulate its tyrosine kinase activity (Coughlin *et al.*, 1988). bFGF is capable of acting as an oncogene when expressed in fibroblasts (Rogel *et al.*, 1988; Sasada *et al.*, 1988). Uncertainty exists as to the principal target of FGF in normal mammary glands. Rudland (1987a) proposed that the effects are restricted to the myoepithelium and stroma, but Karey and Sirbasku (1988) have found that MCF-7 and T47-D cell lines respond.

1.5.5. Prolactin

Prolactin (PRL) has been proposed to have a role in human breast cancer both in terms of a marker of breast cancer risk and due to its ability to stimulate breast cancer cell growth *in vitro* (see section **4.3.1**). PRL is a 22 kDa. polypeptide secreted by the hypothalamic pituitary axis. Recently it has been shown that in addition to the 22 kDa. form, other forms exist. A 16 kDa. form which is extremely mitogenic to breast cancer cells in culture (Mittra, 1980), whilst being lactogenic (Clapp *et al.*, 1988) appears to have some different properties from the intact hormone. It inhibits the proliferation of endothelial cells in culture, while the 22 kDa. form is inactive (Ferrara *et al.*, 1988). Studies by Clapp *et al.* (1989) have revealed that iodinated 16 kDa. fragment specifically

bound to membranes of kidney, liver, brain and skeletal muscle of the rat. Five other novel peptides within the molecular weight range of between 13 - 18 kDa. which appear to be related to PRL have been reported (Sinha *et al.*, 1989). In several mammalian species including man, these peptides have been detected but their physiological properties are still unknown. Polymeric forms of PRL have also been found which appear to have a reduced biological action (Fraser et al., 1989; Jackson *et al.*, 1989).

Some of the better known functions of PRL include osmoregulation and electrolyte metabolism in fish (Loretz & Bern, 1982), pigeon crop sac mucosa growth and differentiation (Horseman, 1987), mammary gland growth and lactation (Topper, 1970). Luteotropic and luteolytic functions in the ovary (Gitay-Goren *et al.*, 1989; Krasnow *et al.*, 1990), growth and secretion of the male sex accessory organs (Bartke & Lloyd, 1970) and maternal and parental behaviour in birds and rodents (Bridges *et al.*, 1985; Horseman, 1987). Recent studies also indicate that PRL has an immunoregulatory role involving T lymphocyte modulation (Russel *et al.*, 1985; Heistad *et al.*, 1986; Bernton *et al.*, 1988; Hartmann *et al.*, 1989).

1.5.5.1. Regulation of Prolactin

The regulation of PRL secretion is controlled largely by inhibitory factors , partial purification of these factors has been achieved but complete characterisation has not. It is recognised that dopamine inhibits secretion both *in vitro* and *in vivo* possibly through a pertussis toxin sensitive G protein which is cAMP independent (Boyd *et al.*, 1988). There is a suggestion that dopamine itself is a physiological PRL release inhibiting factor (PIF) (Neil, 1980). Thyrotrophin releasing hormone (TRH), vasoactive intestinal peptide (VIP) , opiate peptides, enkephalins and β-endorphin are known to cause secretion of PRL. In addition, PRL release is induced by suckling (Neill, 1974), stress (Neill, 1978) and ovarian hormones primarily oestrogen (Veldhuis *et al.*,

1989; Brann *et al.*, 1989). Recent data by Carlson (1989) indicated that dietary factors such as amino acids particularly tyrosine and phenylalanine can play a role in stimulating PRL secretion.

The biochemical mechanisms involved in PRL release appear to involve the interaction of intracellular mechanisms such as cAMP adenlylate cyclase, phospholipid hydrolysis and arachidonate metabolism (MacLeod *et al.*, 1986). However there is some debate as to the role of protein kinase C (PKC). Whilst it is clear that phorbol ester (TPA) addition can stimulate PRL secretion in pituitary cell in culture (Boyd & Wallis, 1989), as studies by Judd *et al.* (1989) have shown that chronic exposure by PKC activators such TPA and phorbol dibutyrate did not effect stimulation of secretion by other prolactin secretagogue such as thyroliberin, angiotensin II and neurotensin.

1.5.5.2. Prolactin Receptor (PRL-R)

PRL belongs to a family of hormones including growth hormone (GH), placental lactogen and proliferin (Linzer & Nathans, 1984). Nicoll (1982) described the complications inherent in the estimation of PRL-R using heterologous ligand binding assays. The main objection is that, in some cases, ovine PRL can bind in addition to the PRL-R other members of the PRL family's receptors resulting in false estimates of concentration and affinity. Little data is at yet available as to the nature of the PRL-R.

Immunoprecipitation studies reveal that the PRL-R in both rabbit and pig mammary tissue is composed of a single PRL binding subunit of 42 kDa. which may be covalently attached to other units of a higher molecular weight 70 - 80 kDa. (Dusanter-Fourt *et al.*, 1987). Ligand crosslinking studies in the rabbit mammary gland reveal both a 34 kDa. receptor and a 78 kDa. receptor which was directly regulated by bivalent cations (Sakai & Khomoto, 1989). Binding of PRL to its receptor appears to be a hydrophobic process (Sakai & Suzuki, 1989). To our knowledge no cloning of the specific genes for PRL-R and establishment of the

amino acid sequence has yet been published. However a cDNA clone for the receptor precursor has been isolated from rat liver (Boustin *et al.*, 1988). The predicted receptor precursor is 310 amino acids which is similar to that of the GH receptor (Leung *et al.*, 1987).

No data is as yet available as to the mechanisms by which the hormone binding signal is transduced. Characterisation of the cDNA clones available has not pointed to the their possible modes of action. To further complicate the elucidation process Davis & Linzer (1989) have demonstrated multiple forms of the receptor which may be part of distinct signal transduction pathways. Few reports are as yet available as to the mechanisms of receptor regulation. This is partly due the lack of antibodies to the receptor and appropriate mRNA probes.

1.5.5.3. Biochemical effects of Prolactin

In addition to its actions on the phosphatidylinositol pathway (see **4.3.9**). PRL may play a role in the adenylate cyclase signal transduction system. Strong evidence has accumulated that PRL acts through a cholera toxin sensitive G protein(G_s) when mitogenically stimulating NB2 lymphoma cell line (Larsen *et al.*, 1988; Too *et al.*, 1989; 1990).

1.5.5.4. Prolactin Summary

To summarise, PRL has many effects within different organisms and target tissues. There is some confusion as to what actually constitutes the native form of the hormone. The precise nature of the receptor and receptor ligand interaction has yet to be elucidated. The biochemical events associated with ligand binding have still to be studied in greater detail.

1.5.6. Other Pituitary Hormones, Steroids and Growth Factors

In vivo growth factors and/or oestrogen are thought to act in concert with other systemic mitogens to promote tumour growth. Dembinski *et al.* (1985)

isolated pituitary derived activity which enhances the mitogenic effects of oestradiol in MCF-7 cells. One pituitary factor already identified is IGF-II (Shiu *et al.*, 1988). In addition pituitary derived GnRH may also directly interact with breast cancer to inhibit its proliferation (Eidne *et al.*, 1985).

MCF-7 cells in culture display growth regulation by a variety of lipid-soluble hormones in addition to oestrogen. These include glucocorticoids, iodothyronines, androgens, and retinoids (for a review see Lippman, 1984). Progesterone induces a specific protein and can be inhibitory *in vitro* (for a review see Rochefort, 1987); other inhibitory hormones include somatostatin (Setyono-han *et al.*, 1987), Interleukin-1 and Interleukin-6, tumour necrosis factor and interferon (for a review see Sporn & Todaro, 1988). Receptors and metabolic effects but little growth response has been shown for other hormones, such as growth hormone, glucagon, and calcitonin (Lippman, 1984). Transferrin, a serum iron delivery molecule, is required for proliferation of normal and malignant mammary cells (Hanover & Dickson, 1985). Its receptor is increased in oestrogen independent breast cancer (Tonik *et al.*, 1986). It is clear that the complexity of growth modulatory hormones observed in studies using *in vitro* breast cancer models, suggest that many serum borne or locally produced modulators of growth may play important regulatory roles *in vivo*. Alternatively, or in addition, growth factors with a similar spectrum of activities could be elaborated by the breast cancer cells themselves.

1.6.1. Epidermal Growth Factor, Transforming Growth Factor Alpha and their Receptor.

Whilst the bulk of this thesis is concerned with the EGF receptor, it is appropriate that the ligands associated with the receptor cited in the literature be outlined. Particular emphasis has been placed on EGF and TGFα. Other ligands for EGF receptor have been described: amphiregulin, isolated recently from serum-free conditioned medium of MCF-7 cells (Shoyab *et al.*, 1989); and the

poxvirus growth factors, especially vacinnia virus growth factor (Buller, 1988).

1.6.2. Epidermal Growth Factor ; Structure.

First identified in mouse submaxillary glands (Cohen, 1962), homologous human EGF (hEGF) originally designated as urogastrone was isolated from urine (Savage *et al.*, 1972; Gregory, 1975). Native hEGF is a peptide of 53 amino acid residues containing three disulphide bridges (see figure **1.1**), with a molecular weight of 6200 amu as assessed by tandem mass spectrometry (Furuya *et al.*, 1989).

Analysis of cDNA clones derived from mRNA transcripts encoding murine EGF and hEGF, indicate that both peptides are derived from proteolytic processing of a larger precursor molecule. The size of this has been estimated to be 1217 and 1207 residues respectively (Gray *et al.*, 1983; Scott *et al.*, 1983; Pfeffer & Ullrich, 1985). The human EGF gene is located on chromosome 4 (Brissenden *et al.*, 1984; Zabel *et al.*, 1985). Predicted cDNA sequence suggests that hEGF precursor is an integral membrane protein having an N-terminal extracellular domain of 1,012 amino acids, a single transmembrane domain of 25 amino acids, and a C-terminal intracellular domain of 150 amino acids. The EGF moiety is in the extracellular domain immediately adjacent to the membrane spanning region. A further eight regions containing partial sequence identity with EGF have also been noted (Gray *et al.*, 1983; Doolittle *et al.*, 1984).

Sequence similarity between the LDL receptor and the murine EGF precursor has led to the suggestion that the precursor may be a receptor for an as yet unknown ligand (Russel *et al.*, 1984). mRNA for prepro-mEGF has been found in many murine tissues, including the submandibular gland, kidney, mammary gland, pancreas, duodenum, pituitary, lung, spleen, brain, ovary, and uterus (Rall *et al.*, 1985). The observation that the precursor is present in the distal tubule in preference to the native form suggests other physiological functions. Studies using NIH 3T3 cells transfected with kidney hEGF precursor cDNA (Mrcozkowski *et al.*, 1988), have shown that the expressed precursor could

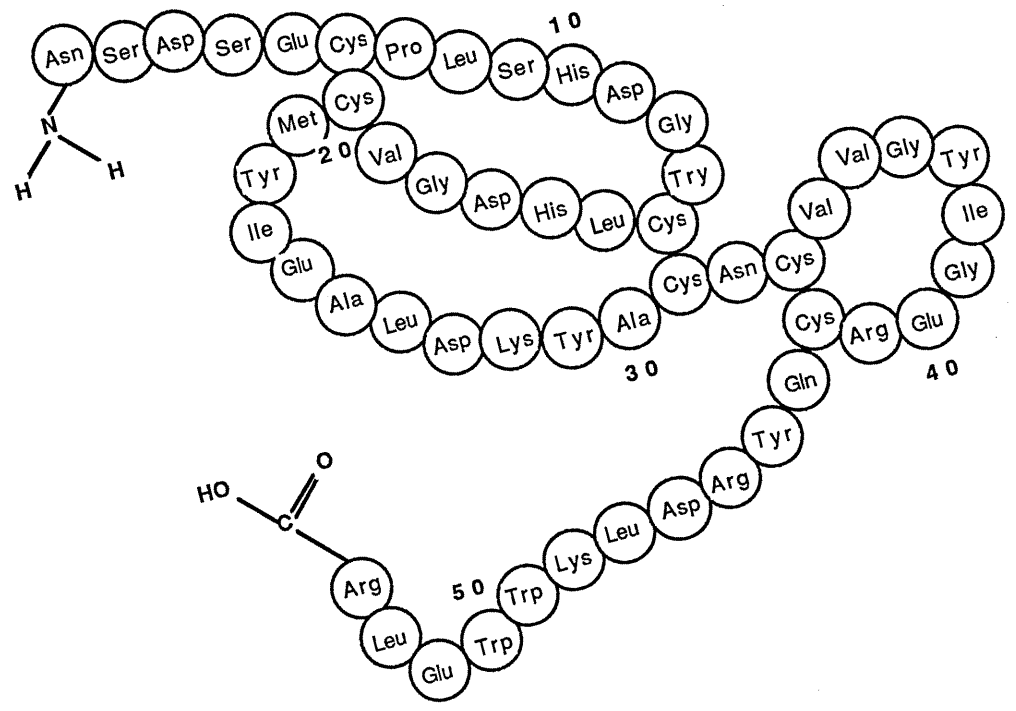


Figure 1.1 Primary Structure of Human EGF

compete with hEGF for receptor binding, could activate receptor, and sustain growth in an EGF dependent cell line (Mroczkowski *et al.*, 1989). Thus proteolytic processing may not be essential for activity.

Structure and function studies of the native form, by Katsura & Tanak (1989), using monospecific polyclonal antibodies directed against specific epitopic regions to hEGF, suggest that region 22-32 is responsible for binding to the receptor and signal transduction, and regions 35-53 stabilise the ligand receptor interaction.

1.6.3. Biological/Biochemical effects of EGF

Since its discovery EGF has been implicated in a number of biochemical and biological responses both *in vivo* and *in vitro*. In contrast to TGF α, little impetus has been placed on EGF action in the breast. Data derived form other model systems may still have a relevance within the breast (for reviews see Carpenter & Cohen, 1979; Stoschek & Carpenter, 1983; Staros *et al.*, 1985; Carpenter *et al.*, 1986).

These include the *in vitro* stimulation of the phosphatidyl inositol (PIP) pathway in mouse mesenchyme cells (Chepenik & Haystead, 1989), in rat hepatocytes (Johnson & Garrison, 1987) and in the A431 human squamous carcinoma cell line often used for EGF receptor studies (Hepler *et al.*, 1987; Pike & Eakes, 1987). Activation of protein kinase C which is associated with increased PIP pathway turnover has also been noted (Moscar *et al.*, 1988). Increases in cytosolic Ca^{++} via the PIP pathway, which appears to be necessary for EGF stimulated mitosis, have also been cited in A431 cells (Mozhayva *et al.*, 1989), in the human hepatocellular carcinoma derived cell line PLC/PRF/8 (Gillligan *et al.*, 1988), in the normal rat kidney cell line NRK-49F (Marks *et al.*, 1988), and rat fibroblasts (Muldoon *et al.*, 1988).

Shelton Earp and colleagues (1986) demonstrated that EGF can stimulate the synthesis of its own receptor in the rat hepatocyte cell line WB, at both the

protein and mRNA levels. Moeller *et al.* (1989) have reported EGF induction of TGFα mRNA in both intact bovine anterior pituitary gland and in tissue culture. Other responses have been noted, these include stimulation of plasminogen activator secretion and mRNA in hepatocytes (Lucore *et al.*, 1988), granulosa cells (Galway *et al.*, 1989), A431 cells (Niedbala & Sartorelli, 1989) and in the human colon carcinoma cell line GEO (Boyd & Brattain, 1989).

Release of arachidonic acid has been observed upon EGF treatment in mouse embryonic cells (Chepenik, 1989) probably via phospholipase A_2 activation (Bonventre *et al.*, 1990), which may be essential for EGF induced mitogenesis (Handler *et al.*, 1990). Ornithine decarboxylase activation has been reported in A431 cells (Yazigi *et al.*, 1989) and in rat colonic explants (Arlow *et al.*, 1990). Stimulation of casein kinase II has been seen in A431 cells (Ackerman *et al.*, 1989,1990). and both the mouse adipocyte cell line 3T3-LI and rat hepatoma cells H4-IIE (Sommercorn *et al.*, 1987).

Whilst EGF has been noted to stimulate synthesis of its own receptor protein in MDA 468 cells (Kudlow *et al.*, 1986). The majority of studies in breast cancer models have centred around its mitogenic effects. Davidson *et al.* (1987) presented evidence that EGF induced mitogenesis was inversely proportional to EGF receptor concentration of the cell line studied.

Addition of EGF has been shown to alleviate antioestrogen inhibition of cell growth in MCF-7 (Cormier & Jordan, 1988) and T-47D cells (Koga & Sutherland, 1987). Recently Koga and colleagues demonstrated alleviation from progestin inhibition (Koga *et al.*, 1989). Loss of both oestradiol and progesterone binding has been observed upon EGF treatment in MCF-7 cells (Cormier *et al.*, 1989). This type of data has led to the supposition that in the heterogeneous tumour mass, the effects of antioestrogen or progestins can be negated by paracrine growth factor production within the stromal or ER negative portion of the tumour.

Little data is as yet available concerning the regulation of EGF in breast cancer and its possible functional significance. Murphy *et al.* (1988) have

demonstrated regulation of EGF mRNA in T47-D cells by progestins suggesting possible hormonal control.

A study by Kurachi *et al.* (1985) demonstrated a direct correlation between increased mammary tumourigenesis and increases in EGF concentration in the submandibular gland of female virgin mice (62.5%, n=48). Removal of the gland (sialoadenoectomy) reduced incidence considerably (12.8%, n=39), reinfusion of sialoadenoectomised mice with EGF resulted in increased tumour incidence. Sialoadenoectomy of mice bearing tumours resulted in rapid sustained cessation of growth, reinfusion resulted in the restoration of tumour growth. Thus within murine models EGF has a role in the promotion and subsequent growth of mammary carcinoma.

1.7. Transforming Growth Factor Alpha

De Larco and Todaro (1978) were the first to isolate TGFα in a spectrum of growth promoting factors from solid tumours, which cause reversible transformation of normal cells. TGFα has been demonstrated in a variety of retrovirally transformed cells, human tumours, and embryonic cells (Ozanne *et al.*, 1980; Todaro *et al.*, 1980; Twardzik *et al.*, 1982).

1.7.1. TGF alpha , Structure

Mature human TGFα is a (see figure **1.2**) single chain polypeptide of 50 amino acid residues (Derynck *et al.*, 1984). With a molecular weight of approximately 6 kilodaltons and is constrained into a three ring structure by disulphide bonds between six cysteine residues (Marquadt *et al.*, 1983; Lee *et al.*, 1985), which are essential for biological activity (Lazar *et al.*, 1989).Further recent studies by Defeo-Jones and colleagues (1989) have revealed that elimination of arginine residue 42 also results in the loss of biological activity, suggesting that this residue may be a key contact point in ligand receptor interaction. Human TGFα shares a 27% amino acid sequence homology with

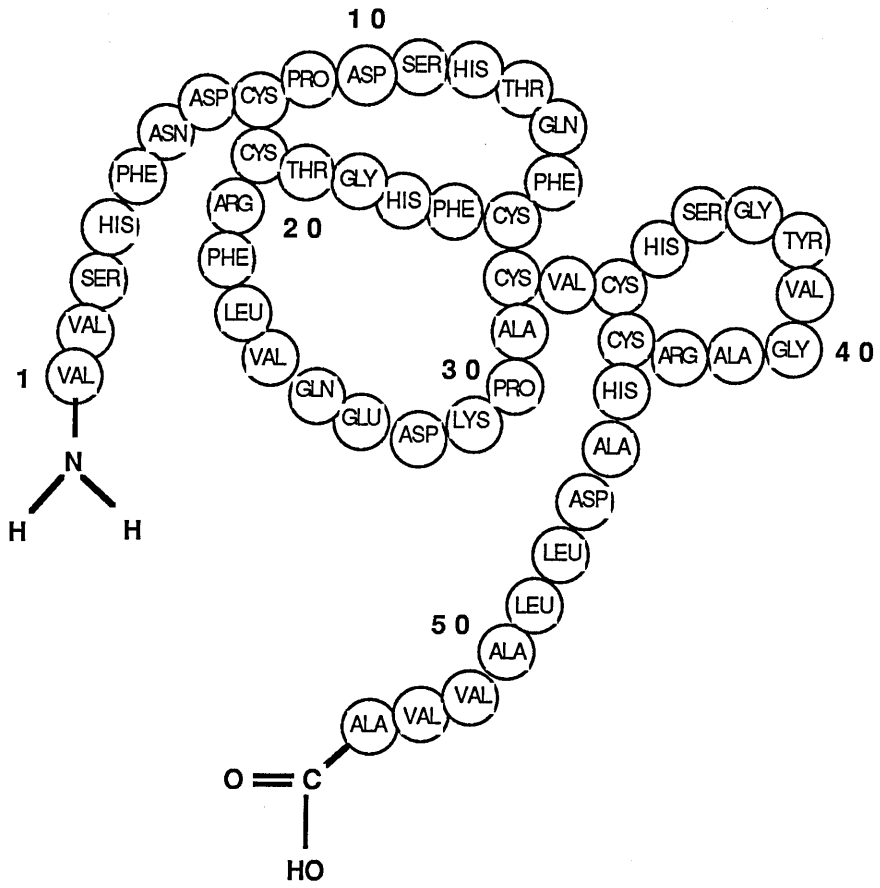


Figure 1.2 : Primary Structure of Human TGF alpha

murine EGF and a 37% homology with human EGF. Three dimensional studies by Kohda and co-workers (1989) indicate that the gross structure of human TGFα resembles that of a "mitten". Receptor ligand interaction occurs in the manner of a "mitten" gripping an object (see figure **1.3**), similar to that proposed earlier for murine EGF (Kohda *et al.*, 1988). The area of greatest sequence heterogeneity is located at the rear of the "mitten" suggesting that the N-terminal polypeptide segment is not involved in receptor binding.

The gene for TGFα is located on chromosome 2 (Brissenden *et al.*, 1985). cDNA cloning has revealed that human (Derynck *et al.*, 1984) and rat (Lee *et al.*, 1985) TGFα are synthesised as highly conserved (>90%) precursor proteins of 160 and 159 amino acids respectively. Both precursors (proTGFα) contain an extracellular N-terminal sequence of 23 unchanged apolar amino acids that may function as a signal peptide, followed by a domain of 74 or 75 amino acids which includes the mature growth factor and possible glycosylation sites, a 23 amino acid hydrophobic transmembrane domain with, finally a cysteine rich sequence of 39 amino acid residues at the COOH-terminal which constitutes the cytoplasmic domain..

It has been proposed that the presence of transmembrane sequence is a sign that pro-TGF α is synthesised as a transmembrane molecule and that the frequent size heterogeneity encountered is due to differential proteolytic cleavages of the external precursor domain and to N- and O- glycosylation of the larger precursor forms (Bringham *et al.*, 1987; Gentry *et al.*, 1987; Teixido *et al.*, 1988). The observation that other integral membrane proteins contain EGF/TGF α like repeats in their extracellular domains, such as *Drosophila Notch* (Wharton *et al.*, 1985) and *Caenorhabditis elegans* lin-12 (Greenwald *et al.*, 1985), both of which are known to play roles in cell-cell interaction, has prompted interest in the biological action of pro-TGFα.

Wong *et al.* (1989) has demonstrated BHK cells transfected with vectors coding for pro-TGFα which are resistant to proteolytic cleavage, resulted in

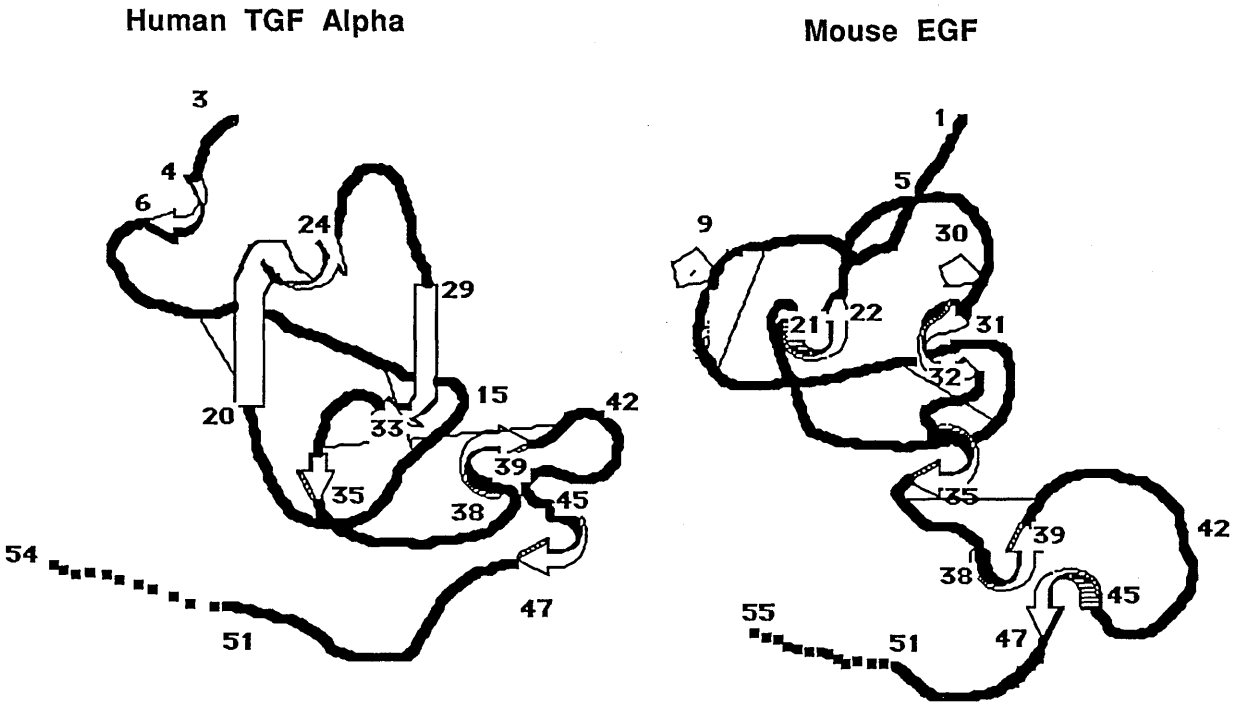


Figure 1.3

. Simplified schematic drawing of the backbone topology of. human TGF alpha. The arrowed ribbons represent the location and direction of ß-sheet strands. The backbone toplogy of murine EGF reported previously is also displayed. Binding of ligand to receptor has been proposed to act in the manner of a mitten grasping an object, with Arg 42 being the thumb and the key to binding.

immunocytochemical identification of pro-TGFα on the cell surface. Coincubation with A431 cells resulted in EGF receptor autophosphorylation. Brachmann and group (1989) using a TGFα precursor which had undergone site directed mutagenesis of two extracellular cleavage sites overexpressed in an EGF receptor negative cell line CHO, revealed immunocytochemical localisation of the mutant TGFα precursor on the cell membrane. Solubilised precursors from this source could induce autophosphorylation in a cell line overexpressing EGF receptors NIH 3T3 and anchorage independent growth in NRK fibroblasts. Coculture of the transfected cell line with cells expressing high levels of EGF receptor, A431 and HERc, resulted in receptor activation as assessed by autophosphorylation.

1.7.2. The role of TGFα in the Transformation Process

Like EGF, TGFα (although less effectively), has been noted to induce c-fos and c-myc expression in CH 10T1/2 cells (Cutry *et al.*, 1988). Brenner and colleagues (1989) have demonstrated mitogenesis in quiescent primary rat hepatocyte cultures, with a transient increase in the proto-oncogene c-jun and increased rates of Na^+ uptake. Studies last year from MacGeady *et al.* (1989) have demonstrated that transfection with a TGFα expression vector results in the transformation of the normal mouse epithelial cell line NOG-8, but not the normal rat fibroblasts. Recent work by Matsui *et al.* (1990) has shown that expression of human cDNA under the control of the mouse mammary tumour virus enhancer/promoter, led to a range of tumour abnormalities including adenoma, adenocarcinoma, lobularhyperplasia and cystic hyperplasia in transgenic female mice. No such abnormalities were observed in transfected male mice. Studies by Jhappan *et al.* (1990) and Sandgren *et al.* (1990) using transgenic mice transfected with an expression vector containing the mouse metallothionein/TGFα fusion protein, reported that where TGFα overexpression occurs epithelial abnormalities can be seen.

1.7.3. TGFα in Human Breast Epithelial cells *in vitro*

Exogenous TGF α is a relatively strong growth promoting agent for the non-transformed 184A1N4 mammary epithelial cell line and for the oestrogen responsive MCF-7, ZR-75-1 and T47-D cell models under serum free conditions (Salomon *et al.*, 1986; Novak-Hoffer *et al.*, 1987; Karey & Sirbasku, 1988; Valverius *et al.*, 1988). It has been shown that EGF or TGFα can partly attenuate anti-oestrogen induced growth inhibition in MCF-7 and T-47D cells (Koga & Sutherland, 1987; Arteaga *et al.*, 1988; Murphy & Dotzlaw, 1989)

A number of breast cancer cell lines have been screened to ascertain if they synthesise and secrete immunoreactive and biologically active TGFα. Biologically active TGFα was originally detected in the conditioned medium (CM) from a number of different MCF-7 clones and in extracts prepared from MCF-7 xenografts (Salomon *et al.*, 1984; Dickson *et al.*, 1985; Dickson *et al.*, 1986). Subsequent demonstration of comparable biological and immunological activity can be found in the CM of ZR-75-1, T-47D and MDA-MB-231 cell lines (Perroteau *et al.*, 1986) which appears represent both the 30 kDa. and the 6 kDa. forms (Dickson *et al.*, 1986; Bates *et al.*, 1988). 17 β-Oestradiol has been shown to induce a dose dependent 3 to 5 - fold increase in the secretion of TGFα protein and mRNA expression in MCF-7 cells over a physiological concentration range known to be growth promoting to these cells (Bates *et al.*, 1988). This has also been demonstrated in both ZR-75-1 and T-47D cells (Murphy & Dotzlaw, 1989) and *in vivo* with differentiated rat mammary tumours (Liu *et al.*, 1987). It should be noted however that antibody blockade of TGFα binding still resulted in oestradiol mediated mitogenesis in the MCF-7 cell line (Arteaga *et al.*, 1988). Further studies by Clarke *et al.* (1989) have shown that the introduction of a TGFα expression vector into MCF-7 cells both *in vitro* and *in vivo* in tumour xenografts did not appreciably affect their ability to respond mitogenically to oestrogens. But the possibility remains that TGFα may still play a role in mediating oestrogen action.

As a rule the highest levels of TGFα activity are found in oestrogen receptor negative breast cancer cell lines such as MDA-MB-231 and MDA-MB-468 (Perroteau *et al.*, 1986; Bates *et al.*, 1988; Zajchowski *et al.*, 1988; Bjorge *et al.*, 1989). Expression of TGFα mRNA and biologically active TGFα protein can also be found in normal non-immortalized populations of human mammary epithelial cell strains (Zajchowski *et al.*, 1988; Valverius *et al.*, 1989). The 184 cell strain secretes TGFα at a level comparable to and even greater than that of some breast cancer cell lines. It has been proposed that mRNA expression of TGFα in the 184 and the 184 A1N4 variant is related to cell proliferation rate (Salomon *et al.*, 1989). This is not the case in several oestrogen receptor positive and negative cell lines investigated (Murphy & Dotzlaw, 1989). Up regulation of TGFα production by the addition of exogenous EGF or TGF α has been observed in both normal 184 A1N4 cells (Salomon *et al.*, 1989) and in the MDA-MB-468 breast cancer cell line (Bjorge *et al.*, 1989) through what appears to be a autoinductive mechanism.

MacNeill *et al.* (1986) have shown that both EGF and TGFα stimulate oestradiol 17-βhydroxysteroid dehydrogenase, increasing the conversion rate of oestrone to oestradiol in cultured human adipose tissue. This may account for the observations that oestradiol concentrations are significantly higher in breast tumours compared to normal tissue (Bonney *et al.*, 1983; Van Landeghan *et al.*, 1985).

1.7.4. TGFα in Human Breast Tissue

Expression of TGFα is not limited to normal or malignant epithelial cells *in vitro*. Immunoreactive TGFα ranging from 1 - 7 ng/mg protein has been found in normal breast tissue, fibroadenomas, fibrocystic lesions, and 22 primary infiltrating ductal carcinomas (Perroteau *et al.*, 1986). Macias *et al.* (1989) found that out of 45 mammary ductal carcinomas, around 31% contained biologically active TGFα. Gregory *et al.* (1989) observed that in positive primary breast tumours the majority of the biologically active peptide is in the 6 kilodalton

form, subsequent tamoxifen treatment resulted in a ten - fold decrease in tumour associated TGFα. Expression of TGFα mRNA has been noted in a variety of human tumours (Derynck *et al.*, 1987). Using slot blot and northern blot analysis Bates and colleagues (1988) demonstrated the presence of TGFα mRNA in 70% of the 40 tumours analysed.

In situ hybridisation studies by Ciardello *et al.* (1989) have demonstrated the presence of TGFα mRNA in the tumour cells of some primary infiltrating ductal carcinomas. Southern blot analysis of these tumours provided no evidence for any major rearrangement or gross amplification of the TGFα gene when compared to patient matched peripheral tissue.

Urinary immunoreactive TGFα has been detected in patients with disseminated breast cancer, which contrasts with the negative findings in the control group (Stromberg *et al.*, 1987). This has led to the proposal that a urinary TGFα assay could complement mammography (Feig, 1984) as a useful method for early breast cancer detection.

1.8. The Epidermal Growth Factor Receptor (Isolation and Structure)

The A431 cell line (Giard *et al.*, 1973) which overexpresses the receptor (EGFR) greatly facilitated the isolation and biochemical characterisation of EGFR including cDNA cloning (Haigler *et al.*, 1978; Stoschek & Carpenter, 1983). Whilst there is no reason to doubt the information derived from this cell line, EGF inhibition of growth is noted in most circumstances (Gill & Khazar, 1981). However, many of the early responses to EGF are representative, possibly exaggerated, of the "normal" mitogenic response (see section **1.6.3**).

Using affinity chromatography Cohen *et al.* (1980) purified EGFR from A431 cells to near homogeneity. The purified receptor had an apparent molecular mass of 150 kDa., but subsequent purification procedures using buffers which eliminated calcium indicated a mass of 170 kDa. (Cohen *et al.*, 1982). This report corresponded to studies using chemically crosslinked [^{125}I] - EGF by other

groups in the field (Das *et al.*, 1977; Hock *et al.*, 1979; Wran & Fox, 1979). It has been reported that most cell homogenates contain a calcium activated protease which cleaves EGFR to a 150 kDa. form (Cassel & Glasser, 1982; Gates & King, 1982). Prior to the isolation of EGFR, it was observed that the addition of EGF to A431 cells resulted in a rapid activation of specific tyrosine kinase activity (Ushiro & Cohen, 1980).

Sequence analysis of tryptic peptides derived from EGFR (Downward *et al.*, 1984) led to cDNA cloning of the receptor and a deduced amino acid sequence of the entire molecule (Ullrich *et al.*, 1984). The mature receptor is composed of three major regions (see Figure **1.4**). A large glycosylated extracellular ligand binding region of 621 amino acid residues, which is anchored by a single transmembrane spanning region of 23 hydrophobic amino acids, followed by the internal cytoplasmic region of 543 amino acids, which contains the kinase domain displaying consensus residues typical of the tyrosine kinase gene family (for a review see Ullrich & Schlessinger, 1990). The Lys-721 residue and consensus sequence Gly-X-Gly-X-Phe-Gly-X-Val- located 15 residues upstream to the Lys residue probably function as part of the ATP binding site (Ruso *et al.*, 1985) in the kinase domain.

1.8.1. Ligand Binding

Quantitative binding experiments with radiolabelled EGF indicate that the stoichiometry of ligand binding to EGFR is 1:1 (Weber *et al.*, 1984). However analysis of ligand binding experiments of [^{125}I] - EGF to intact cells according to Scatchard reveal non linear plots, which are interpreted as an indication of different receptor classes with distinct affinities towards EGF. High affinity EGF receptors with an apparent K_d of 1-3 x 10^{-10}M comprise 5-10% of the total receptors, while the remaining low affinity have an apparent K_d of 2-15 x 10^{-10}M (King & Cuatrecasas, 1982). Whilst Scatchard analysis is an invaluable tool in assessing receptor affinity and number, a few problems should be noted

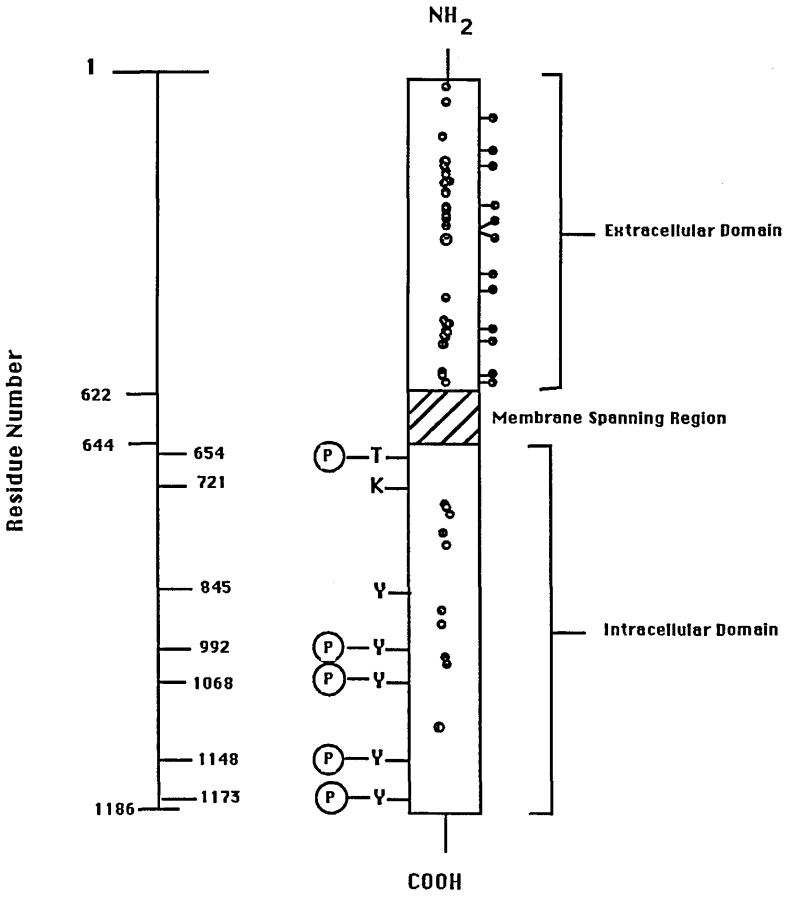


Figure 1.4

Structural features of the Epidermal Growth Factor Receptor. the following symbols are shown:

N- Linked glycosylation sites —●

Cysteine Residues ◐

Tyrosine Residues Y—

Phosphotyrosine Residues (P)—Y—

Phosphothreonine Residues (P)—T—

Lysine Residues K—

which can influence that assessment. In the case of high receptor concentrations it has been demonstrated that unless the receptor levels are 10 - fold less than the Kd, the "apparent" Kd will become a linear function of receptor concentration. Other problems can arise in data extrapolation; heterologous ligand populations can produce non linear plots (Taylor *et al.*, 1975). resolving the curve for the identification of low and high affinity receptors is subject to error (Delean *et al.*, 1981); method of ligand labelling(Carpenter, 1987).

The ligand binding site of EGF receptor has still to be established. Studies using EGF competitive monoclonal antibodies suggest that the region between Ala -351 and Asp- 264 are crucial in ligand binding (Wu *et al.*, 1989). Chimeric receptor studies by Lax and group (1988,1989) proposed that the extracellular area, designated domain III, which encompasses the above region was essential for optimum binding.

1.8.2. Receptor Biosynthesis

The gene for EGFR is located on the short arm of human chromosome 7 (Davies *et al.*, 1980; Kondes & Shimizu, 1983) in the p12-p14 region (Merlino *et al.*, 1985). Normal and transformed cells that express EGFR have two mRNA species of 10 Kb and 5.6 Kb which hybridise with cDNA probes (Xu *et al.*, 1984; Ullrich *et al.*, 1984). Translation of EGFR mRNA *in vivo* is accompanied by co-translational N-linked glycosylation within the lumen of the endoplasmic reticulum (Decker, 1984; Soderquist & Carpenter, 1984; Carlin & Knowles, 1986), which appears to be critical for receptor transport from the endoplasmic reticulum (Slieker *et al.*, 1986). The complete biogenesis time for EGFR, that is the average time required from translation to appearance on the cell surface has been estimated at approximately 3 hours (Stoshchek et al., 1985; Slieker *et al.*, 1986). Half of this time is used for the conversion of the 131 kDa. precursor to the mature 170 kDa. form. An additional 75 mins is required for translocation of the newly

synthesised receptor from the endoplasmic reticulum the golgi complex. (for a review see Soderquist & Carpenter, 1986).

1.8.3. Internalisation and Degradation

Once EGFR has appeared on the cell surface, they do not form an oligomeric species or clusters until EGF is added (Haigler *et al.*, 1979). EGF causes rapid internalisation of its receptor in endocytic vesicles (Carpenter, 1987) and subsequent degradation of receptors in the lysosomes (Dunn *et al.*, 1983; Bequinot *et al.*, 1984). Some evidence suggests that internalisation of ligand receptor complexes is essential for mitogenic signal generation (Decker, 1989). Studies by Honneger *et al.* (1987, 1989) using site directed mutated receptors demonstrated that tyrosine kinase activity, whilst appearing to play no role in the internalisation event, is essential for degradation. Further, that the cytoplasmic domain plays a key role in determining mitogenicity, transforming potential and receptor routing. Recent electron microscope studies suggest that tyrosine kinase activity determines the fate of the internalised receptor within the mutlivesicular body (Felder *et al.*, 1990). The role that internalisation and degradation play in signal transduction is unclear. Cohen & Fava (1985) have shown that internalised ligand receptor complexes display higher tyrosine kinase activity towards exogenous substrates as well as altered substrate specificity.

1.8.4. EGFR Autophosphorylation

In intact cells, EGF induced autophosphorylation of EGFR has been established in three internal residues, Tyr 1068, Tyr 1148 and Tyr 1173 (Downward *et al.*, 1984). Recently a further site has been proposed by Walton and colleagues (1990) that of Tyr-992. Due to the methodological differences, this could also be the site found by Margolis and group (1989a) except that they assessed it as being Tyr 1086. *In vitro* kinetic studies suggest that self

phosphorylation is readily reversible and that the phosphotyrosine bond formed is of a relatively high energy (Hubler *et al.*, 1989). The functional significance of autophosphorylation is unclear, it is possible that autophosphorylation serves an inhibitory function with regard to receptor kinase activity.

1.8.5. Mechanisms of Kinase Activation (Intra vs Intermolecular)

Two models have been proposed to explain how ligand binding to the external binding domain can alter the catalytic properties of the kinase region of the receptor. The "flush chain" model (Staros *et al.*, 1985) requires that ligand binding alters the interaction of the external domain with the transmembrane component resulting in the activation of the kinase domain. The second and more widely cited is the "cluster" theory (Yarden & Schlessinger, 1985) which proposes that ligand binding leads to aggregation of the monomeric receptor species to a least a dimeric form and that this process is intimately involved in kinase activity.

1.8.6. Intermolecular Activation

Intermolecular activation is by far the most popular proposal for EGFR kinase activation in terms of data published. EGF receptors are randomly distributed on the cell surface (Haigler *et al.*, 1979; Schlessinger *et al.*, 1978), and they undergo rapid lateral (Hillman & Schlessinger, 1982) and rotational (Zidovezki *et al.*, 1986) diffusion. Following ligand binding, occupied receptors cluster in clathrin coated pits, internalised ligand and receptor are then degraded by lysosomal enzymes (Carpenter & Cohen, 1979). In the absence of ligand, receptor half life is 10-12 hours but 1 hour in the presence of ligand (Stoschek et al., 1985; Honegger *et al.*, 1987). Two reports provide evidence suggesting that receptor degradation occurs after several rounds of receptor recycling (Dunn *et al.*, 1986; Murthy *et al.*, 1986).

According to the allosteric oligomerisation model proposed by Yarden &

Schlessinger (1987 a,b), monomeric EGF receptors are in equilibrium with the oligomeric form. It is postulated that the oligomeric form has a higher affinity for the ligand. The binding of EGF presumably stabilises the oligomeric species, leading to the activation of the kinase function. Such a mechanism bypasses the energetically unfavourable conformational change proposed for intramolecular activation.

It is well established that EGF induces oligomerisation on the cell surface *in vivo* morphologically (Haigler *et al.*, 1979; Carraway *et al.*, 1989), biophysically (Zidovezki *et al.*, 1986) and biochemically (Cochet *et al.*, 1988). Schlessinger & Yarden (1987a) demonstrated that EGF influenced dimerisation in purified preparations as assessed by non-denaturing gel electrophoresis. The dimers appeared to have a higher kinase activity and ligand binding activity. Transfection studies by Honegger *et al.* (1989) demonstrated cross phosphorylation at identical sites in the kinase negative mutant K721 A (Moolenar *et al.*, 1988) when coexpressed with wild type EGFR. Polyclonal antibodies to EGFR co-immunoprecipitate deletion mutants devoid of epitopes recognised by the antibodies, when transfected with K721 A or wild type receptor. These results suggest that autophosphorylation of solubilised EGFR is mediated by intermolecular cross-phosphorylation, and this is facilitated at least in part by oligomerisation which is not kinase dependent.

1.8.7. Phosphorylation of Exogenous Substrates

The intrinsic tyrosine kinase activity of EGFR has been noted to phosphorylate various exogenous substrates (table **1.4**) either *in vivo* or *in vitro*. Potentially the most interesting of these substrates are the lipocortins and phospholipase C-II. Lipocortins have been reported to inhibit the activity of phospholipase A_2 (PLA_2) (Pepinsky *et al.*, 1986; Wallner *et al.*, 1986), tyrosine phosphorylation of lipocortins relieves this effect (Hirata, 1984). Studies by Goldberg *et al.*, (1990) using cells transfected with a mutant tyrosine kinase

Table 1.4

Proteins reported to be tyrosine phopshorylation substrates for EGFR kinase

Substrate	*in vivo*	*in vitro*
pp 81 (Ezrin)	Hunter & Cooper, 1981 Gould *et al*, 1986	Carpenter *et al*, 1978 King *et al*, 1980
pp 34-39 (Lipocortins)	Cooper & Hunter, 1981 Erikson *et al*, 1981 Guigni *et al*, 1985 Sawyer & Cohen, 1985 Pepinsky & Sinclair, 1986	Gosh-Dastidar & Fox, 1983 Fava & Cohen, 1984 Cohen & Fava 1985 Dee *et al*, 1986
pp 42	Cooper *et al*, 1982 Gilmore & Martin, 1983 Nakamura *et al*, 1983 Cooper *et al*, 1984 Hunter & Alexander, 1985 Kohno, 1985	
Progesterone Receptor		Woo *et al*, 1984
Gastrin		Baldwin *et al*, 1983a
Growth Hormone		Baldwin *et al*, 1983b
Middle T Antigen		Segawa & Ito, 1983
Glycolytic Enzymes		Rice *et al*, 1986 Napier *et al*, 1987
Myosin light Chain		Gallis *et al*, 1983
Erythrocyte band 3		Shiba *et al*, 1986
erb B-2 protein	Richter King *et al*, 1988	
Phospholipase C-II	Margolis *et al*, 1989 Meisenhelder *et al*, 1989	Nishibe *et al*, 1989 Meisenhelder *et al*, 1989

defective receptor, demonstrated that tyrosine kinase activity is essential for PLA_2 activation. This scheme would suggest that treatment with EGF can result in the release of free arachidonate metabolites which have been proposed as being important in the mitogenic response (Berk *et al.*, 1985; Pentland & Needleham, 1986; Murumatsu *et al.*, 1988). Tyrosine phosphorylation of PLC-II has recently been observed upon treatment with EGF or PDGF serving to enhance its enzymatic activity. It has been postulated that this is the link between the phosphatidyl inositol signalling pathway and EGFR (see figure **1.5**).

1.8.8. EGFR Transmodulation

Loss of the high affinity binding component has been observed upon treatment with phorbol esters or PDGF. This results in a decrease in tyrosine kinase activity and a marked increase in the state of phosphorylation at serine and threonine residues (for a review see Schlessinger, 1988).

Whereas it is established that protein kinase C (PKC) mediated phosphorylation of EGFR at THR-654 is observable both *in vivo* and *in vitro* (Iwashita & Fox, 1984; Cochet *et al.*, 1984; Hunter *et al.*, 1984; Davis & Czech, 1985b; Downward *et al.*, 1985), the precise mechanism of modulation is unclear. Site directed mutagenic studies by Countaway *et al.* (1990) suggest that modulation of binding is independent of the major sites of phorbol ester associated phosphorylation, and that THR-654 phoshorylation is required for the inhibition of EGFR autophosphorylation, in accord with earlier studies by Livenh *et al.*, (1988).

EGF itself also regulates its high affinity component. Ligand binding has been noted to result in phosporylation of THR-654 (King & Cooper, 1986; Whitley & Glasser, 1986), which has led to the suggestion that EGFR stimulation of PKC activity is a homologous feedback mechanism of receptor activation (Sturani et al., 1988). Transmodulation of EGFR by IGF-I and insulin has been demonstrated in PKC depleted swiss 3T3 cells (Corps & Brown, 1988).

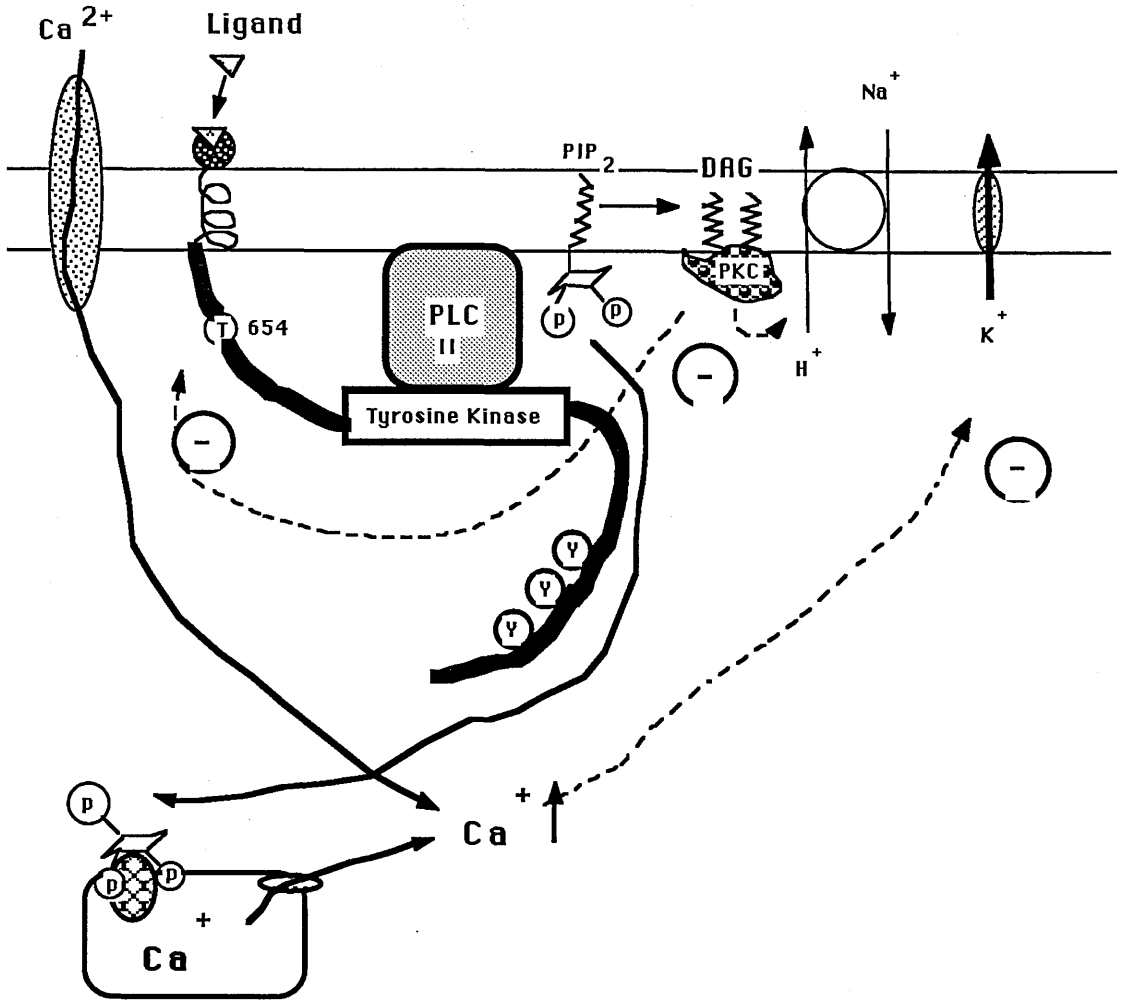


Figure 1.5

Intracellular signaling events associated with ligand activation of the Epidermal Growth Factor Receptor

1.8.9. Regulation of the EGF Receptor

In vivo studies in hypophysectomised mice by Janssen and colleagues (1988, 1989) and Johansson *et al.* (1989) have shown increases in EGFR mRNA upon growth hormone treatment. *In vitro,* retinoic acid has been noted to regulate EGFR levels, increasing binding and immunoprecipitatable protein in foetal rat lung cells (Oberg *et al.*, 1988). In contrast, reduction of both binding and receptor levels was recorded in the human epidermoid carcinoma cell line ME 108 (Zheng & Goldsmith, 1990). To add further confusion, Yung *et al.* (1990) have reported that in several human glioma cell lines tested, retinoic acid treatment resulted in the loss of EGFR phosphotyrosine kinase activity. No loss or increase in receptor binding or concentration was noted.

Loss of both receptor affinity and concentration upon the addition of α interferon in cell culture has been reported in both BHK (Zoon *et al.*, 1986) and A431 cells (Black *et al.*, 1988). Palombella and group (1987) have demonstrated increases in ligand binding and receptor protein levels upon the introduction of tumour necrosis factor in human fibroblasts. In addition to retinoic acid, dexamethasone has been cited as a negative regulator of EGFR mRNA levels in human skin fibroblasts (Oikarinin *et al.*, 1989, and in foetal rat lung cells (Oberg & Carpenter, 1989).

Experimental evidence points to a role for steroids in the regulation of EGFR in breast cancer *in vitro* models. Koga *et al.* (1988) showed that 1,25 Dihydroxy-vitamin D_3 treatment in T47-D and MCF-7 cells resulted in a significant reduction in ligand binding capacity. In contrast studies using the ER negative cell line BT-20 displayed increased EGFR levels (Falette *et al.*, 1989; Frappart *et al.*, 1989). Much more research is needed to explain the differential regulation of EGFR in ER negative and positive cell lines . It should be noted however that 1,25 Dihydroxy -vitamin D_3 treatment led to growth inhibition in the three cell lines studied.

Progestin regulation of EGFR has been well documented in T47-D and MCF-7 cells by Sutherland's group (Murphy *et al.*, 1985; 1986; Koga et al., 1988; 1989) and others (Sarup *et al.*, 1988). In essence much of their research has shown that progestins acting directly or via the glucocortocoid receptor inhibit ligand binding and mRNA levels resulting in a reduction of cell growth. Loss of binding upon treatment with antineoplastic agents cisplatin and vincristine has been shown in MCF-7 cells (Hanauske *et al.*, 1987).

Berthois *et al.* (1989) have demonstrated that incubation with 17β - oestradiol leads to an initial decrease in ligand binding (24-48 hours), followed by increased ligand binding from 3 days onwards, in MCF-7 cells. Addition of 4-OH tamoxifen resulted in the inhibition of ligand binding. These data appear to agree with *in vivo* studies in the rat uterine model system with regard to oestrogen action (Mukku & Stancel, 1985; Gardner *et al.*, 1989). However the Berthois publication is open to question with regard to its methodology. Studies within our group (Rinaldi & Leake, unpublished) have demonstrated that the cell density required to allow continous exponential growth over the time course in the culture vessels the authors cited is not viable in our hands. Further, the authors report double site binding kinetics for MCF-7 cells which contrasts with ourselves and others (Hanauske *et al.*, 1987; Kosano *et al.*, 1988), who have found only single high affinity binding.

1.8.10. Receptor Related Molecules

One of the main reasons that EGFR has been proposed to play a role in the carcinogenic process has been the observations that there exists oncogenic molecules which display tyrosine kinase activity and are homologous to EGFR to some extent. There are several proteins closely related to EGFR . There is an extremely high sequence homology with the sequence of the erb B oncogene of the avian erythroblastosis virus (AEV) (Yamamoto *et al.*, 1983). Chromosomal mapping studies of the EGFR (Merlino *et al.*, 1985) and v-erb B (Spurr *et al.*, 1984)

show that both are located on the same region of chromosome 7. It has been postulated that the gene for EGFR is the proto-oncogene from which viral erb B gene was derived. A sequence of nearly 400 residues in the cytoplasmic domain displays 95% homology (Ullrich *et al.*, 1984). The main difference between the two is a large truncation of the EGFR extracellular binding domain in the v-erb B molecule and a small truncation at the carboxyterminus.

A second retrovirus, the avian leukosis virus (ALV) also utilises a protein related to EGFR. Unlike AEV there is no oncogene within its genome, ALV activates normal cellular sequences by insertion of a provirus into the host genome. Transcription of cellular sequences is under viral promoters. Induction of lymphomas by ALV involves the activation of c-myc sequences. While induction of erythroblastosis involves transcriptional activation of c-erb B sequences (Nilsen *et al.*, 1985).

1.8.11. Neu (HER-2) Oncogene

The product of this gene is a glycoprotein of 185 kDa. which displays 50% sequence homology to EGFR (Bargmann *et al.*, 1986). The neu protein cross reacts with certain antibodies to EGFR (Fendly *et al.*, 1990) and v-erb B probes hybridise with the neu oncogene. The neu protein does not bind to EGF, and it has been proposed to be a tyrosine kinase receptor for an as yet undiscovered ligand (Stern *et al.*, 1986, Akiyama *et al.*, 1986). The neu oncogene has been sequenced cloned and mapped to chromosome 17 (Bargmann *et al.*, 1986; Fukushige *et al.*, 1986). This has allowed the identification of the corresponding proto-oncogene (Yamamoto *et al.*, 1986) designated c-erb B-2. The c-erb B-2 gene has been found to be amplified (Slamon *et al.*, 1987; Guerin *et al.*, 1988, 1989, Zeillinger *et al.*, 1989) and overexpressed (Rio *et al.*, 1987 Guerin *et al.*, 1988, 1989) in about 20% of breast carcinomas and may be a marker of poor patient prognosis.

1.8.12. EGFR role in the Transformation Process

In human malignancies abnormalities involving growth factor receptors most commonly appear to involve gene amplification and/or overexpression of seemingly normal RNAs and proteins (Xu *et al.*, 1984, King *et al.*, 1985a,b; Semba *et al.*, 1985; Liebermann *et al.*, 1985, Fukushige *et al.*, 1986, Yokata *et al.*, 1986, Kraus *et al.*, 1987, Slamon *et al.*, 1987). These findings suggest that overexpression of a normal growth factor may provide a selective growth advantage to a cell progressing along the malignant pathway. Cellular transformation by overexpression of exogenous EGFR in rodent fibroblasts has been shown to result in phenotypic transformation (Velu *et al.*, 1987; DiFiore *et al.*, 1987; Riedel *et al.*, 1988; Haley *et al.*, 1989).

1.8.13. EGFR in Breast Cancer

EGFR has been found in various animal and human tissues (Ozanne *et al.*, 1985). The presence of EGFR in human breast tumours has been reported by several groups using radiolabelled binding techniques. EGFR appears to be present in 33-67% of breast tumours analysed at a concentration range of 1-275 fmoles. mg^{-1} membrane protein with a median ranging from 3-40 fmoles. mg^{-1} membrane protein. As can be seen there is considerable variation in the results cited. This is probably due to a number of factors. Two methods have been used to assess EGFR levels; multipoint scatchard analysis (Perez *et al.*, 1984; Sainsbury *et al.*, 1985; Nicholson *et al.*, 1988; Fekete *et al.*, 1989; Foekens *et al.*, 1989) or single point (Fitzpatrick *et al.*, 1984; Battaglia *et al.*, 1988; Cappelletti *et al.*, 1988; Bauknecht *et al.*, 1989; Grimuax *et al.*, 1989). Both methods have their advantages and disadvantages. Multipoint analysis is the more accurate and allows for resolution of the two possible binding components, but requires in excess of 200 mg tumour tissue. Single point only requires approximately 50 mg, therefore allowing a result to be recorded with a small amount of tissue. This is important if one considers that increased research and early detection has resulted in

investigators receiving increasingly smaller tissue samples for analysis. Variations in results are further compounded by the absence of any standardisation of tissue processing and heterogeneity of material analysed. Despite these reservations, in the majority of studies carried out EGFR presence negatively correlates with that of ER. Follow up studies indicate that it may be marker of poor patient prognosis (Nicholson *et al.*, 1989) and reccurence (Toi *et al.*, 1990).

EGFR levels as assessed by immunocytochemistry would appear to be an ideal method as it uses small amounts of tissue and allows visual assessment of receptor location within the tumour section. Studies reported are subject to wide variations in both the incidence reported (20-60%) and location of receptor. Moller *et al.* (1989) reported staining in 20% of tumours analysed. Further, the majority of staining was present in the myoepithelium and in some cases in the fibroblasts. This contrasts with the findings of Battaglia *et al.* (1988) who reported staining in the majority of tumour cells. Studies by Wrba *et al.* (1988) and Toi *et al.* (1989) have shown staining in 60% and 34% respectively. Both noted a negative correlation with respect to ER status, but were unable to agree on other prognostic markers. In contrast to Toi, Wrba reported no correlation with nodal involvement. Using a novel autoradiographic technique Reubi *et al.* (1988) although finding EGFR in 29% of cases, reported that the majority of staining was present in the tumour cells. Further studies have revealed a negative correlation with ER status (Reubi *et al.*, 1989). But it must be noted that if either recepor concentration or affinity is important in determining patient prognosis, these latter methods would prove inadequate.

2. Materials

2.1 Suppliers

Amersham International; plc, Amersham, U.K.

BDH/Gurr Chemicals Ltd., c/o MacFarlane Robson Ltd., Glasgow, U.K.

Biogenesis Ltd, Bournemouth, U.K.

Biorad Laboratories, Watford, U.K.

Boehringer Mannheim GmbH, c/o BCL, Lewes, U.K.

BRL-Gibco, Paisley, U.K.

Calbiochem, c/o Novabiochem, Nottingham, U.K.

Du-Pont (U.K.) Ltd., Stevenage, U.K.

Falcon, c/o A. & J. Beveridge Ltd., Edinburgh, U.K.

Flow Labs, Rickmansworth, U.K.

FSA Lab Supplies, Loughborough, U.K.

Kodak Ltd., Manchester, U.K.

May & Baker, Ltd., Dagenham, U.K.

Millipore (U.K.) Ltd., Harrow, U.K.

Northumbria Biologicals Ltd., Cranlington, U.K.

Nunc, c/o BRL-Gibco, Paisley, U.K.

Sarstedt Ltd., Leicester, U.K.

Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, U.K.

Sigma Chemical Co. Ltd., Poole, U.K.

Sterilin Ltd., Hounslow, U.K.

Whatman, c/o R. & J. Wood, Paisley, U.K.

2.2.1 Cell Growth Medium

Dulbecco's modification of Eagle's medium (DMEM) was originally supplied as a 10X concentrate by Gibco and diluted to a final concentration of 1X with sterile deionised distilled water. To eliminate the action of phenol red within the experimental system this was replaced by phenol red free DMEM powder

obtained from sigma.

The powder was reconstituted as per manufacturer's instructions to a final concentration of 1X. Methionine free DMEM was also supplied from the same source.

2.2.2 Supplements to Cell Growth Medium

L-Glutamine (200 mM), and Penicillin (10 000 $U.ml^{-1}$) / Streptomycin (10 000 $\mu g.ml^{-1}$) were obtained from Gibco. Foetal calf serum supplies alternated between Gibco and NBL.

2.2.3 Cell Culture Materials

Biofreeze Vials, Costar

Lab Tek 8 well glass slide/tissue culture vessels, Flow Labs

25, 80, 175 cm^2 T/C flasks, Bibby

850 cm^2 plastic tissue culture roller bottles, Bibby

24 well FB plates, Corning

2.2.4 Fine Chemicals

All chemicals used were of analar grade:

Bacitracin, Sigma

Bovine Serum Albumin, Sigma

Calf Thymus DNA, Sigma

Charcoal (Norit A Activated), Sigma

Cycloheximide, Sigma

Dextran, Sigma

Dimethylsulphoxide, May & Baker

Dithiothreitol, Sigma

Dowex 1X8, 200-400 mesh, Sigma

EDTA, Fisons

HEPES, Fisons

Hydroxyappatite, Biorad

Soya Bean Inhibitor, Sigma

2.2.4.1 Electrophoresis/Transblotting Fine Chemicals

Acrylamide, FSA Lab Supplies

Bisacrylamide, FSA Lab Supplies

Glycine, FSA Lab Supplies

Hybond N, Amersham

Mercaptoethanol, BDH

Standard Proteins, Boehringer

SDS, FSA Lab Supplies

TEMED, BDH

2.2.4.2 Enzymes

RNAase A, Sigma

Trypsin, Gibco

2.2.4.3 Detergents

Nonidet P-40, BDH

Triton X-100, Sigma

Tween 20, Sigma

2.2.4.4 Hormones/Growth Factors

Murine Epidermal Growth Factor, Biogenesis

Insulin, Sigma

Human Prolactin, Sigma

Ovine Prolactin, Sigma

Oestradiol, Sigma

2.2.4.5 Stains

Amido Black, Gurr

Bromophenol Blue, BDH

Hoechst 33258 (Bisbenzimide), Sigma

2.2.4.6 Radiochemicals

[^{125}I] - Epidermal Growth Factor, NEN	(specific activity: 100-180 μCi. mg^{-1})
[^{35}S] - Methionine, Amersham	(specific activity: 1200 Ci. mmole^{-1})
[^{125}I] - Human Prolactin, NEN	(specific activity: 20 - 50 μci. mg^{-1})
[^{125}I] - Protein A, Amersham	(specific activity: 77 μCi. μg^{-1})
[^{3}H] - Oestradiol, Amersham	(specific activity: 48.5 Ci. mmole^{-1})

2.2.4.7 Antibodies/Sundries

Rabbit polyclonal Antibody BG 48, Bill Gullick, ICRF

EGFR I mouse monoclonal, Amersham

Flourecsent Labelled goat antimouse IgG, Derek Chalmers, Biochem, G.U.

Sheep serum, SAPU

Pansorbin^{TM}, Calbiochem

2.2.5 Miscellaneous

Ecoscint, Biochem Store

DPX Mountant, PBS Glycerol Mountant, Gurr

RT-30 Tubes, Sterilin

Kodak X-Omat S, KodakHyperfilm, Amersham

Chapter 3. Methods

To facilitate ease of access when referring to the results section, it was decided to subdivide this methodology chapter into three major sections.

3.1 Clinical Research

3.2 Nuclear Localisation Studies

3.3 Cell Culture Studies

3.1 Clinical Research

3.1.1 Buffers

Sucrose/ Glycerol Buffer:

0.25M Sucrose; 1.5mM Magnesium Chloride; 10mM HEPES, pH 7.4 in 50% (v/v) Glycerol

Sucrose Buffer:

0.25M Sucrose; 1.5mM Magnesium Chloride; 10mM HEPES, pH 7.4

HED Buffer:

20mM Hepes; 1.5 mM EDTA, 0.25mM Dithiothreitol, pH 7.4

Dextran Coated Charcoal:

20 mM HEPES; 1.5 mM EDTA; 0.5% (w/v) Charcoal; 0.005% (w/v) Dextran T-70, pH 7.4

EGFR Assay Buffer:

10mM HEPES, 50mM Sodium Chloride, 0.1% (w/v) BSA, pH 7.0

Buffer A:

2mM Potassium dihydrogen orthophosphate; 2mM Sodium dihydrogen orthophosphate; 0.154 M Sodium Chloride, 0.007% (w/v) Bacitracin, pH 7.4

Buffer B:

2mM Potassium dihydrogen orthophosphate; 2mM Sodium dihydrogen orthophosphate; 0.154 M Sodium Chloride, 0.007% (w/v) Bacitracin 0.1% (w/v) BSA, pH 7.4

3.1.2 Tumour collection and storage.

Freshly collected breast tumour tissue was stored in Sucrose/Glycerol buffer at -20°C prior to receptor assays. Receptor assays (full scatchard analysis for ER and single point screen for EGFR) were carried out within 10 days of sample collection. Oestrogen receptor content of tumour tissue is stable under these conditions for at least six months (Crawford *et al.*, 1985). Storage of breast tumour receptor positive control tissue under identical conditions reveal that EGFR content was stable for at least one month (Porteus, 1987).

3.1.3 Tissue Preparation

Tissue preparation was carried out using a modification of the method of Leake & Habib (1987). Stored tissue was rehydrated for 15 mins at 4°C in Sucrose Buffer. Any obvious fat and necrotic tissue was removed. A section of approx. 150-200 mg of tissue was homogenised in freshly made HED buffer at a concentration of 50 mg. ml^{-1}. This involved initial 2 x 10 secs bursts at a setting of 150 on an Ultra Turrax homogeniser (Model TP 18/2) followed by gentle homogenisation using a glass/glass tissue grinder (Kontes Duall Size 21) to ensure

an even suspension. Tissue was kept below 8°C throughout. The resulting homogenate was centrifuged for 10 mins at 800 g, 4°C. The crude nuclear pellet obtained was resuspended in 3 ml HED buffer and retained for oestrogen receptor and DNA estimation. The supernatant was further centrifuged 50,000g for 30 mins, 4°C.

The resulting supernatant (cytosol) was retained for oestrogen receptor and protein estimation. The pellet (membrane fraction) was resuspended in 1 ml EGFR assay buffer, and a single point EGFR screen carried out. To ensure minimum receptor degradation, it should be noted that all manipulations were carried out on ice.

3.1.4 Oestrogen Receptor Assay

Cytosol and nuclear oestrogen receptor levels were measured by the method of Leake *et al* (1981b). Three series of ten RT-30 tubes were set up. Tubes 1-7 received a final concentration range of 8, 12, 20, 30, 60, 80 and 120 x 10^{-10}M [^{3}H] - E_2 in a volume of 50 µl. Tubes 8-10 received 60, 80, 120 X 10^{-10}M [^{3}H] - E_2 plus a 100 - fold excess of Diethylstilboestrol in a volume of 50 µl. Series 1 received 150 µl cytosol, series 2 150 µl nuclear suspension and series 3 150 µl of buffer (to give blanks and totals). The final concentration range of [^{3}H] - E_2 was 2 - 30 X 10^{-10}M. The tubes were then capped with nescofilm and incubated overnight (18 hours) at 4°C.

3.1.4.1 Cytosol Oestrogen Receptor Assay

Separation of bound ligand from free ligand was achieved by transferring the cytosol tubes to an ice bath. There then followed the addition of 200 µl of dextran coated charcoal to each tube, incubating on ice for 15 mins at 4°C, vortexing every 5 mins. The tubes were then centrifuged for 5 mins at 1000g, 4°C, 200 µl aliquots of the supernatant were transferred to scintillation vials to which 4 ml of ecoscint was added. The scintillation vials were then counted on a LKB 1209 rackbeta scintillation counter at a rate of 3 mins per vial, counting efficiency 40%.

3.1.4.2 Nuclear Oestrogen Receptor Assay

100 µl aliquots of nuclear suspension were added to 5 ml portions of 0.9% (v/v) saline immediately prior to pouring onto a pre-wetted filter disc (2.5 cm. dia.) held in a millipore filtration apparatus. The tube which had contained the saline was washed with a further 5 ml of saline and this also poured into the funnel.

The chimney of the apparatus was rinsed with 5 ml saline and the filter rinsed with a final 5 ml. The filter was then dried before transferring to a scintillation vial. 4 ml of ecoscint was added to each vial, and the vials allowed to stand for 5 hours at room temperature. Vial radioactivity was assessed by the method of **3.1.4.1** at a counting efficiency of 40%.

3.1.4.3 Analysis of Data

Scatchard analysis (Scatchard, 1949) of ligand binding assay data derived was carried out using a BBC microcomputer programmed as described by Leake *et al.* (1987b).

3.1.5 Protein Estimation

Determination of tumour cytosolic fraction protein content was by the method of Lowry (1951). The standard curve range used was 0 - 0.7 mg. ml^{-1} Bovine Serum Albumin (BSA).

3.1.6 DNA Estimation

Determination of tumour nuclear fraction was by the method of Burton as modified by Katzenellenbogen and Leake (1974), extraction efficiency of DNA by this method was estimated as 95%. The standard curve range used was 0 - 350 µg. ml^{-1} Calf Thymus DNA.

3.1.7 EGF Receptor Assays

Initially all tumour membrane preparations were screened by a single point assay (**3.1.7.1**). The remaining sample was stored at -70°C until the result of the single point screen was known. Full multipoint analysis (**3.1.7.2**) was carried out within a month if the screen resulted in a value for EGFR of greater than 10 fmoles. ml^{-1} of membrane fraction. EGFR was assumed be stable with respect to

concentration and affinity over this time period. Studies using placental membranes prepared and stored under identical conditions show no losses in receptor concentration (see fig. **3.1**) or affinity (see fig. **3.2**) even after 6 months storage at -70°C.

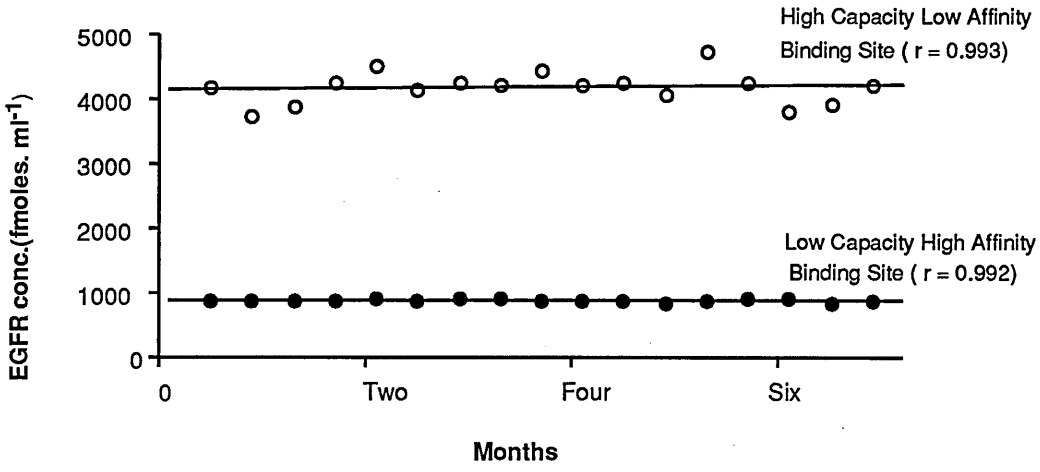
3.1.7.1 Single Point Screening Assay

This assay was performed in duplicate. To eppendorf polytubes (precoated with 0.1% BSA (v/v) in PBS) 50 µl of tumour membrane preparation was added. To one set of duplicate polytubes 50 µl of [^{125}I] - EGF (specific activity: 100-180 µCi. µg^{-1}) at a final concentration of 1 nM in EGFR assay buffer was added. Non specific binding was assessed by incubation of a further set of polytubes with [^{125}I] - EGF at the same final concentration but with a 100 - fold excess of unlabelled EGF.

Assay incubation was carried out at room temperature (21°C) for 1 hour. The binding reaction was terminated by plunging the tubes into an ice bath . Immediate addition of 1 ml ice cold EGFR assay buffer was followed by centrifugation at 20 000 g for 15 mins at 4°C. After centrifugation the supernatant was aspirated and discarded into radioactive waste. The pellet was resuspended in 100 µl of 5N sodium hydroxide and transferred to gamma counting tubes.
The tubes were then counted for 60 secs per vial using an LKB 1275 minigamma counter. Total binding varied between 1% and 15% of the total counts added, non-specific between 0.5% and 3% of the total counts. Specific binding was estimated by subtracting non-specific counts from the total bound counts.

Figure 3.1

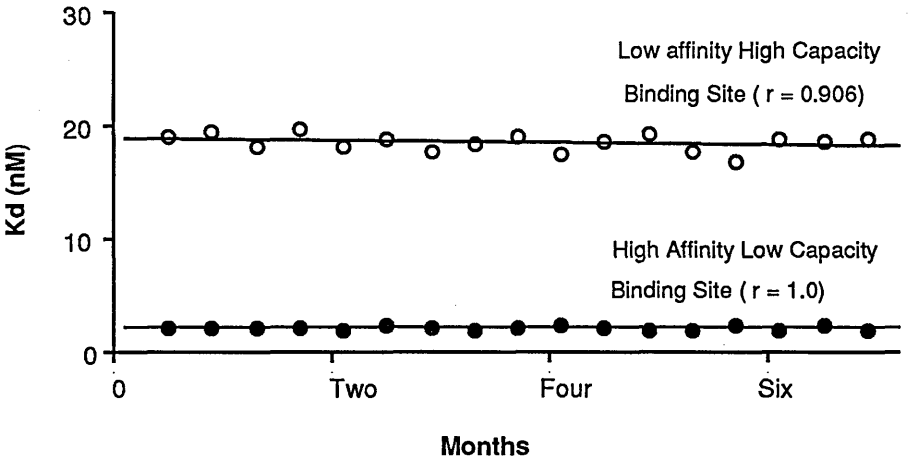
Stability of EGFR binding in Placental Membrane preparations



Stability of EGFR binding in placental membrane preparations stored at -70°C over a six months period. Note that no loss in either binding component can be observed. Full multipoint analysis of breast tumour membranes was carried out within one month of single point screen. Assessment of EGFR levels was carried out by the method outlined in section **3.1.7.2.**

Figure 3.2

Stability of EGFR affinity in Placental membrane preparations



Stability of EGFR affinity in placental membrane preparations stored at -70°C over a six months period. Note that no loss in either affinity component can be observed. Full multipoint analysis of breast tumour membranes was carried out within one month of single point screen. Assessment of EGFR affinity was carried out by the method outlined in section **3.1.7.2.**

3.1.7.2 Multipoint Analysis ("In house Assay ")

Multipoint Analysis was carried out with twelve points of increasing concentrations of 50 µl of [^{125}I] - EGF at the final concentration of 0.086, 0.208, 0.416, 0.616, 1.67, 5.0, 8.334, 13.34, 15.0, 16.7 nM. Non specific binding was ascertained by incubating three aliquots containing in addition to the top three labelled concentrations a 100 - fold excess of unlabelled EGF. Assay incubation, separation, and counting conditions were as for **3.1.7.1.**

3.1.7.3 Calculation of Results

All calculations were according to Scatchard (1949). The calculations were performed manually, the plot coordinates were resolved on an apple MacIntosh Plus computer using the MacGraph program.

3.1.7.4 EORTC EGF Receptor Assay

This assay was devised by Benraad & Foekens (1989) and has since recently been revised (Benraad & Foekens, 1990).

To a series of RT-30 tubes, increasing amounts of [^{125}I] - EGF (10 µl in buffer B) at the following final concentrations 0.15, 0.4, 0.7, 0.8, 1.0, 1.4, 2.75, 3.3, 5.5 nM was added along with 30 µl of buffer B. Determination of non-specific binding was assessed by adding to three tubes 10 µl of the top three concentrations of label with 20 µl of 5 x 10^{-6}M unlabelled EGF and 10 µl of buffer B. 100 µl of sample membrane preparation was added to each tube. The tubes were then vortexed and incubated for 1 hour at room temperature.

Separation of bound from free ligand was achieved by the addition of 100 µl

of hydroxyapatite (HAP) slurry (2:1 v/v in buffer A), incubation for 30 mins at 4°C, followed by centrifugation at 1000 g for 2 mins at 4°C. The supernatant was aspirated and the HAP pellet was washed twice with 1 ml aliquots of buffer A. Following the final wash the pellets were counted using an nuclear enterprise (NE 1612) turbo gamma counter. Adsorption of labelled EGF to the hydroxyapatite slurry in the absence of membrane sample was estimated as 0.3% ± 0.12% of the total counts added. Because this adsorption factor was constant, it was not taken into account in the final calculations of specific binding.

3.1.7.4.1 Calculation of Results

Results were calculated manually according to the method of Chamness & MacGuire (1978). Data coordinates were resolved as described in section **3.1.7.3.**

3.2 Nuclear Localisation Studies

3.2.1 Buffers

HE/Triton Buffer:

20 mM HEPES; 1.5 mM EDTA; 1% (v/v) Triton X-100; pH 7.4

Laemmli Sample Buffer:

0.0625 M Tris-HCL (pH 6.8); 10% (v/v) (w/v) sucrose; 5% (v/v) β-mercaptoethanol; 2% (w/v) SDS; 0.002% (v/v) bromophenol blue. Dispensed into suitable aliquots and stored -20°C until required.

Stacking Buffer:

0.125 M Tris-HCL (pH 6.8); 0.4% (w/v) SDS

Resolving Buffer:

0.375 M Tris-HCL (pH 8.8); 0.4% (w/v) SDS

Acrylamide Stock:

30% (w/v) Acrylamide; 0.8% (w/v) N'N'-methylene bis-acrylamide

Persulphate:

1.5% (w/v) ammonium persulphate, made fresh on the day.

Electrode Buffer Stock (10 x):

0.25 M Tris; 1.92 M Glycine; 0.1% (w/v) SDS

Transblot Electrode Buffer:

25 mM Tris; 0.192 M Glycine; 0.01% (w/v) SDS; 20% (v/v) Methanol.

TBST:

10 mM Tris-HCL pH 8.2, 150 mM Sodium Chloride, 0.05% (v/v) Tween 20

All solutions once prepared could be stored for a maximum of 4 weeks at 4°C unless otherwise stated.

3.2.2 Preparation of Tissue

The methodology for initial tissue preparation is described in section **3.1.3** with the following changes incorporated in the preparation of the membrane fraction. Buffer B at the same volume as before replaced EGFR assay buffer. The nuclear fraction was resuspended in 3 ml HE/Triton buffer and centrifuged at 3000 g for 10 mins at 4°C. The supernatant was discarded and the pellet washed a furter two times by this method. Finally the "washed" nuclear pellet was resuspended in 2 ml buffer B.

3.2.3 Salt Extraction of Nuclear Fraction

Aliquots (1ml) of the "washed" nuclear fraction were salt extracted with 2M sodium chloride for 30 mins on ice by the addition of an equal volume of 4M sodium chloride. Following incubation the suspension was centrifuged at 3000 g for 10 mins at 4°C and the resulting pellet resuspended in buffer B (1ml).

3.2.4 Scatchard Analysis

Scatchard Analysis of the fraction quoted in results section **4.2** was by the EORTC method (Section **3.1.7.4**).

3.2.5 SDS/Polyacrylamide Gel Electrophoresis

The methodology involved in SDS/Polyacrylamide gel electrophoresis (SDS/PAGE) is essentially the discontinuous Tris/Glycine buffered system described by Laemmli (1970).

3.2.6 Preparations of Samples for Electrophoresis

Samples for analysis were mixed 1:1 (v/v) with Leammli sample buffer and boiled for 3 - 5 mins before electrophoresis.

3.2.7 Gel Preparation

For analytical purposes, gels were cast using a home made apparatus in slabs 19 cm x 9. 5 cm x 0.15 mm. Gels were prepared as follows:-

7.5% Slab Gel

Constituent	Stacking Gel (ml)	Resolving Gel (ml)
Distilled H_2O	11.5	23.0
Buffer	5.0	5.0
Acrylamide Stock	2.5	10.0
Persulphate	1.0	2.0
TEMED	0.02	0.03

Following the addition of TEMED the resolving gel was poured, and propan-2-ol added to ensure an even surface during the setting of the gel. Propan-2-ol was then discarded and traces were removed by washing 3 or 4 times with distilled water. The top of the resolving gel was dried with filter paper and the stacking gel added.

3.2.8 Electrophoretic Conditions

SDS/PAGE was performed at a constant current of 40 ma - 60 ma per gel until the bromophenol blue tracker dye was 0.5 cm from the bottom of the gel. The electrode buffer was a dilution of the 10 x stock described in **3.2.1**.

3.2.9 Coomassie Blue Staining

Where appropriate the gels were stained for protein in 0.1% (v/v) coomassie brilliant blue R250, 50% (v/v) methanol and 10% (v/v) acetic acid for 1 hour at room temperature. Destaining of the gels was accomplished by washing the gels several times in a 10% (v/v) methanol and 10% (v/v) acetic acid solution.

3.2.10 Determination of M_r by SDS/PAGE

The M_r values of polypeptides resolved by SDS/PAGE was determined by calibrating with a set of standard proteins.

These proteins include:-

Trypsin Inhibitor M.W. 20 100

Lactate Dehydrogenase M.W. 36 500

Glutamate Dehydrogenase M.W. 55 400

Phosphorylase b M.W. 97 400

α_2-Macroglobulin M.W. 170 000

Relative Mobility was calculated as the ratio :-

$$R_f = \frac{\text{Distance migrated by the protein}}{\text{Distance migrated by the tracking dye}} \quad \text{for each protein}$$

A plot of R_f against log (M_r) of the standards yielded a curved line that was used for calibration. Standards and samples were analysed on the same gel.

3.2.11 Western/Immunoblotting/Immune Replica Analysis

The immunoblotting procedure was employed to permit the immunological detection of polypeptides after their electrophoretic transfer onto nitrocellulose paper (Towbin *et al.*, 1979; Batteiger *et al.*, 1982).

Polypeptides were resolved by SDS/PAGE (Section **3.2.5**) and transferred electrophoretically onto nitrocellulose paper (Hybond N) at 400 ma for 3 hours or 40 ma for 16-20 hours using a Bio-rad Trans-Blot^{TM} cell. Blotting methodology was according to Ong *et al.* (1990) using the polyclonal antibody BG 48 raised against the synthetic peptide 2E, residues 985-996 (Kris *et al.*, 1985).

Following transfer to nitrocellulose the blots were blocked in TBST containing 2% (w/v) non-fat dried milk for one hour at room temperature (21°C). The blot was then washed five times with TBST for a total of one hour. Polyclonal antibody BG 48 at a dilution of 1:200 in TBST was added for 16 hours at 4°C. The blot was then washed five times as before. [^{125}I] - Protein A at concentration of 0.1 μCi. ml^{-1} was added in TBST, incubation was allowed to proceed for one hour at room temperature. Removal of unbound radiolabel was achieved by a further five washes in TBST. Finally the blots were air dried and exposed for one week to Kodak x-omat S film with intensifying screens at -70°C.

To identify the quality of transfer and to compare the molecular weight standards the blot was stained for 5 mins with 0.1% (w/v) Amido Black dissolved in 45% (v/v) methanol, 10% (v/v) acetic acid followed by destaining with 90% (v/v) methanol/2% (v/v) acetic acid (Schaffner & Weissmann, 1973).

3.2.12 5' Nucleotidase Assay

The activity of 5' nucleotidase in each fraction was estimated by the method of Lowry & Lopez (1946). The relative activity of each fraction assayed in

triplicate, was quoted as the value obtained at OD_{660nm} against a standard curve range of 0 - 25 µg KH_2PO_4 (anhydrous) per milligram of fraction protein.

3.2.13 Alkaline Phosphatase Assay

Alkaline phoshatase activity was estimated by the method of Engstrom (1961). relative activity was assessed by triplicate estimation of the OD_{600nm} per mg fraction protein. No standard curve range was used.

3.3 Cell Culture Studies

3.3.1 Buffers & Serum Preparation

Dialysed Heat Inactivated Dextran Coated Charcoal Stripped Serum (dialysed HIDCCFCS)

100 ml of FCS was dialysed against four 1 litre changes of Hank's modified buffer over 48 hours at 4°C. Following dialysis the serum was transferred to a glass container and heat inactivated for 45 mins at 56°C. The serum was then cooled to 4°C and added to a pellet of dextran coated charcoal (0.25% Charcoal(w/v), 0.0025% Dextran (w/v) in 25 ml PBS-A which had been centrifuged and the supernatant removed). This solution was allowed to stir at 4°C for 30 mins before centrifuging at 10 000 g for 30 mins at 4°C. The supernatant was filter sterilised through 0.2 micron filters.

Dulbecco's Phosphate Buffered Saline (PBS-A)

170 mM Sodium Chloride; 3.4 mM Potassium Chloride; 10 mM Di-Sodium hydrogen orthophosphate, 2 mM Potassium dihydrogen orthophosphate, pH 7.2.

Versene

125 mM Sodium Chloride; 2.7 mM Potassium Chloride; 6.3 mM Di-Sodium hydrogen orthophosphate; 3.2 mM Potassium dihydrogen orthophosphate; 0.5 mM EDTA.

Trypsin/Versene

40 ml Versene : 10 ml 0.25% (w/v) Trypsin Solution

ETN Buffer

10 mM EDTA; 10 mM Tris-HCL; 100 mM Sodium Chloride, pH 7.0

Phosphate Buffered Saline (PBS)

170 mM Sodium Chloride; 10 mM Di-Sodium hydrogen orthophosphate;
2 mM Potassium dihydrogen orthophosphate, pH 7.2

Tris Buffer

50 mM Sodium Chloride; 10 mM Tris-HCL, pH 7.3

NP-40 Solution

0.5% (v/v) NP-40 in Tris Buffer

Hank's Modified Buffer

Calcium Chloride 1.3 mM; Potassium Chloride 5.4 mM; Magnesium Chloride 0.5 mM, Magnesium Sulphate 0.5 mM, Sodium Chloride 137 mM, Sodium hydrogen carbonate 4 mM, Sodium dihydrogen orthophosphate 0.4 mM, pH 7.2 - 7.4

Hank's/BSA/Glucose (HBG)

Hank's modified buffer 100 ml; 2% (w/v) BSA; 10 mM Glucose

HBG/LiCl

HBG 20 ml; 800 µl of 250 mM LiCl

3.3.2 ZR-75-1 Cell Line

All results cited in section **4.3** were from studies using the ZR-75-1 oestrogen dependent breast cancer cell model (Engel *et al.*,1978). This cell line was derived from the malignant ascitic effusion of a metastatic breast tumour.

3.3.3 Routine Cell Culture

ZR-75-1 cells were routinely grown at 37°C in a 5% CO_2 atmosphere. All aseptic manipulations were performed within the confines of a laminar air flow cabinet (Flow Labs).

Initially routine subculture was performed using Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), HEPES, pH 7.3 (10 mM), Insulin (5 µg. ml^{-1}), penicillin (1000 iu. ml^{-1})/streptomycin (100 µg. ml^{-1}) and Glutamine (2 mM). Cell doubling time of ZR-75-1 cells in culture when grown routinely was estimated as 14 hours by the Hoechst DNA method, and 15.7 hours by trypan blue exclusion. Cell medium was changed every two to three days. The majority of routine subculture was carried out using phenol red free DMEM supplied as a powder, reconstituted as per manufacturers instructions. Supplementation was as above, however no glutamine was required. Stocks of cells were grown routinely in 175 cm^2 plastic

tissue culture flasks seeded at a concentration density of 4 x 10^6 cells per flask in 40 ml DMEM + 10% (v/v) FCS. For the large scale immunoprecipitation experiments (section **4.3.7.1**) 850 cm^2 plastic tissue culture roller bottles were used, seeded at a density of 2 x 10^7 cells per bottle in 100 ml DMEM + 10% (v/v) FCS. Most experiments used 175 cm^2 flasks or 24 well F.B. plates seeded at a cell density of 11-15 x 10^4 cells per well. Cells were subcultured by washing twice with sterile PBS-A followed by the addition of trypsin/versene. Incubation at 37°C was allowed to proceed until detachment from the substrate layer was observed. The required concentration of medium was added and the suspension thoroughly resuspended. The cells were then seeded as described above.

3.3.3.1 Mycoplasma Screening

ZR-75-1 cells were tested for contamination every 2 - 3 months. Cells were grown in glass slide/tissue culture vessels for 48 hours. The medium was aspirated and the wells washed with three changes of PBS-A.

Fixation was achieved by the addition of acetone:methanol (1:1) cooled to -20°C for 4 - 5 minutes. After air drying, the cells were stained with Hoechst 33258 stain (100 ng. ml^{-1}) for 10 mins at room temperature. The slides were then washed in three changes of PBS-A and mounted in a PBS/Glycerol mountant. The cells were examined under a fluorescent microscope for visual evidence of infection. If infection was observed, all viable cell stocks were destroyed. The cell culture unit was thoroughly cleaned by successive treatments with Hypochlorite, Savlon and 70% (v/v) ethanol. New cell stocks were then ordered from the European animal cell culture collection.

3.3.3.2 Preparation of Frozen Cell Stocks

Frozen stocks of ZR-75-1 cells were prepared as follows:
Cells were grown in 175 cm^2 flasks in DMEM + 10% (v/v) FCS until they were 80 - 90% confluent. Cells were harvested in trypsin/versene, then recovered by centrifugation at 1000 g for 5 mins at 4°C and resuspended in 1 ml 90% FCS (v/v) + 10% (v/v) DMSO per flask. 1.5 ml aliquots were transferred to biofreeze vials. Cells were frozen in two stages, an overnight incubation at -70°C followed by long term storage at -170 °C under liquid nitrogen.

3.3.3.3 Recovery of Frozen Cells

Cells were recovered from the -170°C freezer, thawed rapidly to 37°C in a water bath and transferred to a Sterilin vial containing 10 ml of prewarmed medium (DMEM + 10% (v/v) FCS). The vial was then centrifuged at 1000 g for 5 mins at 4°C and the supernatant discarded. The pellet was resuspended in 5 ml of medium which was transferred to a 25 cm^2 flask containing 10 ml of medium.

3.3.4 Experimental Methodology

3.3.4.1 DNA Synthesis Experiments

Cells were plated down overnight in DMEM + 10% (v/v) FCS in 24 well F.B. plates. This medium was removed and the cells washed with two changes of sterile

PBS-A (1 ml). Experimental medium DMEM + 5% (v/v) Dialysed HIDCCFCS ± hormones/growth factor were added to a final volume of 0.5 ml per well.

At appropriate incubation times, wells were harvested by the removal of the medium, washing twice PBS-A (1 ml). To each well 100 µl aliquots 0.2% (w/v) SDS in ETN buffer was added. Samples were solubilised by incubation at 37°C for 30 minutes. Solubilised samples were transferred to RT-30 tubes and stored at -20°C until the day of DNA assay.

3.3.4.2 Hoechst DNA Assay

The assay methodology is essentially that described by Leake & Habib (1987). To each tube 2.4 ml of ETN buffer containing Hoechst 33258 (100 ng. ml^{-1}) and RNAase (5 µg. ml^{-1}) was added. The tubes were then vortexed and incubated at 37°C for 15 mins in the dark. Fluorescent enhancement at 450 nm was measured using a Hitachi-Perkin Elmer MPE-2A fluorescent set at an excitation wavelength of 360 nm with both slit widths set at 5 nm. The results obtained were compared to a standard curve range of calf thymus DNA of 0 - 50 µg. ml^{-1}.

3.3.4.3 Total Membrane EGF Receptor Assays

Cells were plated down overnight in DMEM + 10% (v/v) FCS in 175 cm^2 tissue culture flasks. This medium was removed and the cells washed with two changes of sterile PBS-A (10 ml). Experimental medium DMEM + 5% (v/v) Dialysed HIDCCFCS ± hormones/growth factor were added to a final volume of 20 ml per flask. At appropriate incubation times flasks were harvested by using a rubber policeman to resuspend the cells into the medium of the flask. The suspension was then decanted into centrifuge tubes and centrifuged at 1000 g for 5 mins at 4°C. The

supernatant was discarded and the pellets washed twice with 1 ml ice cold PBS-A. The pellet was finally resuspended in 0.5 ml EGFR assay buffer (see section **3.1.2**). 100 µl was retained for DNA estimation (see section **3.3.4.2**) and the remaining suspension assayed for EGF receptor content (see section **3.3.4.3**).

3.3.4.4 Preparation of Samples for Hoeschst DNA Assay

100 µl aliquots of cell suspension were centrifuged at 1000 g for 5 mins at 4°C. The supernatant was discarded and 100 µl of 0.2% (w/v) SDS in ETN buffer added. The pellet was solubilised at 37°C for 30 mins. DNA estimation was by the hoechst method (see section **3.3.4.2**).

3.3.4.5 Total Membrane EGF Receptor Assay Methodology

The remaining cell suspension from section **3.3.4.3** was sonicated for 5 secs on ice using a Dawe 7532 A sonicator at a setting of 5. The sonicated cells were transferred to eppendorf vials and centrifuged for 10 mins at 15 000 g at 4°C. The supernatant was discarded and the pellet resuspended in 0.4 ml of EGF assay buffer. Duplicate 100 µl aliquots were added to equal volumes of [^{125}I] - EGF in EGF assay buffer at a final concentration of 1ng (c.p.m. 200,000). To determine the non-specific binding, duplicate 100 µl aliquots were added to 1ng [^{125}I] - EGF containing a 100 - fold excess of unlabelled EGF. This resulting labelled ligand suspension was incubated for 1 hour at room temperature. Binding was terminated by the addition of 1.0 ml ice cold EGF assay buffer followed by immediate centrifugation at 10 000 g for 15 mins at 4°C. The supernatant was aspirated and the pellet solubilised in 100 µl of 5N NaOH which was transferred to gamma counting tubes and counted. All calculations were as in section **3.1.7.1** final values were quoted as EGFR concentration. mg^{-1} DNA.

3.3.4.6 Cell Surface Binding Experiments

Cells were plated down overnight in DMEM + 10% (v/v) FCS in 24 well F.B. plates. This medium was removed and the cells washed with two changes of sterile PBS-A (1 ml). Experimental medium DMEM + 5% (v/v) Dialysed HIDCCFCS ± hormones/growth factor was added at a final volume of 0.5 ml per well. Following incubation, the plates assayed for EGFR by the whole cell binding assay technique (see **3.3.4.7.**)

3.3.4.7 Whole Cell EGF Receptor Binding Assay

The medium of each well was removed and the cells washed twice with 1 ml ice cold PBS-A. To quadruplicate sets of wells 100 µl aliquots of 0.5 ng [^{125}I] - EGF (final conc.) in DMEM + 0.1% (w/v) BSA was added. To a further set of quadruplicate wells, 100 µl aliquots of 0.5 ng [^{125}I] - EGF plus a 100 - fold excess of unlabelled EGF in DMEM + 0.1% (w/v) BSA was added. The wells were then incubated at 37°C for 30 mins in a 5% CO_2 atmosphere. Binding was terminated by the addition of 1 ml ice cold PBS-A. This was aspirated and the wells washed twice with 0.5 ml aliquots of ice cold PBS-A. 100 µl aliquots of 5N NaOH were added to each well, after 15 mins incubation at 37°C the extract was transferred to gamma tubes and counted.

The technique for full scatchard analysis was essentially the same. A complete 24 well F.B. plate was used for each assay. To 15 wells increasing concentrations of [^{125}I] - EGF was added (0.01 - 5.0 ng, 0.017 - 8.35 nm depending on the specific activity 100-170 µCi. µg^{-1}). To determine non specific binding unlabelled EGF (at 100 - fold excess) was added to the wells containing the five highest concentrations of labelled EGF.

The remaining four wells were retained for DNA estimation (see section **3.3.4.1** & **3.3.4.2**).

3.3.5 Indirect Immunofluorecence Studies

Cells were plated down overnight having been seeded at a cell density of 1 - 2 x 10^4 cells per well into 8 well glass slide/tissue culture vessels. Following overnight incubation the wells were aspirated and washed twice with PBS-A, then 0.25 ml of DMEM + 5% (v/v) dialysed HIDCCFCS ± hormones was added. 48 hours later the wells were aspirated and washed twice with PBS-A.

3.3.5.1 Indirect Immunofluorescence Method

This technique is essentially that proposed by Waterfield *et al* (1982). The cells were fixed by the addition of 200 µl acetone:methanol at -20°C per well for one minute and allowed to air dry. PBS containing 3% (v/v) sheep serum (200 µl) was then added for 20 mins at room temperature. This was then removed and primary antibody (200 µl/well) EGFR 1 (1:300 in PBS + 0.1% (w/v) BSA) added. The wells were then incubated for two hours at room temperature in a humidified chamber. The slides were then washed five times with 0.5 ml aliquots of PBS + 0.1% (w/v) BSA. Fluorescent labelled goat antimouse IgG (200 µl/well) diluted 1:100 (approx. 20 µg. ml^{-1}) in PBS + 0.1% (w/v) BSA was then added and incubated as before. Following incubation the wells were then washed five times with PBS + 0.1% (w/v) BSA.

The cells were then counterstained by the addition of 100 ng. ml^{-1} hoechst 33258 stain for five mins at room temperature. The slides were then dehydrated by sequential one minute dips in graded alcohols (50, 70, 90, 100% (v/v)) followed by

clearance in xylene. The slides were then mounted with DPX mountant and immunofluorescence examined using a LIETZ fluorescent microscope.

3.3.6 Immunoprecipitation Studies

Cells were plated down overnight in 850 cm^2 roller bottles. Following incubation the medium was aspirated and cells were washed twice with 25 ml sterile PBS-A. To each roller bottle 25 ml Methionine free DMEM containing 0.5% (v/v) dialysed HIDCCFCS + 10 μCi. ml^{-1} [^{35}S] - Methionine (Specific Activity: 1420 Ci/mM) following 24 hours incubation, hormones were added. Cells were incubated at 37°C and harvested for at specific time points. The cells were harvested by removal of the medium and washing twice with PBS-A. To each bottle 15 ml of trypsin/versene was added and the cells incubated until cell detachment from the substrate. To prevent further trypsinisation 15 ml of DMEM + 10% (v/v) FCS was added and the cell suspension thoroughly mixed. The suspension was then decanted into centrifuge tubes and centrifuged at 5000 g for 10 mins at 4°C. The supernatant was discarded and the pellet washed twice with 5 ml Tris buffer. Finally to each pellet, 0.5 ml of NP-40 solution was added and the cells incubated at 37°C for 30 mins. Following incubation the extract was centrifuged at 5000 g for 10 mins at 4°C. The supernatant was transferred to eppendorf tubes and stored at -70°C until the day of immunoprecipitation.

3.3.6.1 Immunoprecipitation Methodology

This permits the isolation and identification of small amounts of a particular protein in a complex mixture by means of its interaction with an antibody. Immunoprecipitation of antigens is usually performed in two steps.

First, specific antibody is added to a detergent lysate containing radiolabelled antigens. Next, the immunocomplexes are bound to a second agent which can be an immunoglobulin directed against the first antibody (double antibody precipitation system) or chemically fixed protein A bearing strains of *Staphylococcus Aureus* bacteria. The isolated immunocomplexes can then be resolved by SDS/PAGE and be detected by autoradiography.

3.3.6.2 Preparation of Pansorbin^{TM}

3 ml of 10% (w/v) *S.Aureus* bacteria (Pansorbin^{TM}) was washed twice with 12 ml of NP-40 solution by centrifugation at 1000 g for 5 mins at 4°C. The final pellet was resuspended in 3.0 ml of NP-40 solution, to which 100 µl of 10% (w/v) BSA was added and stored on ice.

3.3.6.3 Immunoprecipitation

Removal of nonspecific binding was achieved by adding 10 µl of washed Pansorbin^{TM} to 100 µl of NP-40 solubilised cell extract with gentle mixing for 30 mins at 4°C, followed by centrifugation at maximum speed in MSE microcentaur for 4 mins at 4°C. The supernatant was transferred to fresh eppendorf polytubes and the procedure carried out a further two times.

Whilst "clearing" the samples, 90 µl of primary antibody EGFR I at a dilution of 1: 100 was preabsorbed to 900 µl of washed Pansorbin^{TM} plus 10 µl of 10% (w/v) BSA for one hour at room temperature. Following incubation the preabsorbed antibody was dispensed into 20 x 50 µl aliquots and centrifuged for 30 secs at maximum speed at 4°C. The supernatant was discarded and the pellet placed on ice. To the appropriate polytube cleared samples were added and the tubes vortexed and placed on ice for one hour. The tubes were then centrifuged as

before and the supernatant discarded. The pellets were resuspended in 0.5 ml NP-40 solution, centrifuged as before and the supernatant discarded.There then followed two washes with 0.5 ml SDS buffer and 0.5 ml Tris buffer respectively. Following this final step the pellets were stored at -70°C until SDS/PAGE analysis.

Prior to analysis by SDS/PAGE (see section **3.2.5**) the pellets were boiled for 3 mins in 100 µl of sample buffer. Following SDS/PAGE the gel was dried at 80°C under vacuum for 2 hours and subjected to autoradiography using hyperfilm for one month at -70°C.

3.3.6.4. Densitometric Analysis

Densitometric Analysis of the autoradiograph produced was by using a Bio-rad model 620 video densitometer.

3.3.7 Conditioned Medium Studies

Cell culture was essentially that outlined in section **3.3.4.6.** After 48 hours incubation in the presence of hormones/growth factor the conditioned medium (CM) was removed. The CM was then filter sterilised, charcoal stripped without heat inactivation or dialysis (see section **3.3.1**) and added to fresh medium + 5% (v/v) dialysed HIDCCFCS at final concentration of 50:50 (v/v). This medium was then added to fresh medium and subjected to the following treatments where appropriate.

(a) Trypsin Treatment

Trypsin was added to a final concentration of 10 µg. ml $^{-1}$ and incubated for 30 mins at 37°C. The trypsinisation reaction was terminated by the addition of two - fold excess soya bean inhibitor.

(b) Heat Inactivation

The CM was incubated for 30 mins at 56°C.

(c) Cycloheximide Treatment

Cycloheximide was added to the CM: fresh medium preparation to a final concentration of 40 nM.

The treated CM: fresh medium preparations were then added to unstimulated cells and EGF receptor contents (see section **3.3.4.7**) and DNA contents (see section **3.3.4.2**) assessed after 48 hours incubation at 37°C in 5% CO_2 atmosphere.

3.3.8 Prolactin Receptor Assays

Cell culture methods were the same as those outlined in section **3.3.4.6**. Prolactin receptor estimation was essentially the same as that proposed by Shiu (1979) with the modifications proposed by Sakai & Suzuki (1989). In the E_2 dose response studies (see Fig. **4.3.9**) and in the time course studies (see Fig. **4.3.10**) estimation of receptor levels was by the single point method. To remove endogenous PRL, each well was washed twice with 4M MgCL (0.5ml) at room temperature, followed by two washes with ice cold assay buffer (10 mM Sodium phosphate, 150 mM Nacl, 10 mM EDTA, 0.1 (w/v) BSA). To washed quadruplicate wells, 0.5 ml of [^{125}I] - hPRL in assay buffer at a final concentration of 1nM ± a 100 - fold excess of unlabelled hPRL was added. Incubation was carried out for six hours at room temperature. The cells were then washed twice with ice cold PBS-A and the wells extracted and counted as outlined in section **3.3.4.7**.

Scatchard analysis was performed by the same techniques as above using a concentration range of nine points in duplicate of 0.01 - 5 nM [^{125}I] - hPRL.

Estimation of non specific binding was by the top three concentrations plus a 100 - fold excess of unlabelled hPRL.

3.3.9 Inositol Phosphate Assays

After plating on 24 well F.B. plates in DMEM and 10% (v/v) FCS for 24 hours the medium was then removed and the cells washed twice with sterile PBS-A. 1 ml of DMEM and 1% (v/v) dialysed HIDCCFCS with either 1μCi. ml^{-1} (total Inositolphosphates (InsPs)) or 5 μCi. ml^{-1} (individual InsP.) of myo- [2-^{3}H] - inositol was added. The cells were then incubated for 48 hours. Labelling medium was aspirated and the cells incubated in 1 ml Hanks buffered saline (pH 7.4) for 3 minutes at 37°C. This was followed by an incubation in 1 ml Hank's/BSA/Glucose (HGB) containing 10 mM lithium chloride for 20 minutes. Finally the cells were incubated with 200 μl of HGB and lithium chloride containing prolactin at the final concentrations and times indicated. For assays of total phosphates, incubations were terminated by direct addition of 500 μl of ice cold methanol. After scraping, the cell debris was transferred into a 2 ml plastic polytube, each well was washed again with a further 200 μl of methanol and the two washes pooled. Phospholipids were extracted from the cell debris by addition of 300 μl of chloroform followed by vortexing and standing on ice for 30 minutes.

Following the addition of 300 μl of ice cold distilled water the samples were centrifuged at 10 000 g for 3 mins at 4°C to split phases , 1ml of the upper aqueous/ methanolic phase was transferred to 5 ml scintillation vials and 3 ml of Optiphase Hi-safe scintillation fluid was added and the total inositol phosphate fractions counted on an LKB scintillation counter, counting efficiency 65%.

Assays of individual phosphate fractions followed a different methodology. The wells were aspirated and 150 μl of ice cold 10% (v/v) perchloric acid added, inositol phosphates were extracted on ice for 20 mins followed by scraping and

removal of all debris into an eppendorf tube. The 24 well F.B. plates were then washed with a further 150 µl of distilled water, which was pooled with the acidified extraction and centrifuged at 10 000g for 10 mins at 4°C

The supernatants were neutralised by the addition of 1.5 M potassium hydroxide/60mM HEPES in the presence of universal indicator and the precipitated potassium perchlorate removed by centrifugation at 10 000g for 10 mins. The supernatants were analysed by ion exchange chromatography on Dowex 1x8 formate column.

Neutralised supernatants were diluted in 3ml of 5 mM sodium tetraborate/0.5 mM EDTA (pH 6.7) and applied to 1 ml columns of Dowex formate 1x8. 200-400 mesh prepared in glass wool plugged pasteur pipettes. The columns were then washed with 12 ml of water followed by 12 ml of 60 mM ammonium formate/5 mM sodium tetraborate to remove free inositol and glycerophosphoinositols respectively. Inositol mon-, bis-, tri- and tetraphosphates were eluted with sequential 10 ml washes of 0.1M formic acid containing 0.2, 0.4, 0.8 and 1.2 M ammonium formate respectively. Aliquots of each fraction (3 ml) were mixed with Optiphase Hi-safe scintillant and the radioactivity determined by liquid scintillation counting.

Chapter 4 Results

Results Chapter 4.1

4.1 Introduction

Although there are studies available for EGF receptor levels as assessed by ligand binding assay for other parts of the U.K. and in other countries. No data were available for Scotland at the time of conception of this project.

All assays were carried out between the period of November 1987 and January 1990 from biopsies received from patients in hospitals throughout the Strathclyde region. The majority came from the Glasgow Western Infirmary.

This chapter reports data on the ligand binding assay system used to assess EGF receptor levels, and presents the results in relation to the oestrogen receptor status of the biopsies.

4.1.1 Optimum Time course of [^{125}I] - EGF binding

Prior to assay of breast tumours, it was decided to assess the time course of total binding of labelled EGF to its receptor. Incubation was carried out at 4°C, room temperature (21°C) and 30°C by the methodology outlined in section **3.1.7.1**, using as positive control tissue, freshly processed placental membranes, prepared as described in methods section **3.1.3**.

Following the addition of [^{125}I] - EGF at a final concentration of 1nM to placental membranes at a final concentration of 50 µg protein, it was found that maximum total binding occurred from 19 hours at 4°C (see figure **4.1.1.1**), 60 minutes at room temp (21°C) (see figure **4.1.1.2**), and 40 mins at 30 °C (see figure **4.1.1.3**). On the basis of these findings, assay incubation time course was set at room temperature for one hour.

The rationale behind choosing 1nM [^{125}I] - EGF was based on reports by other workers who have reported Kd values for EGFR in human placenta in the region of 10^{-10} and 10^{-9} M. The concentration chosen above does not of course saturate the lower affinity site, but it does avoid influencing the binding kinetics which can be caused by a significant amount of binding to non receptor sites seen at high ligand concentrations.

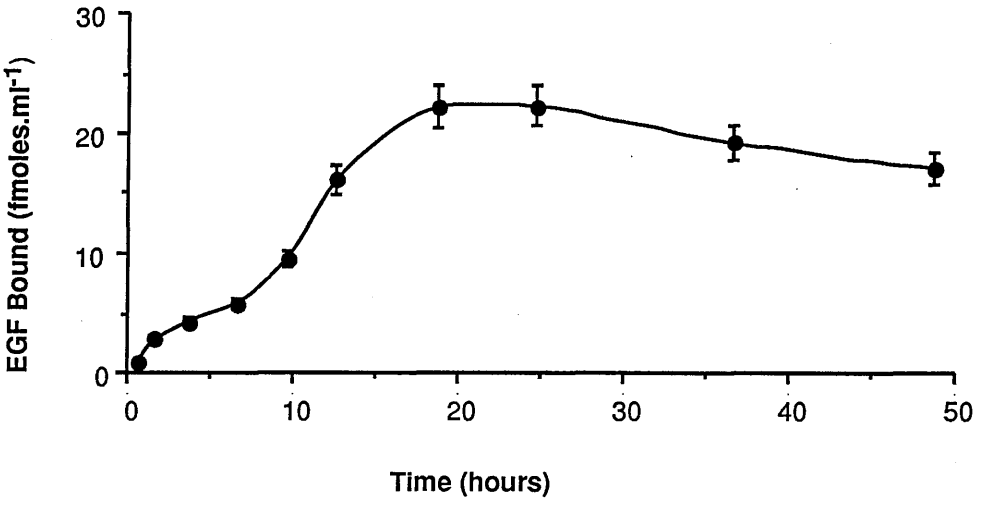


Figure 4.1.1.1

Time course of [^{125}I] - EGF binding to placental membranes at 4°C

Each point represents the mean of quintiplicate assays (SD< 7%). Each assay involved 50 µg total protein content of human placental membrane incubated with 1 nM [^{125}I] - EGF at 4°C for the times shown

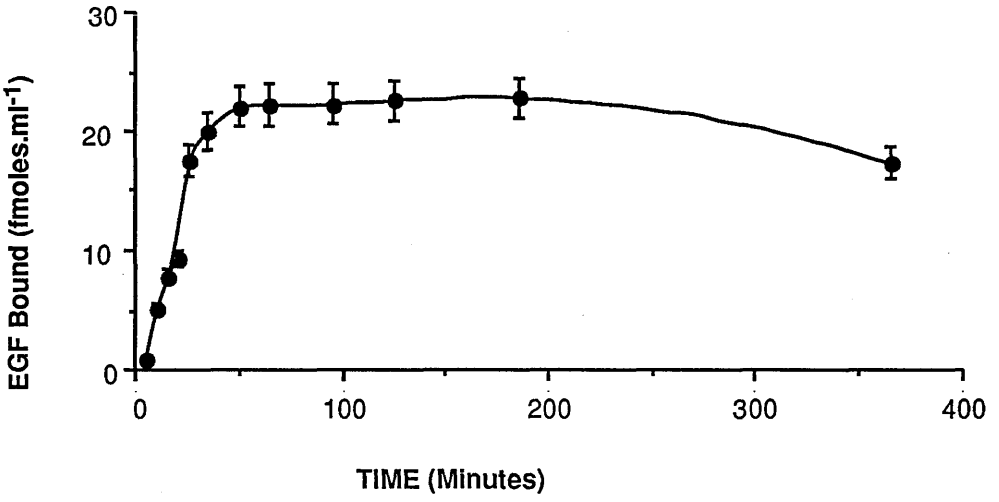


Figure 4.1.1.2

Time course of [^{125}I] - EGF binding to placental membranes at 21°C

Each point represents the mean of quintiplicate assays (SD< 5%). Each assay involved 50 µg total protein content of human placental membrane incubated with 1 nM [^{125}I] - EGF at room temp 21°C for the times shown.

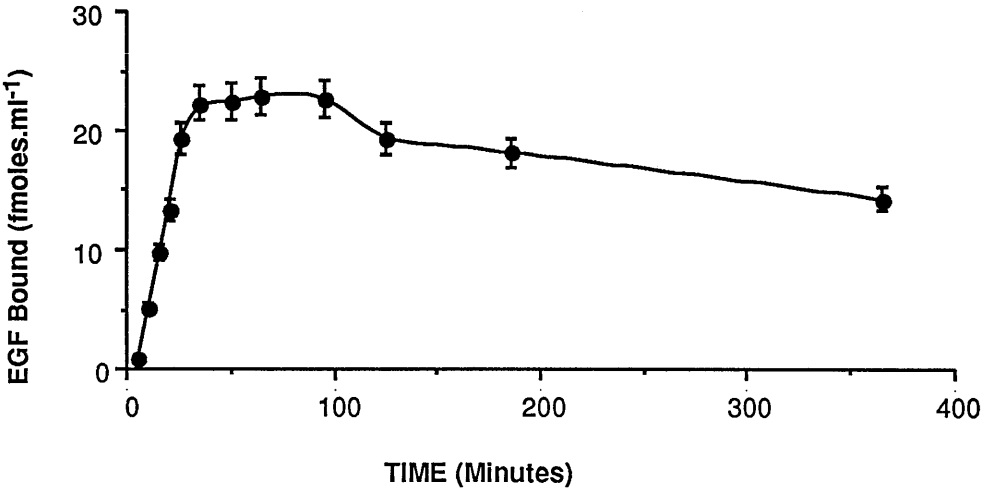


Figure 4.1.1.3

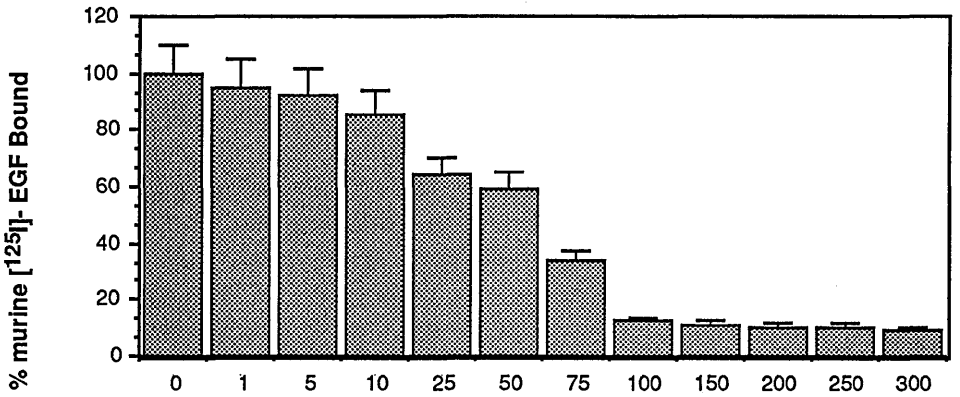
Time course of [^{125}I] - EGF binding to placental membranes at 30°C

Each point represents the mean of quintiplicate assays (SD< 6%). Each assay involved 50 µg total protein content of human placental membrane incubated with 1 nM [^{125}I] - EGF at 30°C for the times shown.

4.1.2 Determination of non-specific binding (NSB)

In any ligand binding assay system, a portion of total binding is not specific. The most common method of assessing NSB is to compare total binding of labelled ligand to that of binding derived in the presence of an excess of unlabelled ligand. The optimum concentration of excess unlabelled ligand is that which competes for the maximum amount of EGFR without competing for [^{125}I] - EGF binding to proteins other than receptor. Incubation was carried out at room temperature using the methodology outlined in section **3.1.7.1.**

[^{125}I] - EGF (1nM final concentration) was displaced by the addition of increasing concentrations of unlabelled murine ligand (see figure **4.1.2.1**). Residual (NSB) binding was deemed to be that retained at a concentration of 100 - fold excess or greater. On the basis of these findings it was decided to incorporate NSB estimation at a concentration of 100 - fold excess of unlabelled ligand both for single point screening and full multipoint analysis. To assess the biological equivalance between the murine labelled assay system and the human ligands EGF and TGFα. Exchange of labelled murine EGF by increasing concentrations of unlabelled human EGF and TGFα was carried out (see figure **4.1.2.2**). Plots derived from experimental data displayed similar displacement kinetics to those seen with murine EGF (see figure **4.1.2.1**). 50% displacement values were 74 nmoles. litre^{-1} for human EGF and TGFα and 62.5 nmoles. litre^{-1} for murine EGF.



Each point represents the mean of quintiplicate assays (SD <10 %)

Fig. 4.1.2.1

Displacement of murine [^{125}I] - EGF from placental membrane.

Human placental membrane was exposed to 1nM murine [^{125}I] -EGF for one hour at room temperature in the presence of increasing concentrations of unlabelled murine EGF.

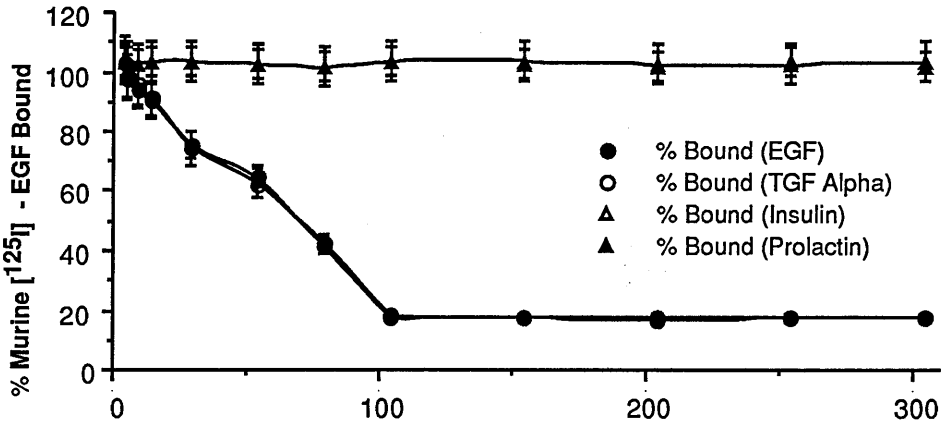


Figure 4.1.2.2

Displacement of murine [^{125} I] - EGF by human EGF and TGF Alpha

Human placental membrane was exposed to 1 nM murine [^{125} I] - EGF for one hour at room temperature in the presence of increasing concentrations of unlabelled human EGF and human TGF Alpha. Specificity of displacement was compared against displacement of 1nM murine [^{125} I] - EGF by ovine Prolactin and Bovine Insulin.
Note that the displacement kinetics of both human EGF and human TGF Alpha are similar to those of murine EGF alone.
(see figure **4.1.2.1**)

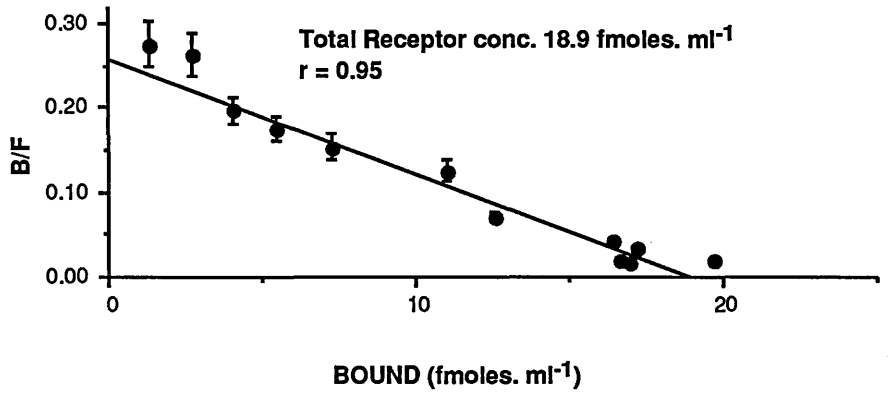
4.1.3 Comparison of Assay Methods

One of the major problems in EGFR estimation by ligand binding assay has been the variations in methodology cited in the literature (see Introduction **1.8.13**) e.g. whether there was single or multiple concentrations of labelled ligand with NSB estimated by an excess variable concentration of unlabelled ligand. Further, the methods of separating bound from free ligand differ, preparation of tissue sample, time and temperature of incubation etc.

Due to these variables it was decided to compare our method (see section **3.1.7.2**) with the common methodology proposed by the European Organisation for the Research and Treatment of Cancer (EORTC) study group (see section **3.1.7.4**) using the lyophilised placental membrane quality control standard provided. Each vial was reconstituted in 2ml deionised distilled water. The purpose of the EORTC method was to establish whether a common methodology could lead to similar conclusions from all centres on the clinical value of EGFR determination in different human cancers.

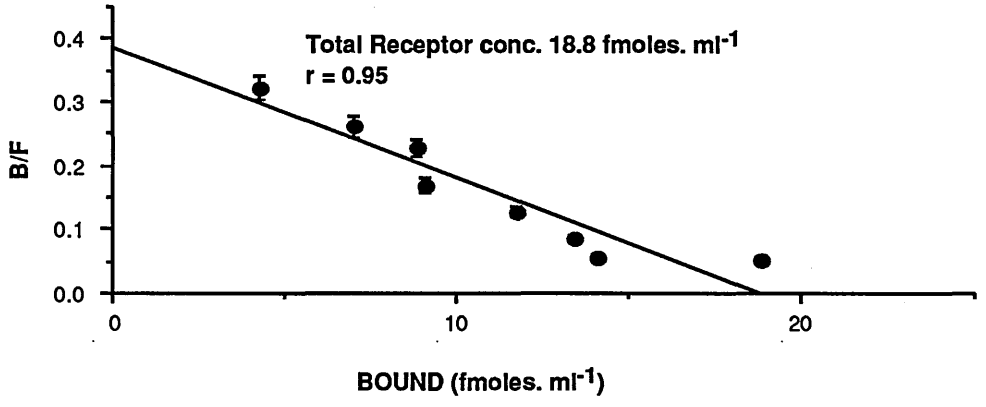
As can be seen total receptor concentration determined by our method , 18.9 fmoles. ml^{-1}, was very similar to the result recorded using the EORTC protocol, 18.8 fmoles $.ml^{-1}$. The Kd values recorded showed some variation 0.064 nM " in house " compared to 0.049 nM EORTC (see figure **4.1.3.**). No low affinity binding component was observed by either method. This may be a result of the lyophilisation process. It should be noted that our results using the EORTC protocol correlated with the median point of the values reported by all members of the EORTC receptor study group in the first two rounds of EGFR assay quality control.

" In house " Assay Protocol



Each point is the mean of triplicate assays (SD <10%)

EORTC Assay Protocol



Each point is the mean of quintuplicate assays (SD <6%)

Fig. 4.1.3

Comparison between "In house" and EORTC assay protocols for EGFR determination using lyophilised human placental membrane

4.1.4 Single Point Screening and Multipoint Scatchard Analysis.

Initial screening of breast tumour membrane preparations (see methods section **3.1.3**) was by the single point method (see methods section **3.1.7.1**). On the basis of the screen a positive result was recorded if the membrane fraction contained greater than 10 fmoles specific binding $.ml^{-1}$ of membrane suspension. No specific $[^{125}I]$ - EGF binding was observed in membrane fractions derived from pathologically normal breast epithelium (n = 4).

Wherever sufficient material was available full multipoint binding analysis (see methods section **3.1.7.2**) was carried out on samples found to be positive in the single point screen. Multipoint analysis was also carried out on random negative samples to ensure that the single point screen was correctly indentifying EGFR positive samples. Correlation coefficent for the single point screen and Scatchard values recorded for the low capacity high affinity binding site was r = 0.775 (see figure **4.1.4**).

4.1.5 Multipoint Analysis

Figure **4.1.5** refers to an example scatchard plot of a patient biopsy received for ER/EGFR analysis from the Glasgow Western Infirmary. Initial two point screening revealed EGF binding levels of 92 fmoles. ml^{-1}. Subsequent analysis demonstrated " Classical " double binding site kinetics. A high affinity low capacity binding component of 120 fmoles. ml^{-1} and a low capacity component of 400 $fmoles.ml^{-1}$.

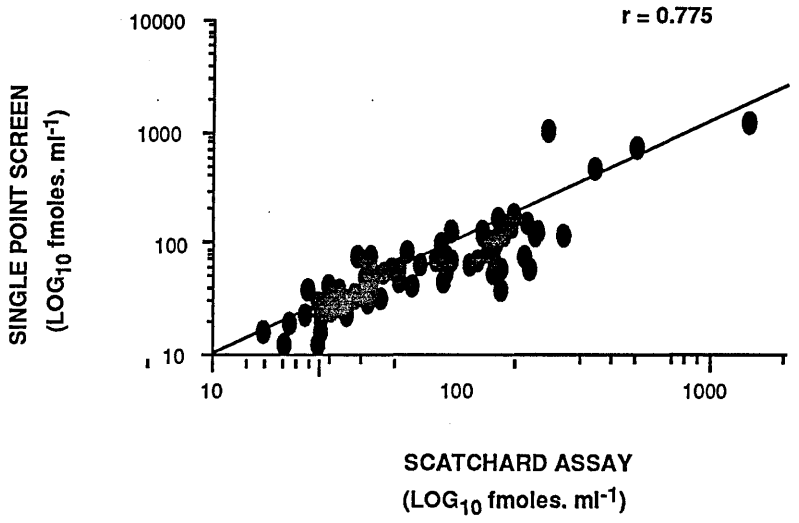


Fig 4.1.4

Log plot of receptor levels as assessed by single point screening assay versus that of high kd receptor levels by multipoint analysis .

Values for EGFR determined by the single point screen were compared with the high Kd receptor values (i.e. low Kd receptor values were ignored for this purpose because the single screen concentration was only 1 nM) determined by Scatchard analysis.
Correlation was carried out using the Statworks package on an Apple Macintosh Plus system.

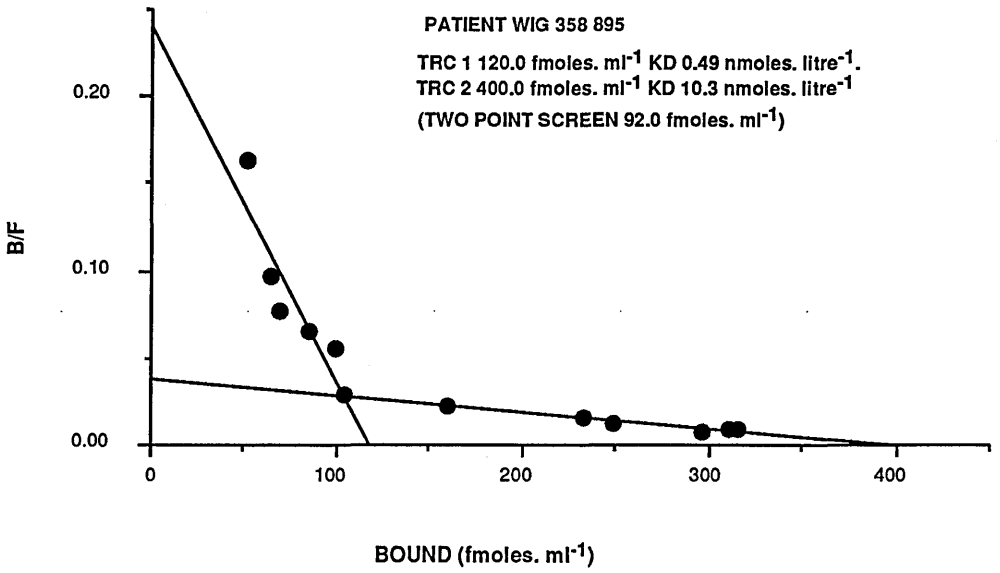


Fig 4.1.5

Example Scatchard binding data derived from membrane preparation of abreast tumour biopsy receieved for ER/EGFR analysis.
The assay conditions were those of the " in house " method.

Data was computed manually and the curve fits resolved on a Apple Macintosh plus system using the Cricket Draw package.

4.1.6 Oestrogen Receptor Levels in EGF Receptor Negative Tumour Preparations

199 patients were screened for EGFR presence by the single point method, 124 were found to be EGFR negative i.e. had levels of EGF binding of < 10 fmoles. ml^{-1}. Within this group, 99 (79.8%) were ER positive i.e. contained levels of oestrogen binding of greater than 20 fmoles. mg^{-1} protein for the cytosolic fraction and 150 fmoles. mg^{-1} DNA for the nuclear fraction as determined by ligand binding assay (see methods section **3.1.4**). These data are summarised in table **4.1.1**. As can be seen from table **4.1.2**, ER values recorded display wide variations of receptor concentrations, in both cytosolic and nuclear fractions. This is probably due at least in part to tumour heterogeneity.

4.1.7 Analysis of EGF Receptor Positive Group.

Of the 75 patients deemed who, by the single point screen had tumours which were EGFR positive, 73 full multipoint plots were carried out (Table **4.1.3**). Scrutiny of the data revealed 65 plots which satisfied our criteria of a linear regression coefficient of greater then 0.7 for each binding component. All 65 contained high affinity, low capacity binding. Low affinity, high capacity binding was observed in 60. Wide variations were again recorded with respect to concentrations and affinities.

Scrutiny of initial follow up data (mean 16.9 months from date of biopsy) reveal six deaths out of the 199 patients analysed. Because of the relatively small sample pool, it is common practice that no statistical analysis is attempted until a median five year follow up period has elapsed.

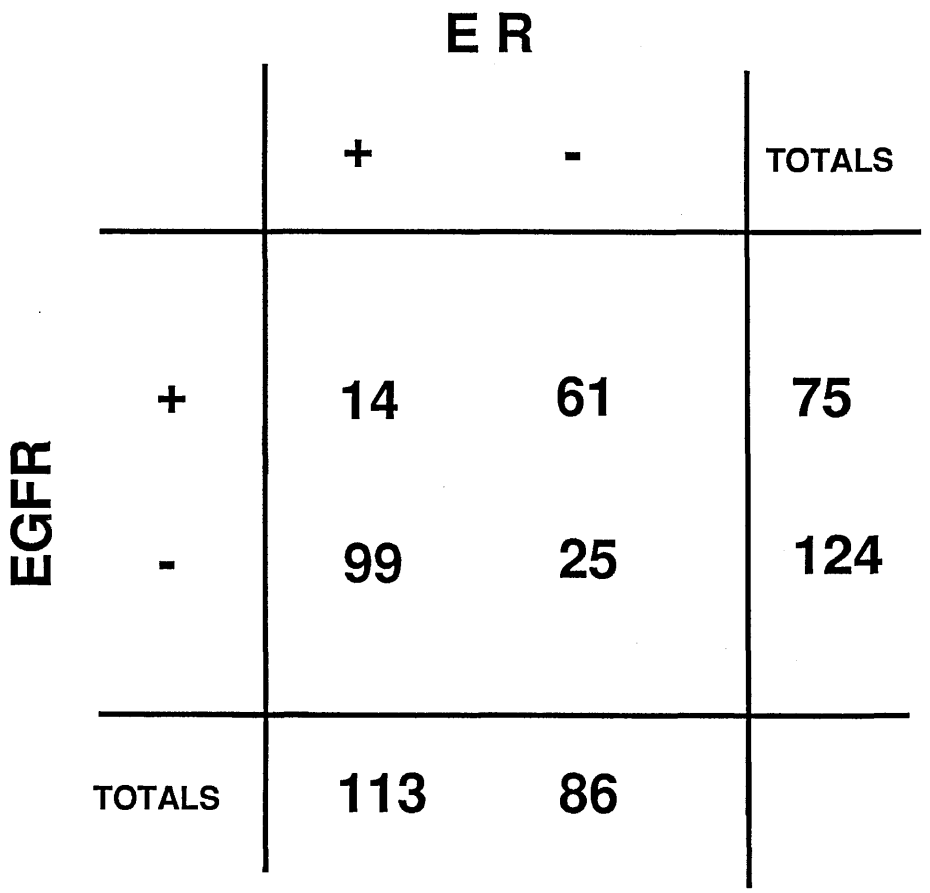


Table 4.1.1

Tabulation of Oestrogen Receptor and Epidermal Growth Factor Receptor ocurrence in 199 breast cancer patients in the West of Scotland

	CYTOSOL	NUCLEAR
Total Receptor Concentration	(fmoles. mg^{-1} Protein)	(fmoles. mg^{-1} DNA)
Range	19.7 - 1156.0	163.0 - 9680.0
Mean	156.9	1662.5
SD	215.5	1986.9

Age range : 33 - 94 ; Mean 61.7.

Table 4.1.2

ER Scatchard Analysis of the EGFR -ve group

Cut-off values for ER were 20 fmoles. mg^{-1} protein for cytosol (one tumour of 19.7 fmol. mg^{-1} was taken as = 20. 150 fmoles. mg^{-1} DNA was taken for the nuclear fraction.

	High Affinity	Low Affinity
Number of Observations	65	60
Total Receptor Concentration (fmoles.mg^{-1} DNA)		
Range	69.6 - 28201.0	360.6 - 34466.0
Mean	1581.24	5223.76
SD	3647.64	6233.48
KD Values (nmoles. litre^{-1})		
Range	0.010 - 1.4	0.108 - 38.7
Mean	0.362	8.892
SD	0.351	7.503

Table 4.1.3

Scatchard Analysis of EGFR positive group

73 full scatchards were carried out in the EGFR +ve group.
Age range : 32 - 91 ; Mean: 59.5, no correlation was found between age and EGFR content. 14 (19.2%) patients were also ER positive, no correlation was found between ER and EGFR concentrations in this group.
Further, the ER concentrations covered the same range as in the EGFR negative patient group (Table 4.1.2).

4.1.8 Results Conclusion/ Discussion

The data in this chapter show that approximately 38% of human breast cancer biopsies assayed (75/199) contained appreciable levels of EGFR, and in this group only 7% of the total population contained ER. This contrasts with the 99/124 positive for ER in the EGFR negative group (see also table **4.1.1**). EGFR was estimated by ligand binding parameters. Our initial single point screen correlated reasonably well with the high affinity low capacity sites calculated from subsequent multipoint scatchard analysis. The high affinity low capacity and low affinity high capacity values recorded (of the 73 saturation analyses carried out , 65 yield high affinity and 60 yield low affinity binding sites) are comparable with those reported by Nicholson *et al.* (1988). This contrasts with the reports of Fitzpatrick *et al.* (1984), Battaglia *et al.* (1988) and Cappelleti *et al.* (1988), who have found only a single high affinity binding site . This discrepancy is probably due mainly to differences in methodology although there may be some contribution from tumour heterogeneity, but this would hardly explain 60/75 tumours with low affinity sites as observed here.

Whilst confirming the inverse correlation between ER and EGFR, it was interesting to note that, in the group containing both receptors, no correlation was observed with respect to receptor concentrations suggesting that these tumours may be of a polyclonal origin. It is hoped that double staining immunocytochemical studies of this group will yield data concerning location of the two receptors within the tumour or total tissue cell population. One thought provoking observation was noted in the case of two patients who presented with disease in both breasts. EGFR and no ER was detected in one breast, ER and no EGFR in the other. This fits in with the results by Davidson *et al.* (1987) who have reported the absence of ER gene expression in human breast cancer was associated with increased levels of EGFR protein and mRNA. This suggests that in the ER positive/ EGFR negative breast, tumour growth is regulated in an

endocrine dependent manner, and in the case of the ER negative/ EGFR positive breast, tumour growth was regulated in an autocrine/paracrine manner.

In contrast to the results seen in breast cancer biopsies in which the majority contain either EGF or ER but not both. Some breast cancer cell lines commonly used as *in vitro* models show an interdependence of steroids and growth factors. Bates *et al.* (1988) using the endocrine dependent breast cancer model (MCF-7) demonstrated regulation of TGFα production by oestradiol, TGFα mRNA was found in three other ER positive cell lines and in 70% of patients analysed.

On binding to its ligand, EGFR usually undergoes auto- phosphorylation, internalisation, and degradation (Yarden & Schlessinger, 1985). These events cause a decrease in receptor binding (down modulation) and can lead to a situation where ligand binding techniques cannot detect EGFR. Up regulation of EGFR mRNA and protein in the MCF-7 and T-47D cell lines has been demonstrated in the presence of progestins (Murphy *et al.*, 1986; Murphy *et al.*, 1989).

Studies within our lab have shown that prolactin at physiological levels can increase EGFR protein, ligand binding and mitogenic activity in the ZR-75-1 cell line (see results Chapter **4.3**).

These reports may oversimplify the growth control mechanism in ER positive breast cancer. Oestradiol controls the autocrine/paracrine production of the ligand TGFα. Progesterone, whose own receptor (PR) is induced by ER DNA binding (Sarup *et al.*, 1988) controls the levels of EGFR. Cormier *et al.* (1989) have demonstrated that the addition of EGF to ER dependent breast cancer cells leads to a decrease of progesterone binding. Other workers have found that EGF treatment abolishes ER induction of PR (Katzenellenbogen *et al.*, 1987).

Via these events EGF/TGFα could regulate its own receptor not only in terms of ligand binding and modulation, but also at the level of its own receptor production due to its ability to down regulate PR. Because EGFR is in a state of constant modulation, only low levels (undetectable ?) need be in operation for

efficient mitogenic function. This latter possibilty might explain the apparent contrast between cell lines and solid tumours.

Loss of endocrine function in this scheme would at first glance lead to a total loss of EGFR levels, due to loss of PR control and/or ER controlled ligand production. But it is possible that loss of ligand by ER depletion leads to a decrease of ligand binding and therefore internalisation, degradation events. Alternatively, there may be an "over production" of receptor in an attempt to pick up the reduced levels of TGFα available. Another intriguing possibilty may be that loss of ER/PR control leads to uncontrolled production of EGFR and TGFα. This could possibly be the case in EGFR positive/ER negative breast tumours.

It is interesting to note that studies by Dickson *et al.* (1987) have shown that the addition of EGF to breast cancer cell lines followed an inverse pattern of stimulation. Cell lines containing relatively small amounts of EGF binding sites (less than 43,000 sites per cell) were mitogenically stimulated whereas cell lines containing (greater than 90,000 sites per cell) displayed no response or were inhibited. This also correlated with the ER status of cell lines investigated.

It is tempting to speculate that high EGFR levels may be a reflection of loss of endocrine control. In this group, tumour cell mitogenesis is no longer dependent on EGF/TGFα activating EGFR. Other mitogenic factors could be of greater importance.

Another possibility may be that the basal intrinsic tyrosine kinase activity of EGFR is at a level sufficient to promote EGFR mitogenic function irrespective of ligand binding. This possibility in has not been thoroughly investigated.

Results Chapter 4.2

4.2.1 Nuclear Localisation Studies

It is accepted that the EGF receptor is localised mainly in the cell membrane. However there are some indications that the receptor is present in other areas of the cell. Studies have revealed that EGF binding can be observed in the triton insoluble portion of A431 Cells (Weigant *et al.*, 1986). Indirect immunofluorescence studies have revealed perinuclear localisation of the EGF receptor upon EGF treatment in A431 cells and murine 3T3 cells (Murthy *et al.*, 1986). The insulin receptor, another tyrosine kinase cell surface receptor, has been demonstrated to undergo nuclear translocation in the nuclei of isolated rat hepatocytes (Podlecki *et al.*, 1987).

Studies by Rakowicz-Szulczynska *et al.* (1986, 1989) have shown chromatin binding of EGF, NGF and PDGF in cell lines containing the appropriate surface receptors. However no data were available for the solid tumours. This section of this thesis deals with preliminary studies on the nuclear localisation of the EGF receptor within a pooled breast tumour homogenate.

4.2.2 Results

30 gm of unselected breast tumour tissue were processed by the methods outlined in section **3.2.2** & **3.2.3.** to yield both membrane and nuclear fractions.

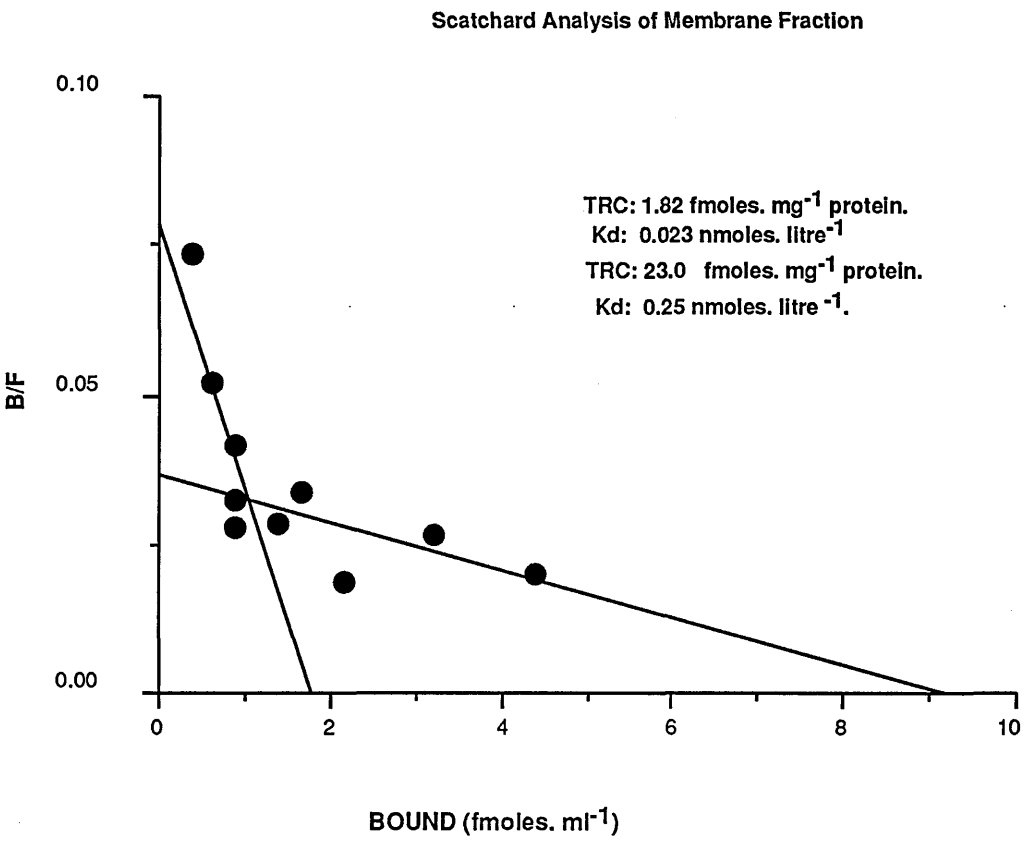
4.2.3 Scatchard Analysis of Membrane Fraction

Scatchard analysis of the membrane EGFR component revealed double site kinetics (Figure **4.2.1**). A high affinity low capacity binding component of Kd 0.023 nmoles. litre^{-1} and a receptor level of 1.82 fmoles. ml^{-1} (2.6 fmoles. mg^{-1} membrane protein), plus a low affinity binding component high capacity binding component of Kd 0.25 nmoles. litre^{-1} and a receptor level of 9.1 fmoles. ml^{-1} (23 fmoles. mg^{-1} membrane protein) were detected. These values vary somewhat from the studies outlined in table **4.1.2** with respect to the affinity values noted. This could be partly due to the difference between the in house and the EORTC method used in these studies with regard to receptor affinity (see figure **4.1.3**), or alternatively may be the result of bulk processing of combined tumour biopsies.

4.2.4 Scatchard Analysis of Nuclear Fraction

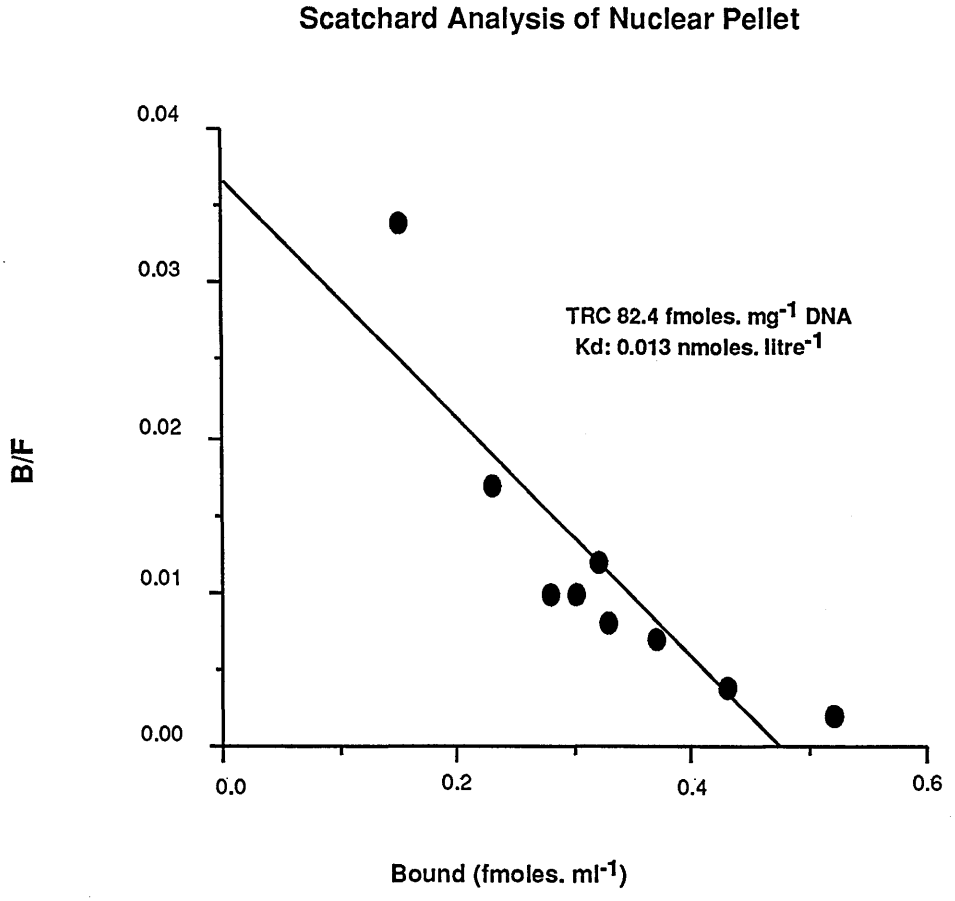
Analysis of the nuclear fraction (Figure **4.2.2**) revealed a single high affinity low capacity binding component, Kd 0.013 nmoles. litre^{-1}, with receptor levels of 0.47 fmoles. ml^{-1} (82.4 fmoles. mg^{-1} DNA). Salt extraction (see methods **3.2.3**) of the nuclear fraction (see figure **4.2.3**) resulted in some changes in the single high affinity low capacity binding component, Kd 0.022 nmoles. litre^{-1}, with receptor levels of 0.96 fmoles. ml^{-1} (72.3 fmoles. mg^{-1} DNA).

Fig. 4.2.1



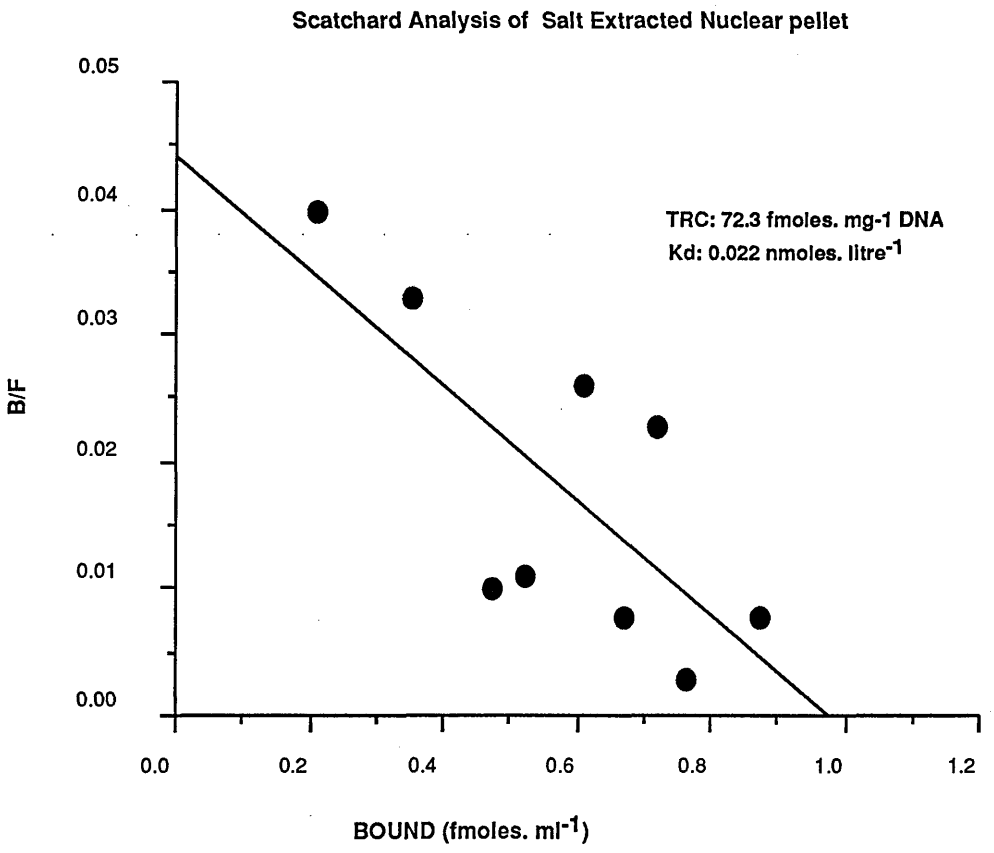
A bulk membrane fraction was prepared from 30 gm of unselected breast cancer biopsy material.
A fraction (0.7 mg/ml membrane protein) was assayed for EGFR levels using the EORTC method (see methods **3.1.7.4**)

Fig. 4.2.2



The nuclear fraction was prepared as described in methods **3.2.2** and assayed for EGFR levels as described in methods **3.1.7.4.**

Fig 4.2.3



A portion (2 ml) of the nuclear pellet was salt extracted with 2M NaCL for 30 mins at 0°C.
The pellet was then assessed for EGFR binding by the EORTC method described in **3.1.7.4.**

4.2.5 Western Blotting of Tumour Fractions

Western blotting (see methods **3.2.5 - 3.2.11**) of the tumour fractions reveal that the nuclear localised form of the receptor appears to be the same molecular weight as that of the membrane bound form as assessed by autoradiography (figure **4.2.4**).

4.2.6 Membrane Marker Enzymes

To ensure that there is no contamination of the washed nuclear fraction by plasma membranes. The relevant fractions were assayed for two known membrane enzyme markers, 5' nucleotidase and alkaline phosphatase (see methods **3.2.12** and **3.2.13**). As can be seen from table **4.2.1** whilst there is appreciable enzymic activity in both the plasma membrane and unwashed nuclear fractions, there is no activity in the washed nuclear fraction for either enzyme.

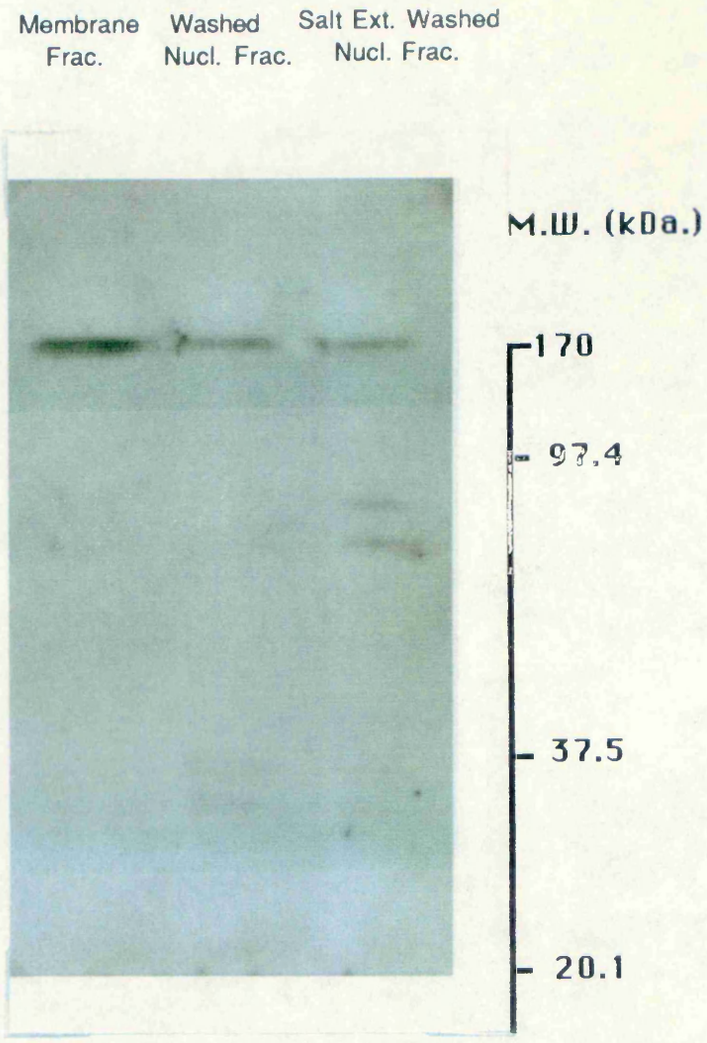


Figure **4.2.4**

7.5% PAGE/Western Blot/Autoradiogram of Breast Tumour Pool see methods **3.25 - 3.2.11**

Table 4.2.1 Plasma Membrane Marker Analysis of Tumour Fractions

Membrane Marker	Membrane Fraction	Nuclear Fraction	Washed Nuclear Fraction
Alkaline Phosphatase (OD 600 nm. mg $^{-1}$Protein)	0.038	0.021	0.00
5' Nucleotidase (µg. KH_2PO_4 std. mg^{-1} Protein	6.75	15.23	0.00

Table 4.2.1
Marker enzymes were assayed by the methods outlined in **3.2.12** and **3.2.13.**
Each value cited is the mean of triplicate determinations.

4.2.7 Result Conclusion/Discussion

Three tentative conclusions may be derived from this study :-

(a) There appears to be a single high affinity low capacity binding class of the EGF receptor located within the chromatin complex in breast tumours containing surface EGF receptors

(b) High salt extraction of the chromatin complex (although affecting receptor affinity and concentration to some degree by appearing to double both values (See Figures **4.2.2** and **4.2.3**) did not eliminate binding. Therefore the nature of receptor/chromatin interaction is likely to be highly specific.

(C) The chromatin bound receptor form appears to be the same molecular weight as the membrane bound. This suggests that the chromatin bound receptor form is not significantly processed before binding to the chromatin.

Whilst the above to data appears to confirm the early observations that there may be nuclear localised form of the EGF receptor. It should be noted that previous reports have not attempted to assess possible contamination of the nuclear fraction by plasma membrane, the main site of EGF receptor localisation. From our results, no activity of the two membrane associated enzymes, Alkaline phosphatase and 5'Nucleotidase was observed in the nuclear fraction. However it is possible that there may be contamination from the transmembrane portion of the membrane. In retrospect assessment of a transmembrane associated enzyme, such as Adenylate cyclase would have been a valuable indicator of transmembrane contamination.

Although it generally accepted that EGF initiates its mitogenic activity through specific cell surface receptors, followed by activation of the protein tyrosine kinase in the cytoplasmic domain of the receptor, it is not known what secondary processes are required for their ultimate effect. Translocation of EGF into the nucleus has been demonstrated by a few authors (Johnson *et al.*, 1980a, Johnson *et al.*, 1980b, Savion *et al.*, 1981, Rakowicz-Szulczynska *et al.*, 1986). Johnson *et al.* (1980b) demonstrated that in addition to mitogenesis, EGF alters the activity of several genes and modifies the chromatin structure. Studies by Rakowicz-Szulczynska *et al.* (1989) have shown that EGF can bind to a single *EcoRI* fragment. Further, binding of EGF appears to confer a DNAase II protection effect. This promotes the possibility that EGF receptor complex may bind within the specific promoter/enhancer regions of unknown genes.

The interaction of growth factor receptors with the genetic apparatus of solid tumours is an exciting prospect. It is outwith the scope of this thesis to investigate further these observations. However it is tempting to speculate on possible avenues of investigation, one important clinical application could be the assessment nuclear localisation of EGF, PDGF, IGF I & II receptors in solid tumours and their possible prognostic significance. Nuclear localisation studies of tyrosine kinase oncoproteins such as Neu and verb B could lead to greater understanding of their role in cell mitogenesis. Further, the potential for blocking growth signals at the nuclear level gives hope for improved anti-cancer agents.

Results Chapter 4.3

4.3.1 Introduction

Regulation of the EGF receptor by oestrogen and progesterone has been reported in cell culture systems used as models for oestrogen dependent carcinoma (see Introduction **1.8.9**). This section of the thesis deals with the possible involvement of other hormones in EGF receptor regulation and in particular with that of Prolactin .

Prolactin (PRL) has been shown to have a role in both the induction and growth of rodent mammary tumours (Welsch, 1987), and is mitogenic both *in vivo* (Kiss *et al.*, 1987; Muldoon, 1987) and *in vitro* (Manni *et al.*, 1985 & 1987). PRL has been found to promote the growth of human mammary carcinoma cells in culture (Malarkey *et al.*, 1983; Simon *et al.*, 1984; Vonderhaar & Biswas, 1987). The precise function of PRL in human breast cancer is unclear, but it may contribute to breast cancer risk due to its ability to stimulate breast epithelial growth (Bruning, 1987). Elevated plasma PRL has been reported in women considered to be high risk. These studies are confounded by many variables such as blood sampling, stage of menstrual cycle, etc (Wang *et al.*, 1987). Reports have suggested that the presence of PRL receptor (PRLR) is a significant prognostic factor in the relapse free survival of oestrogen receptor (ER) positive patients (Bonnetre *et al.*, 1987). This is in addition to the findings of Murphy *et al.* (1984), that there is significant linear correlation between PRLR and ER in cell lines in long term tissue culture and breast biopsies. Using the established breast cancer cell line ZR-75-1 (Engel *et al.*, 1978) which contains relatively small amounts of EGF receptor (Fitzpatrick *et al.*, 1984; Davidson *et al.*, 1987), we studied the effect of PRL on cell growth and EGF Receptor (EGFR) synthesis .

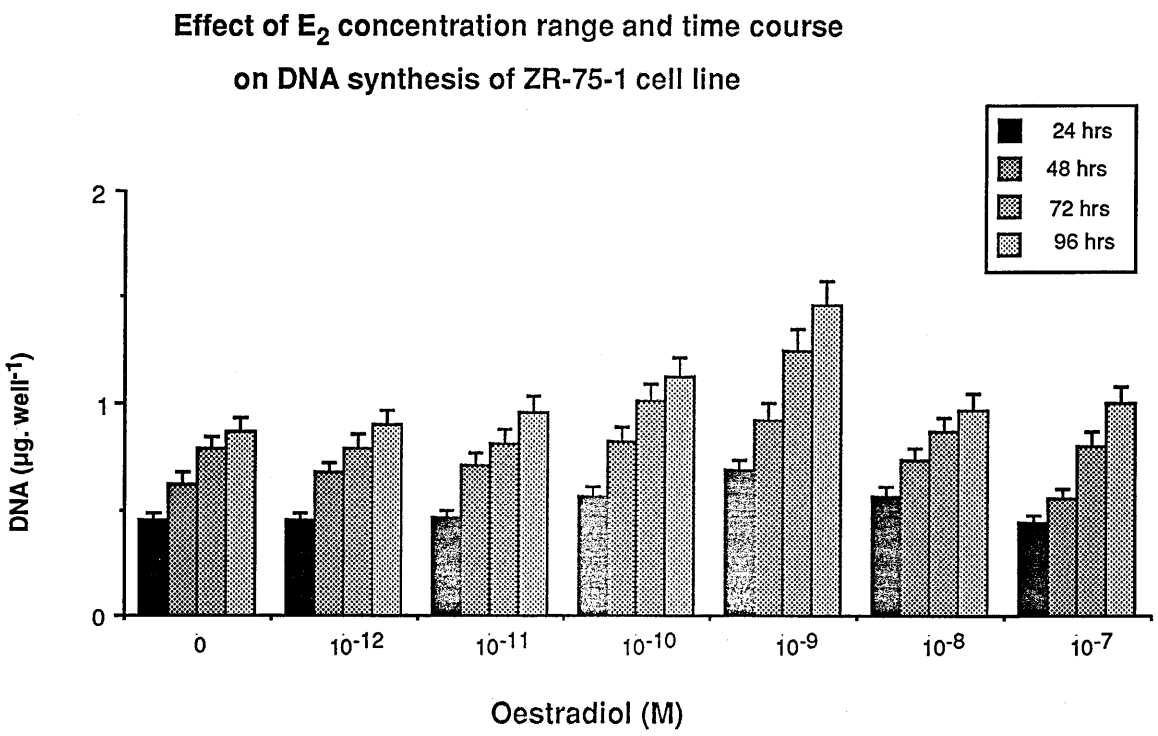
4.3.2 Hormone / Growth Factor action on DNA synthesis.

Our preliminary observations were concerned with the effect of β-Oestradiol (E_2), ovine Prolactin (oPRL) and murine Epidermal Growth Factor (EGF) on DNA synthesis of ZR-75-1 cells using the hoechst DNA assay technique (Methods **3.3.4.1** & **3.3.4.2**). Initial studies involved ascertaining the cellular response of the above mitogens over a range of concentrations and time courses (see Figures **4.3.1** - **4.3.3**).

From the results of the above experiments, it was decided to use the following optimal mitogenic concentrations of E_2 $1x10^{-9}M$ and EGF 5 ng. ml^{-1} for assessing the effects of hormone/growth factor combinations on DNA synthesis of ZR-75-1 cells. oPRL 1μg. ml^{-1} was also included at the stated concentration, whilst oPRL did not appear to be mitogenic over 48 hours it was mitogenic over 72 - 96 hours (see Figure **4.3.2**)

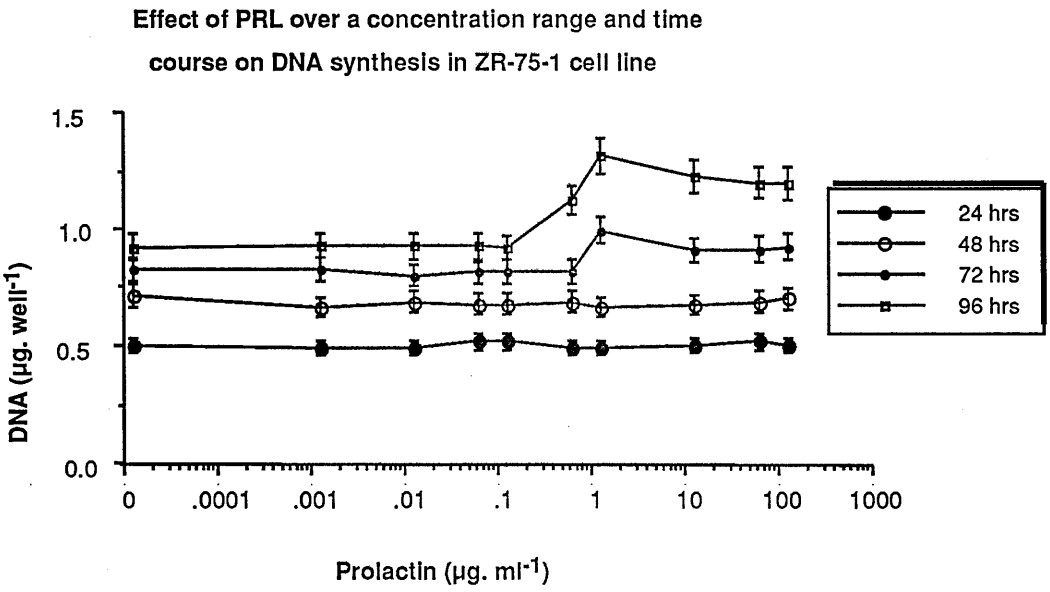
The results of the combination experiments are summarised in figure **4.3.4,** as can be seen oPRL synergistically raised the mitogenic effect of EGF. Cell growth in terms of DNA levels were more than twice that of control values after 48 hours. EGF and E_2 had comparable effects on DNA synthesis at approximately 1.4 times control values. All three in combination boosted DNA synthesis 2.5 times higher than control values.

Figure 4.3.1



N = 6 experimental determinations. Each determination is the mean of quadruplicate wells. Cell DNA content was assessed after incubation times noted above by the method of Leake and Habib (see Methods **3.3 4.2**). Note in figs.**4.3.1 - 4.3.6** DME medium contained Phenol red (15 mg/l).

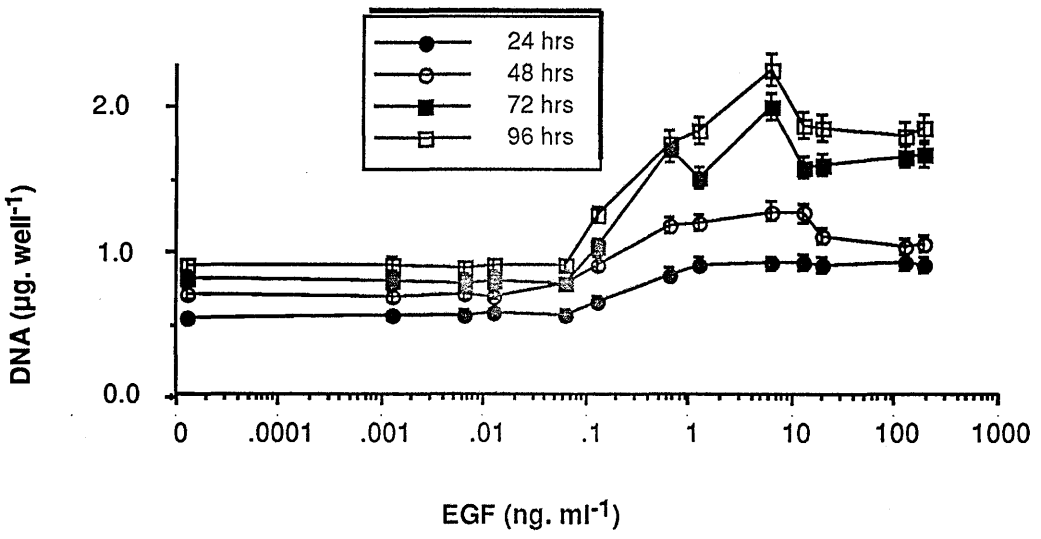
Figure 4.3.2



N = 6 experimental determinations. Each determination is the mean of quadruplicate wells. Cell DNA content was assessed after incubation times noted above by the method of Leake and Habib (see Methods **3.3.4.2**). Note in figs.**4.3.1 - 4.3.6** DME medium contained Phenol red (15 mg/l).

Figure 4.3.3

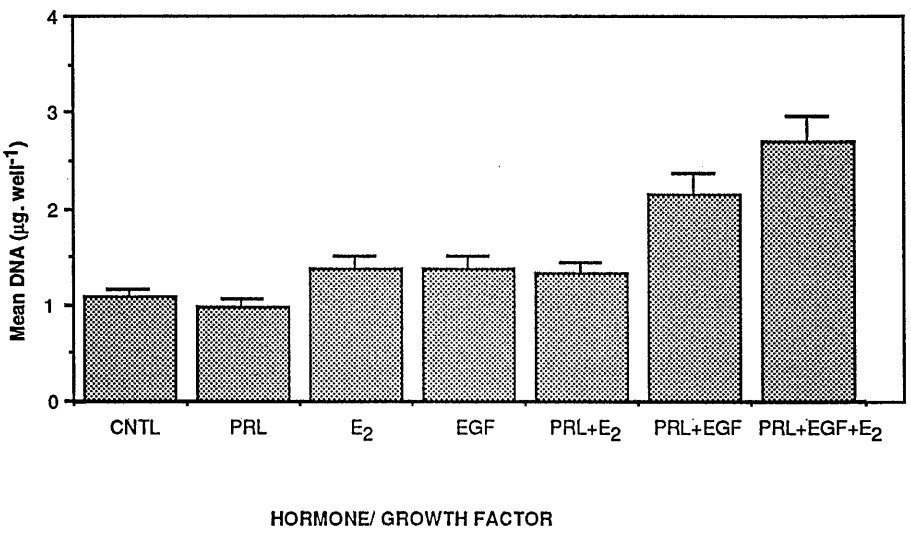
Growth response to EGF over time and concentration range in the ZR-75-1 cell line



N = 6 experimental determinations. Each determination is the mean of quadruplicate wells. Cell DNA content was assessed after incubation times noted above by the method of Leake and Habib (see Methods **3.3.4.2**). Note in figs.**4.3.1- 4.3.6** DME medium contained Phenol red (15 mg/l).

Figure 4.3.4

Effect of E_2, oPRL and EGF on DNA synthesis in ZR-75-1 cells



N = 6 experimental determinations. Each determination is the mean of quadruplicate wells. Cell DNA content was assessed after 48 hours incubation by the method of Leake and Habib (see Methods **3.3 4.2**). Note in figs.**4.3.1- 4.3.6** DME medium contained Phenol red (15 mg. litre^{-1}). Student T-test analysis of the data presented above recorded levels of significance at < 0.001, where mitogenesis is observed.

4.3.3 Dosage response of oPRL on [^{125}I] - EGF binding

On the basis of the findings recorded in figure **4.3.4**, it was decided to assess the effect of oPRL on EGF binding to its receptor in the total membrane fraction(by the methods outlined in **3.3.4.3 - 3.3.4.5**) as a function of (a) oPRL concentration and (b) over a time course.

(a) The binding of [^{125}I] - EGF was found to increase in a dose dependent manner upon oPRL addition with optimal binding at around 1μg. ml^{-1} oPRL over a 48 hour time course (Figure **4.3.5**). The conclusion derived from these experiments was that in all subsequent studies of oPRL effects on the EGF receptor , the concentration of oPRL used was set at 1μg. ml^{-1}.

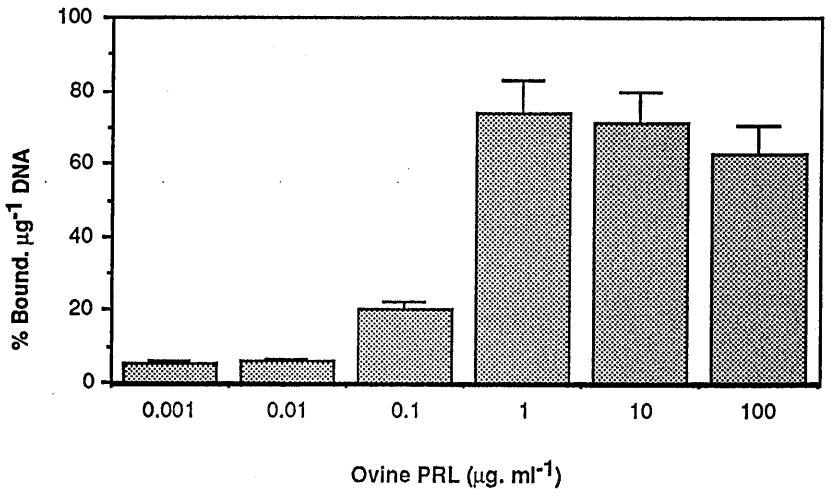
(b) The addition of oPRL resulted in a maximal 3-4 fold increase in EGF receptor levels in the total membrane fraction reached and maintained between 4-6 days (Figure **4.3.6**). EGF receptor levels were seen to decrease at 6-8 days , this was associated with the onset of confluence in the culture vessels.

Cells grown in the presence of oPRL and 40 nmoles. litre^{-1} Cycloheximide (sub-lethal concentration) display little or no receptor levels although cell growth continued at the control levels.

These findings suggest that the increase in receptor concentration is a protein synthesis associated event and not merely an increase in receptor capacity for the ligand.

Figure 4.3.5

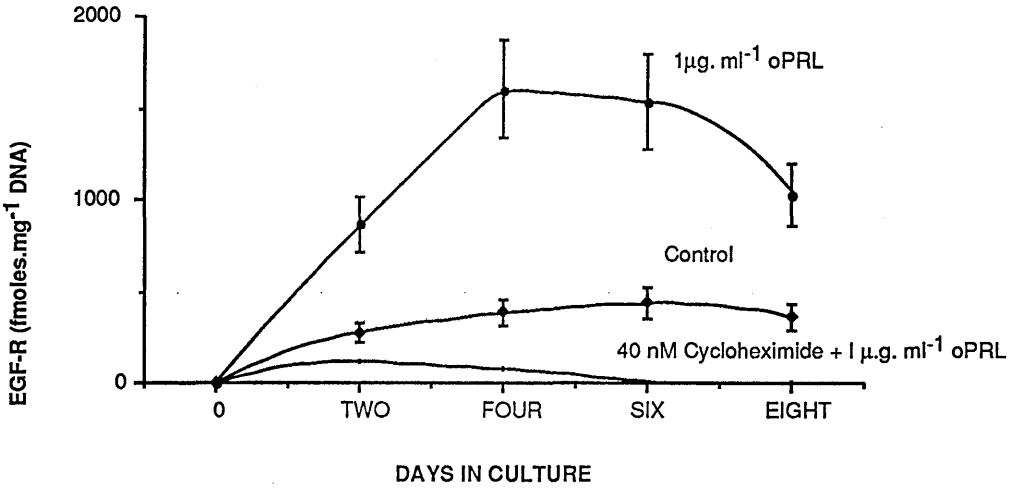
Dose Response of oPRL on [^{125}I] - EGF binding over 48 Hrs



N = 6 expts. Each determination is the mean of quadruplicate flasks. Ligand binding was assessed by methods **3.3.4.5.** Values quoted as percentage increase over control.

Figure 4.3.6

Induction of EGF Receptor by 1μg. ml^{-1} oPRL



N = 6 experiments, each expt is the mean of quadruplicate flasks. EGF-R concentration was assessed using the membrane binding assay technique (methods **3.3.4.5**).

4.3.4 Effect of Hormones/Growth Factors on cell surface binding of EGF

To further extend the study, it was deemed necessary to assess that the increases in EGF binding in the total membrane fraction was realised at the cell surface. Cell surface binding experiments were carried out as outlined in methods section **3.3.4.7.**

Several key changes in methodology were also incorporated at this stage. It was decided to use phenol red free media (see methods **3.3.3**) to remove any possible oestrogenic effects of phenol red (Welshons *et al.*, 1988) in the experimental system. It should be noted however that we detected no effects of phenol red on the oestrogen receptor levels in this cell line (see Figure **4.3.7**) Further, the use of human prolactin (hPRL) at a physiological concentration of 40 ng. ml^{-1} replaced the pharmacological levels of ovine prolactin.

The results display an interesting pattern (see Figure **4.3.8**). Whilst hPRL still stimulated increases in binding at the cell membrane (53% above the control values) and addition of E_2 cause a small but consistent stimulation (26% above the control values).Which was not observed using phenol red containing medium (data not shown). The addition of hPRL and E_2 in conjunction resulted in the greatest increases (151% above the control values). Co-culture with EGF resulted in a decrease in binding as would be expected from the down regulation effect of EGF. However residual binding of 37% above the control values was still observed in the hPRL + E_2+ EGF treated cells.

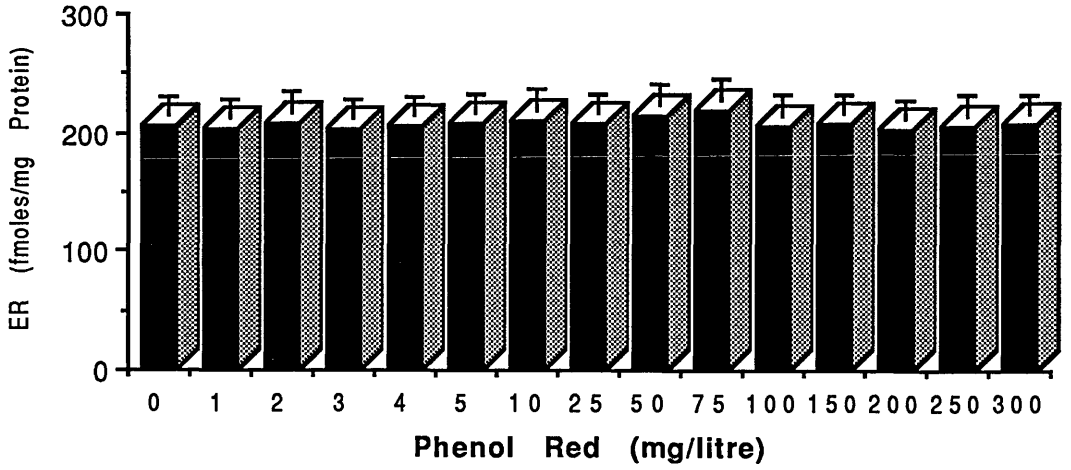
Scatchard analysis of the hPRL + E_2 treated cell (Figure **4.3.9**) revealed that the stimulation of ligand binding was purely a consequence of an increase in receptor concentration and did not reflect a change in receptor affinity.

Figure 4.3.7

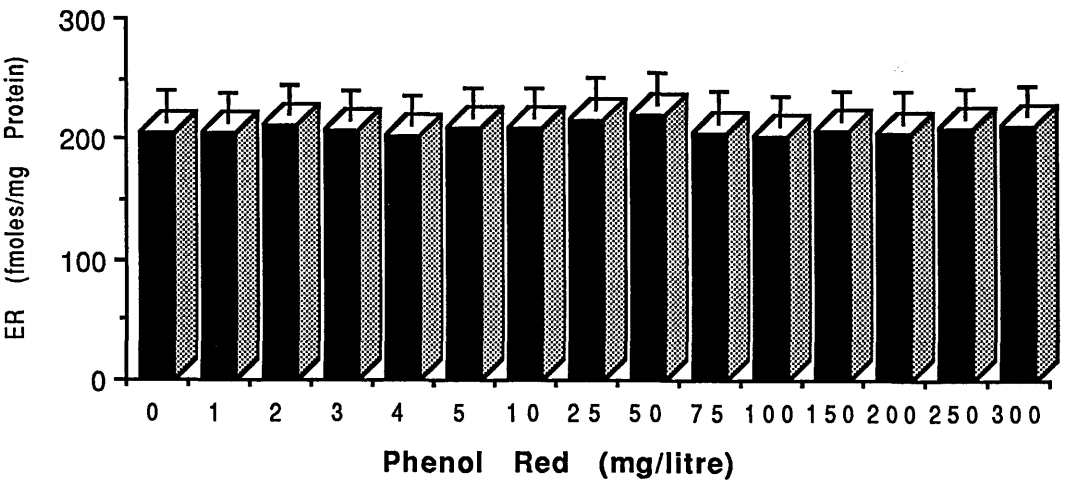
Effect of Phenol Red concentration range and time course on Oestrogen Receptor Levels in ZR-75-1 cell line

Oestrogen receptor levels were detected by ligand binding assay (Offord, 1988), the opposite series of histogram plots are example binding data derived at 24, 48 and 96 hours. No effects were observed at the above time points, no effects were noted even after 2 weeks in culture. Full scatchard analysis revealed no effects with regard to receptor affinity.

Effect of Phenol Red on ER levels in ZR-75-1 cell line after 24 hours



Effect of Phenol Red on ER levels in ZR-75-1 cell line after 48 hours



Effect of Phenol Red on ER levels in ZR-75-1 cell line after 96 hours

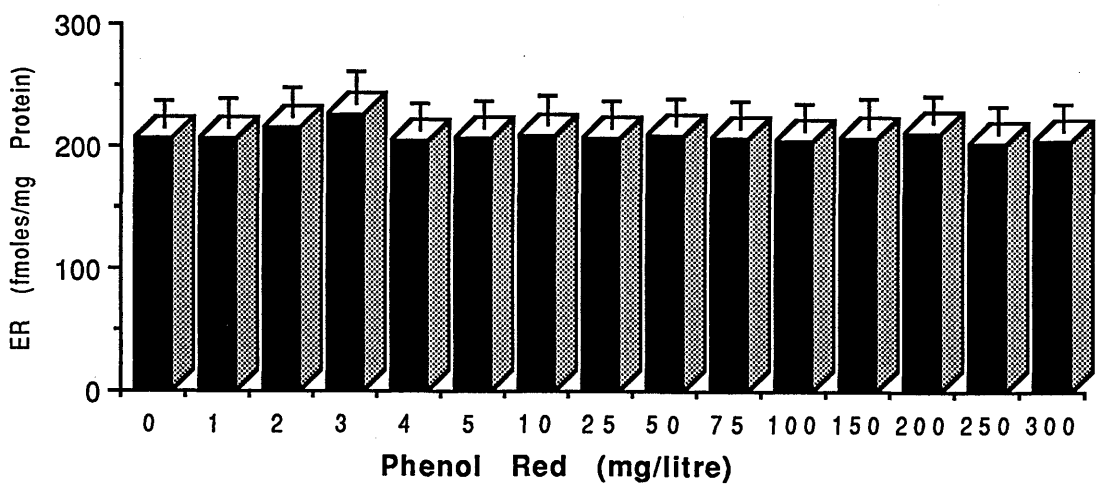
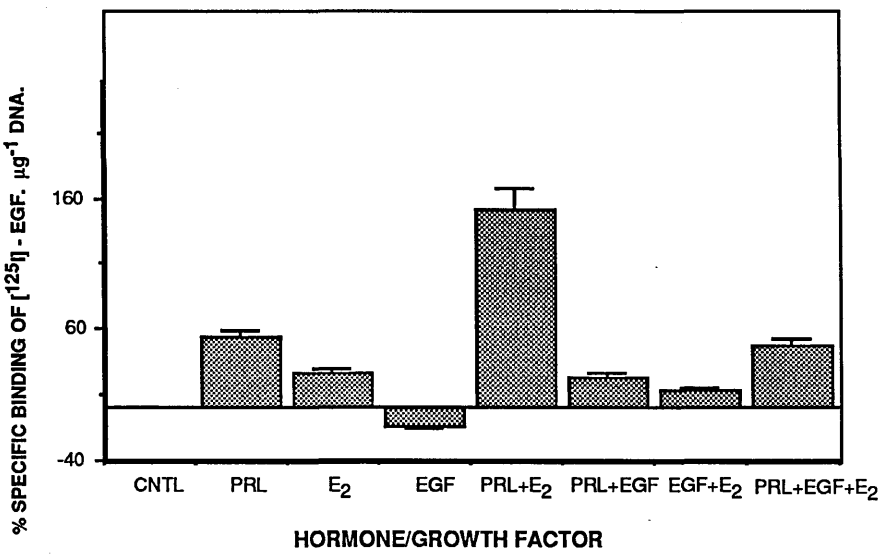


Figure 4.3.8

EFFECT OF E_2, hPRL AND EGF ON WHOLE CELL BINDING OF [^{125}I] - EGF

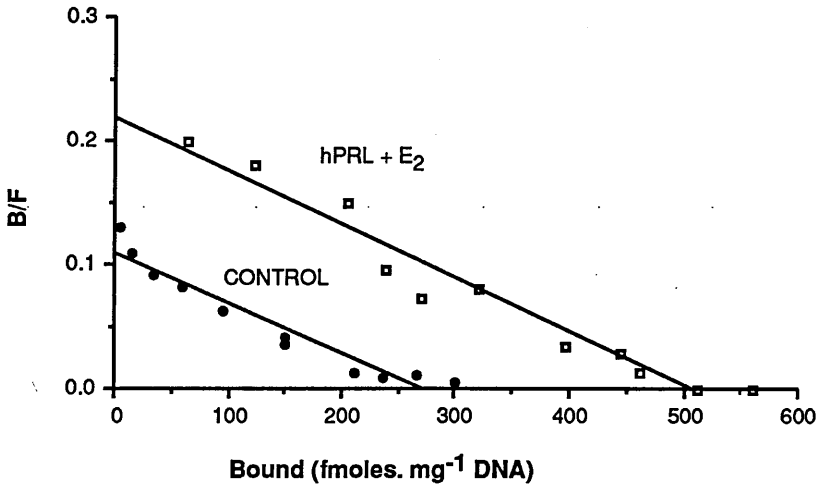


N = 6 experiments, each expt. is the mean of quadruplicate wells.

Hormone/Growth factor effects were assessed after 48 hours. Binding estimated as the percentage increase/decrease compared to control values using the whole cell binding assay technique (see methods **3.3.4.7**).

Figure 4.3.9

Scatchard Analysis of Whole Cell Binding of [^{125}I] - EGF



Example scatchard analysis plot of whole cell binding in hPRL + 10^{-9}M E_2 treated cells compared to control cells.

4.3.5 Indirect Immunofluorescence studies

From the findings of the experiments discussed in sections **4.3.1-4.3.4** it was decided to assess the effect of hPRL and E_2 on the EGF receptor by other parameters, in particular, immunological techniques utilising the commercially available EGFR 1 monoclonal antibody.

Initial studies used indirect immunofluorescent techniques outlined in methods **3.3.5** & **3.3.5.1.**. Figure **4.3.10.1** displays the surface morphology of ZR-75-1 cell surface morphology when grown on glass/slide culture vessels. No apparent difference between surface morphology was observed with that of plastic culture vessels. Cells were stained by the indirect immunofluorescent technique and counterstaining was acheived with Hoechst dye. Figure **4.3.10.2** displays a counterstain pattern typical of ZR-75-1 cells. These experiments were carried out four different occasions and the photographs represent a random selection of results obtained. During each experiment, A431 cells where cultured to provide a positive control for the experiments (Figure **4.3.10.4**).

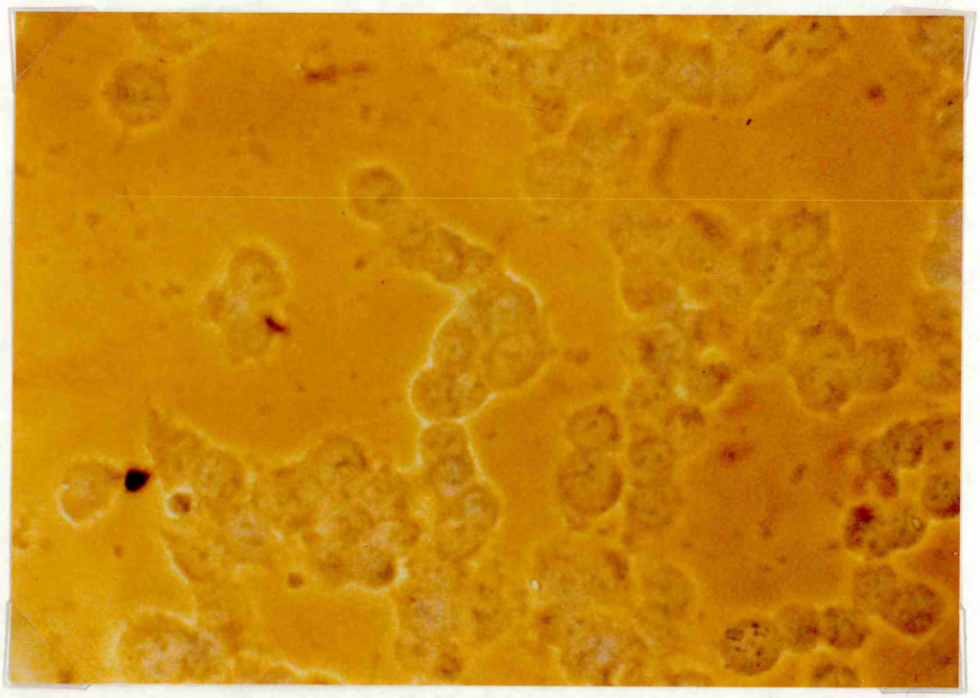
Incubation with the primary antibody resulted in relatively low fluorescence in hormone untreated ZR-75-1 cells (Figure **4.3.10.6**) when compared to the positive A431 cells. This is only to be expected in a cell line containing low levels of the receptor. Treatment with hPRL alone (Figure **4.3.10.7**), and in conjunction with E_2 (Figure **4.3.10.8**) after 48 hours incubation resulted in visual increases in staining intensity. Unfortunately it was not possible to quantify the fluorescent staining observed. The process of photographing slides and the production of prints has reduced the visual impact of these experiments. However these results appear to confirm the earlier bindings studies (see Figures **4.3.1 - 4.3.9**), in that more cells express sufficient EGFR after stimulation (relative to controls) as seen by retention of specific flourescence.

Figure 4.3.10.1

Surface morphology of ZR-75-1 cells in culture (x 200 magnification)

Figure 4.3.10.2

Example Hoechst counterstain of ZR-75-1 cells grown in these experiments (x 200 Magnification)



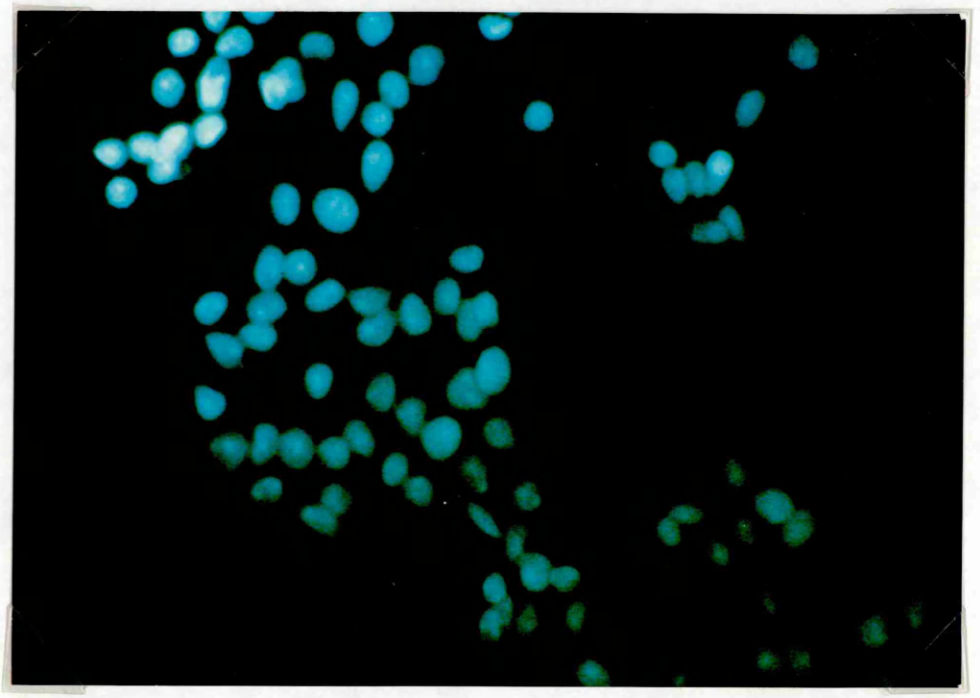
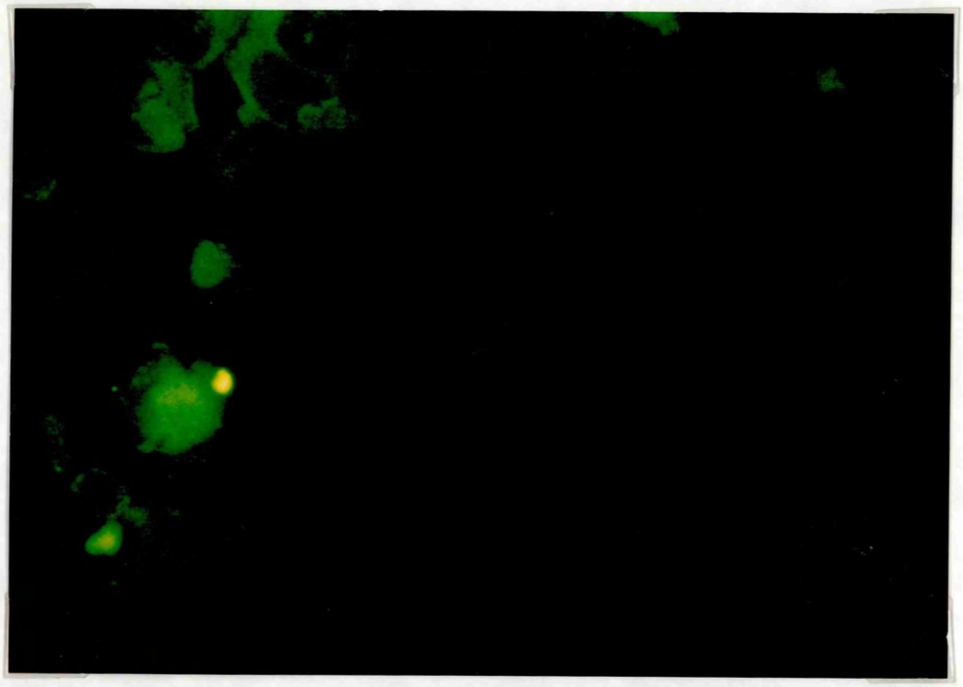


Figure 4.3.10.3

Non Specific Binding Fluorescence of A431 cells (x 400 magnification)

Figure 4.3.10.4

Fluorescence of A431 cells when incubated with EGFR 1 Monoclonal Antibody (x 400 Magnification)



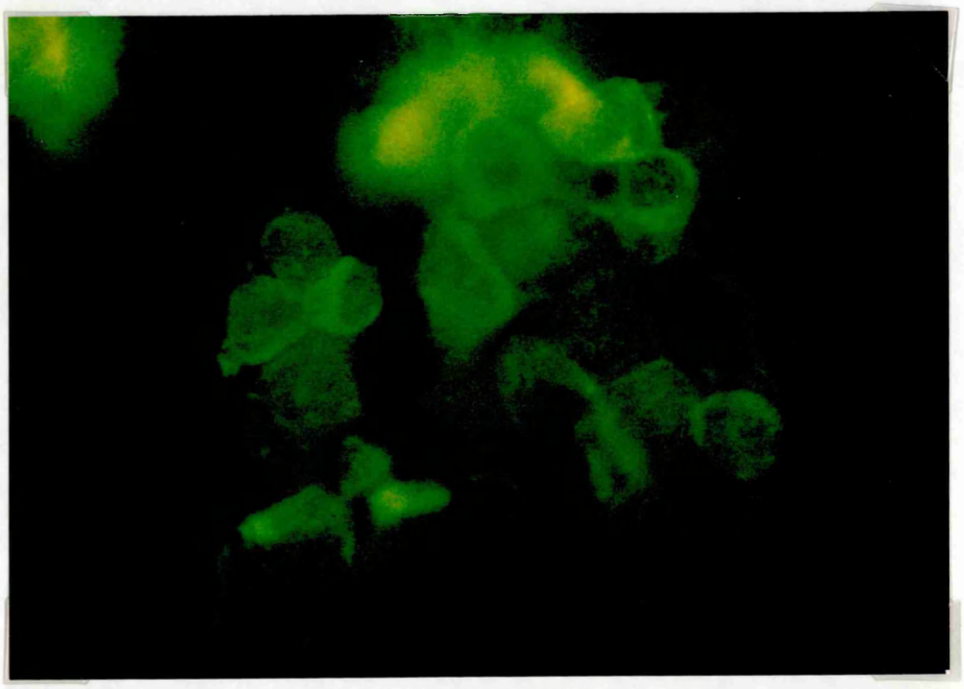
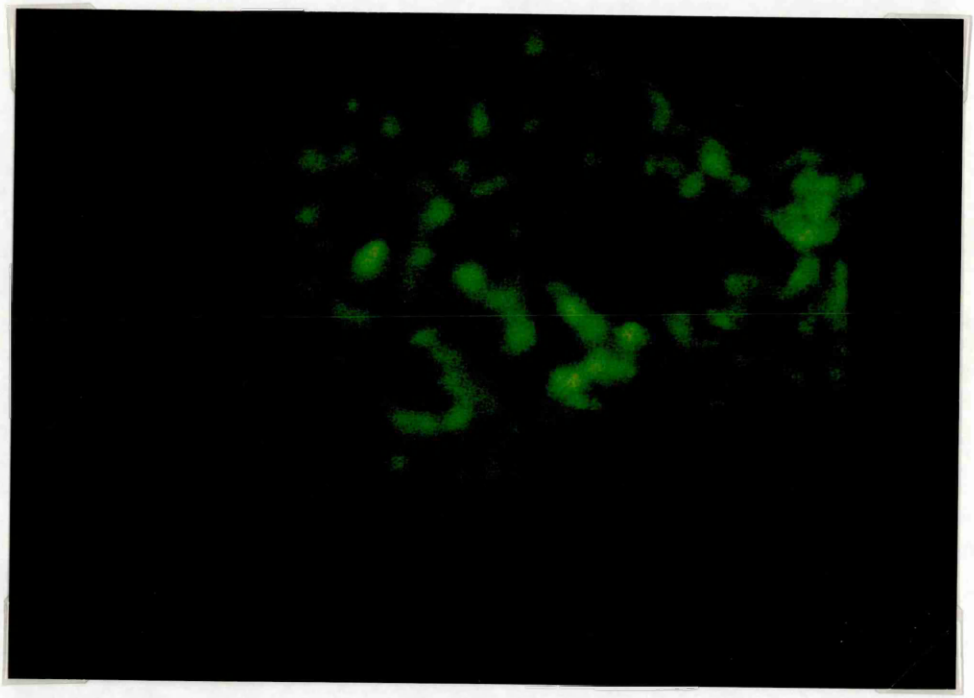


Figure 4.3.10.5

Example Non Specific Binding Fluorescence of ZR-75-1 cells (x 200 Magnification)

Figure 4.3.10.6

Example Fluorescence of Control ZR-75-1 cells when incubated with EGFR 1 Monoclonal Antibody (x 200 magnification)



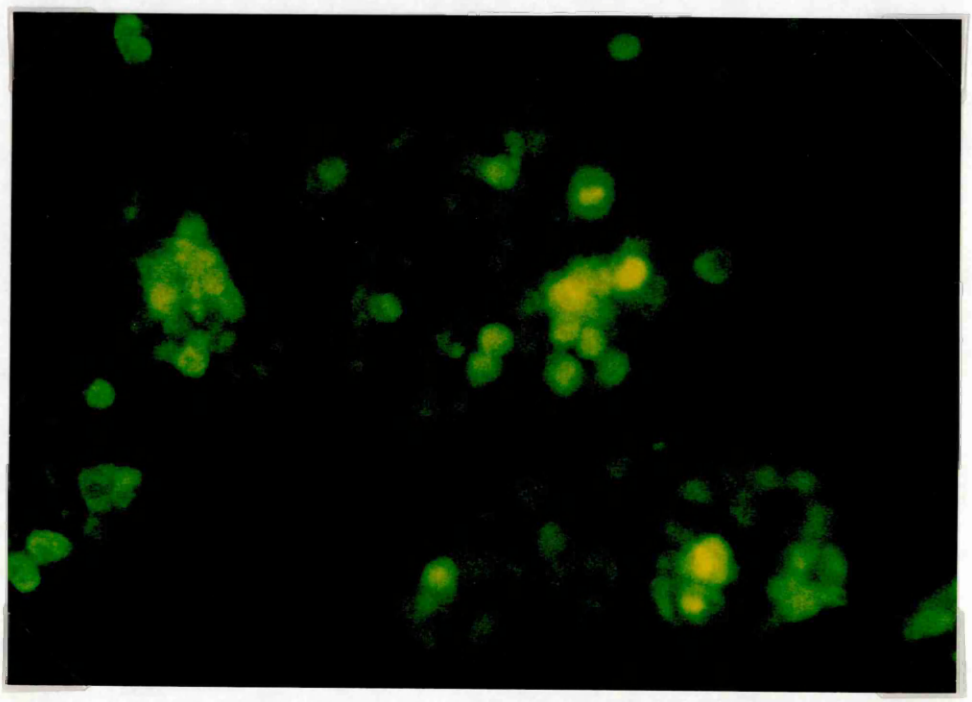
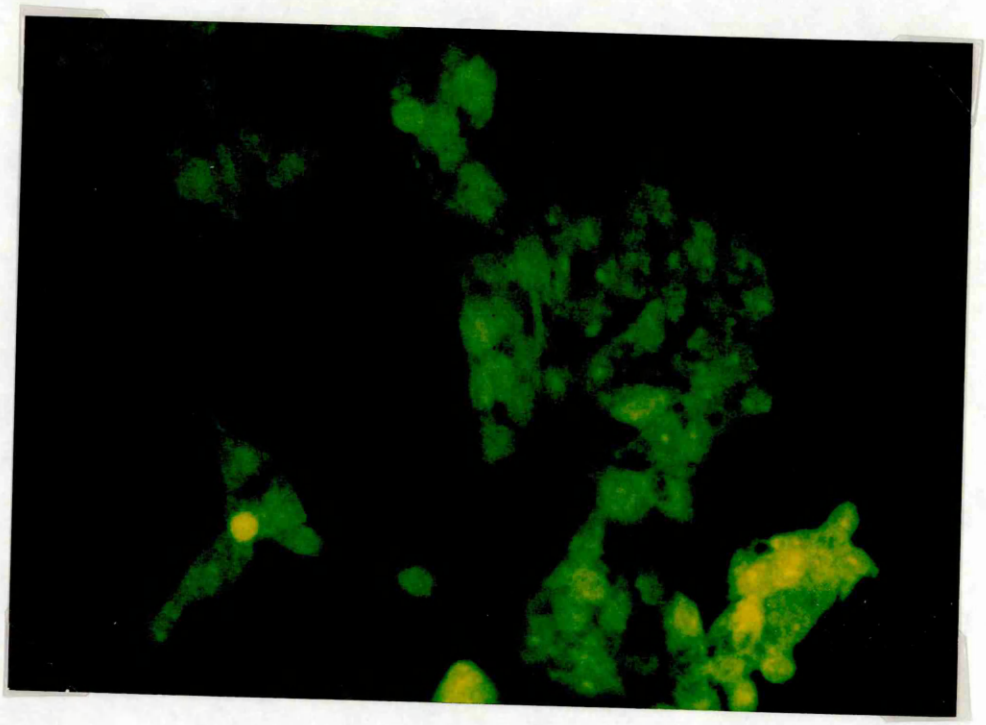


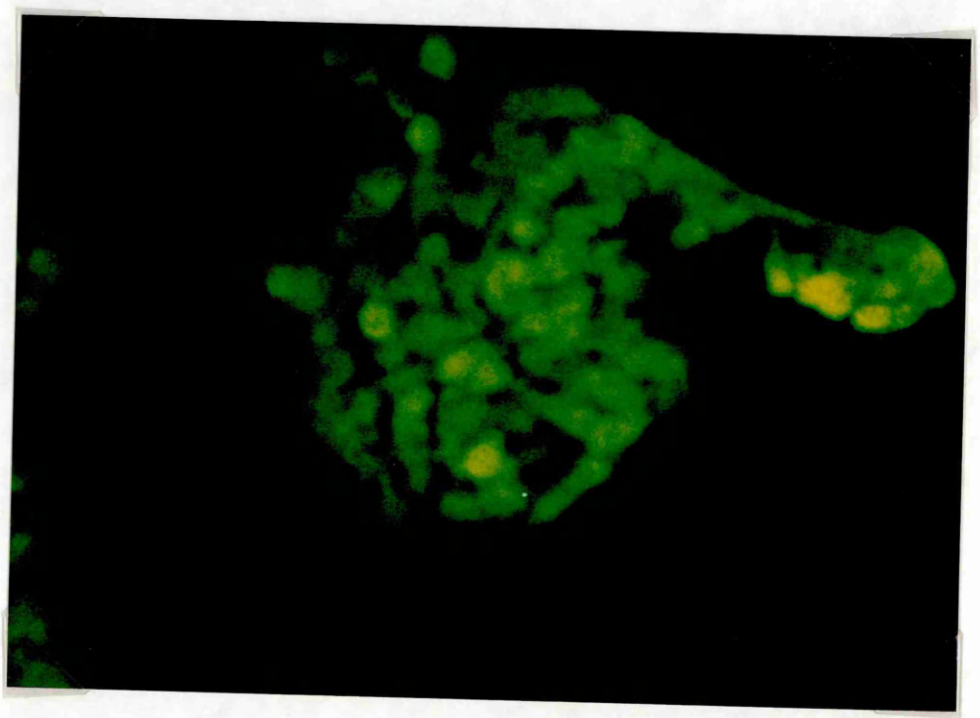
Figure 4.3.10.7

Example Fluorescence of hPRL treated ZR-75-1 cells when incubated with EGFR 1 Monoclonal Antibody (x 200 magnification)

Figure 4.3.10.8

Example Fluorescence of hPRL + E_2 treated ZR-75-1 cells when incubated with EGFR 1 Monoclonal Antibody (x 200 magnification)





4.3.6 Immunoprecipitation Studies

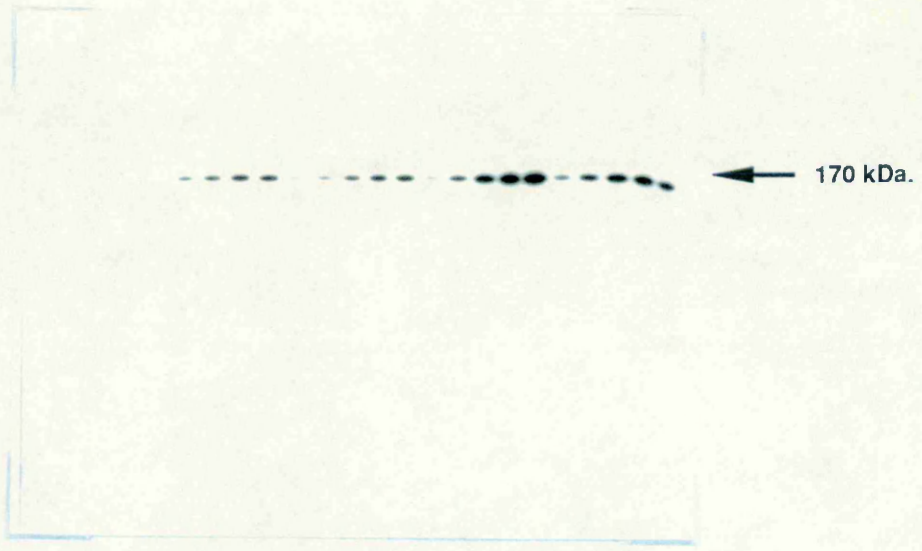
Whilst increased staining for EGF receptor was observed in the previous section, it was impossible to quantify these results on the microscope used. With this in mind it was decided to assess the effects of hPRL and E_2 on the immunoprecipitatable levels of incorporated [^{35}S] - methionine labelled EGF receptor. The experimental protocol is outlined in section **3.3.6.**

Figure **4.3.11.1** is an example autoradiogram derived from after one month exposure at -70'C. There is some curling at the ends of the gel. Of four separate immunoprecipitation experiments, Figure **4.3.11.1** was the one which gave the best result in terms of low background staining. Increases in immunoprecipitated receptor were seen as as a function of time i.e. 6 - 24 hours. The greatest increases were observed with hPRL alone and with hPRL + E_2 treated cells. E_2 appeared to have no effect in this assessment of EGF receptor levels. Subsequent densitometric analysis (Table **4.3.1** and Figure **4.3.10.3**) revealed that EGF receptor content is increased with hPRL + E_2 treatment to 60% above control values after 24 hours. E_2 alone had little effect although some stimulation was observed after 48 hours.

It should be noted that these experiments were carried out at low serum concentrations (0.5% (v/v) Dialysed HIDCCFCS). cell division in control and hormone treated cells were comparable. Therefore the increases in immunoprecipitated EGF receptor were not a function of cell division. Note that in Figure **4.3.10.2** shows no increase from 24 - 48 hours. This effect is probably due to the low serum conditions of the experiment.

Figure 4.3.11.1

Autoradiogram of immunoprecipitated EGFR in ZR-75-1 cells



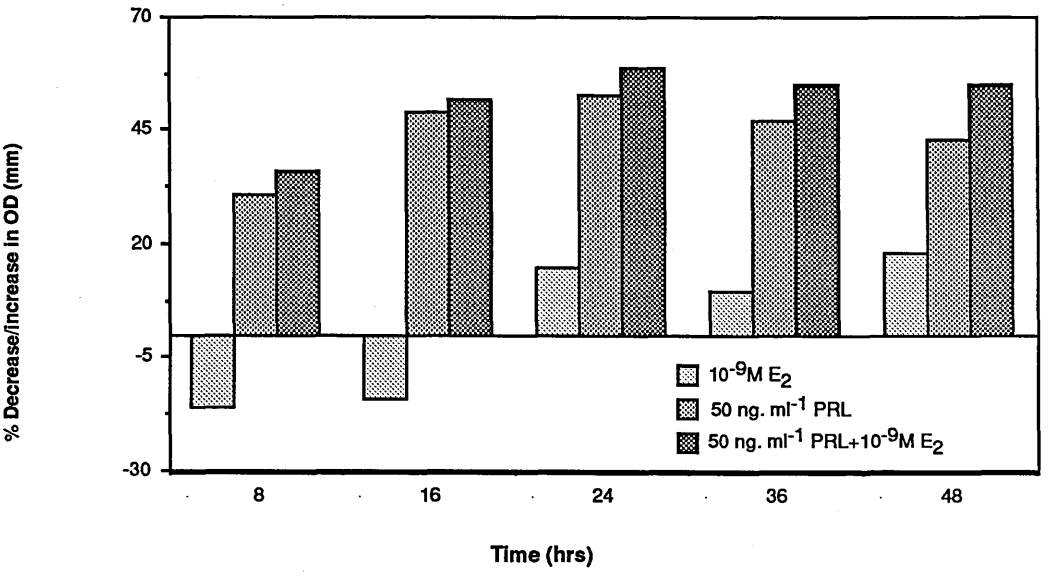
Lane Number

Time (Hours)	Control	10^{-9}M E_2	50 ng.ml^{-1} PRL +10^{-9}M E_2	50 ng.ml^{-1} PRL
8	1	6	11	16
16	2	7	12	17
24	3	8	13	18
36	4	9	14	19
48	5	10	15	20

At the specific time points above, cells were harvested and immunoprecipitation of the [^{35}S] - Methionine labelled EGF receptor carried out using the EGFR 1 monoclonal antibody.
The preparations were then subjected to SDS/PAGE followed by Autoradiography (see methods **3.2.5 -3.2.10** and **3.3.6 - 3.3.6.4**). The above table dispays time point according to lane number assigment.

Figure 4.3.11.2

Densitometric Analysis of Figure 4.3.11.1.



Values quoted as % decrease/increase over control values.

4.3.7 Conditioned Medium Studies

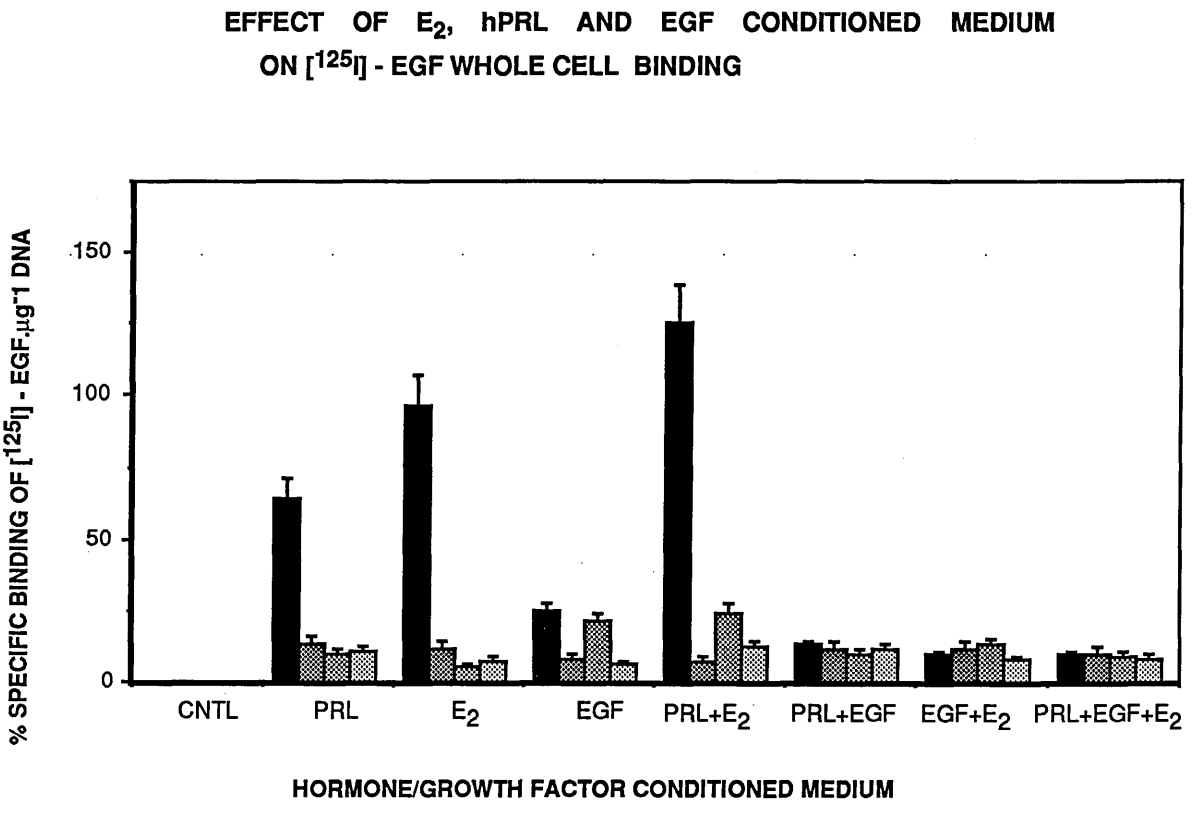
This section of the thesis deals with the possible mechanisms by which PRL may induce the EGF receptor. Initial studies were concerned primarily with a possible autocrine/paracrine approach.

Crude conditioned medium studies (see methods **3.3.7**) reveal that media conditioned (CM) by cells exposed hPRL alone and in conjunction with E_2 stimulates the binding of EGF to its receptor. Treatment with cycloheximide, heat or trypsin appeared to negate this response. An interesting finding in this series of experiments was that E_2 CM also increased specific EGF binding to its receptor 94% over control compared to 62% for hPRL CM. Again this effect was negated by cycloheximide, heat or trypsin treatment. All data is presented in Figure **4.3.12**.

It was outwith the scope of this lab to assess the "carry over" of any of these hormone/growth factors. However dextran coated charcoal treatment of the CM, a prerequisite of CM preparation (See methods **3.3.7**) has been demonstrated to remove both E_2 and oPRL biological activity (Manni *et al.*, 1986). Treatment with EGF appeared to inhibit any stimulation observed. However it is possible that the "carry over" of EGF is responsible for down regulation of EGF binding observed in the CM containing EGF.

Isolation and purification of the factor or factors involved in these responses was considered. However the necessary prerequisite for a study of this nature is serum free culture. Unfortunately all attempts to culture ZR-75-1 in serum free conditions where unsuccessful. It was with regret that this avenue of investigation was abandoned.

Figure 4.3.12



N = 6 experiments, each expt. is the mean of quadruplicate wells after 48 hours incubation (■). Stimulation of binding following 48 hours incubation was negated by Cycloheximide (▩), Heat inactivation (▩) and Trypsin (▩) pretreatment of the conditioned medium. See methods section **3.3.7** for details of Trypsin, Heat Inactivation and Cycloheximide treatments.

4.3.8 Mechanism of Oestrogen Involvement.

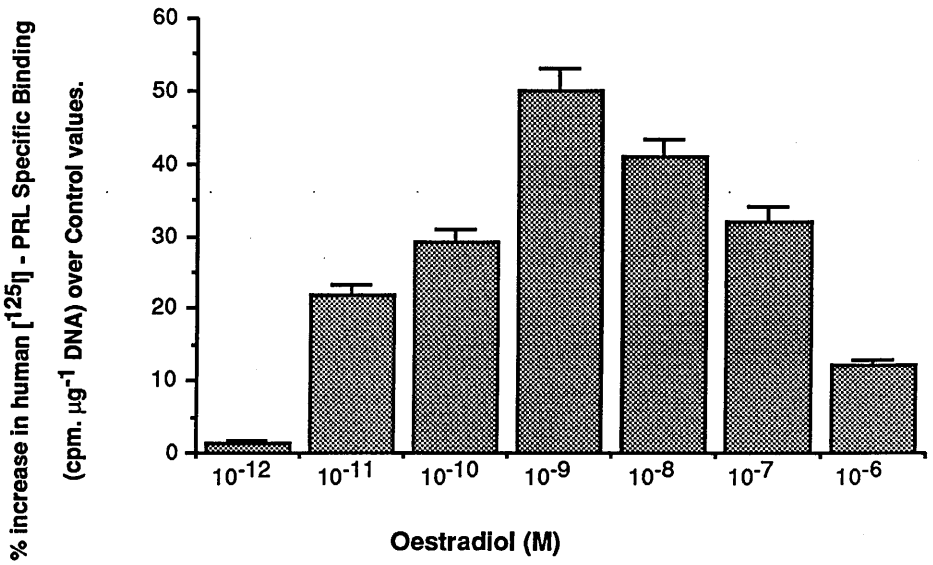
It is well established that E_2 upregulates TGFα mRNA in some breast cancer cell culture models (see Introduction **1.7.2 - 1.7.4**). This may play a role in the responses noted. However it has been demonstrated that oestrogen and progesterone both play a role in the stimulation of PRL production in human endometrial cells (Huang *et al.*, 1987), and that PRL can stimulate the production of its own receptor (Barash *et al.*, 1988). With these studies in mind it was decided to study the effect of E_2 on the PRL receptor in the ZR-75-1 cell line using the methodology outline in methods **3.3.8.**

These studies found that E_2 increased the specific binding of PRL to its receptor both as a function of E_2 concentration (Figure **4.3.13**) and time (Figure **4.3.14**). Subsequent Scatchard analysis reveals that E_2 treatment causes a doubling of PRL levels with no relative changes in receptor affinity (0.75 nmoles. $litre^{-1}$) after 48 hours (Figure **4.3.15**). It should be noted that that these experiments were only initial findings i.e. only three experiments were carried out.

A tentative conclusion that may be derived is that enhancement of EGF receptor levels in cells treated with hPRL and E_2 is due at least in part to E_2 upregulation of PRL receptor levels. In the light of the CM studies where it was observed that E_2 CM alone can increase EGF receptor levels, it is possible that in addition to E_2. E_2 CM induces the PRL receptor levels raising the possibility (not yet tested) that TGFα might stimulate PRL receptor synthesis. Alternatively, it is much more likely that this situation is more complex and other mechanisms are involved.

Figure 4.3.13

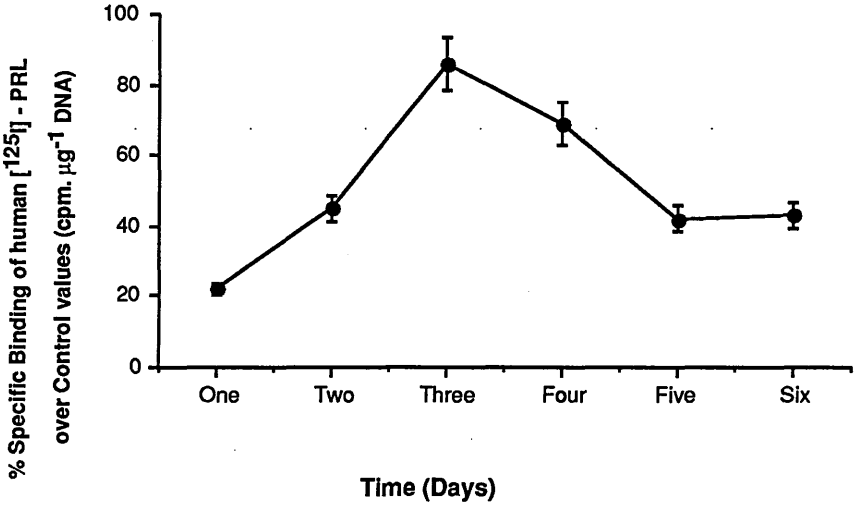
E_2 dose response of specific human [^{125}I] - Prolactin binding after 48 hrs.



The above histogram plot is the mean results of quadruplicate expts (S.D. ± 6.3%). PRL binding was determined by a single point binding assay , 1 nmole. litre^{-1} human [^{125}I] - PRL ± 100 - fold excess of unlabelled human PRL (see methods **3.3.8**).

Figure 4.3.14

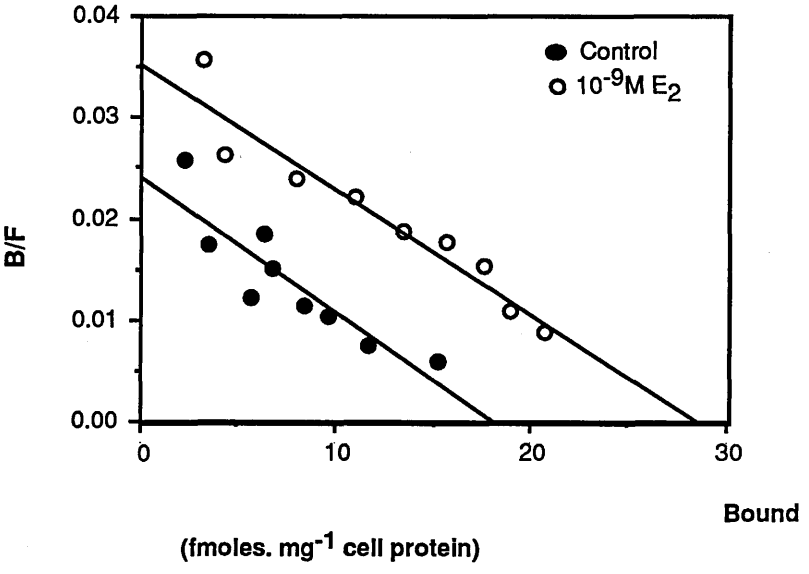
Time course study of the effect of 10^{-9}M E_2 on human [^{125}I] - Prolactin binding



The above plot is the mean results of quadruplicate expts (S.D. ± 5.5%). PRL binding was determined by a single point binding assay , 1 nmole. litre^{-1} human [^{125} I] - PRL ± 100 fold excess of unlabelled human PRL (see methods **3.3.8**) over time course indicated.

Figure 4.3.15

E_2 (10^{-9}M) Induction of Prolactin Receptor



Comparative Scatchard analysis of human [^{125}I] - PRL binding in ZR-75-1 cells after 48 hours exposure to 10^{-9}M E_2 (see methods **3.3.8**)

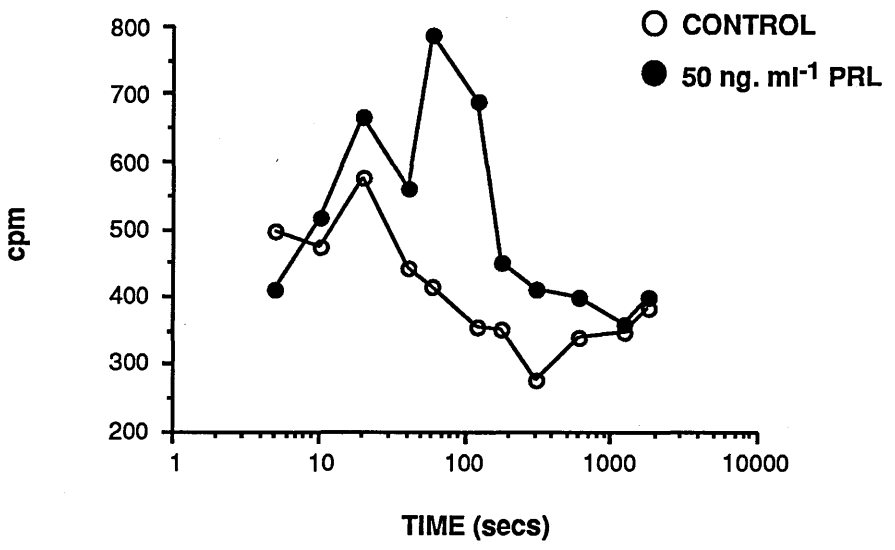
4.3.9 Prolactin and the Phosphatidylinositol Pathway

It has been noted that stimulation of the phosphatidylinositol pathway (PIP) by phorbol esters can lead to increases in EGF receptor protein and mRNA (Shelton-Earp *et al.*, 1988). During the period of research involved in this thesis, it was demonstrated that addition of prolactin could stimulate the flux of the PIP in mouse mammary explants (Etindi & Rillema, 1988). With this in mind it was decided to assess the effects of hPRL on the PIP in ZR-75-1 cells using the methodology outlined in methods **3.3.9**.

Studies reveal that treatment of cells with 50 ng. ml^{-1} hPRL resulted in small increases in secondary messengers, inositol triphosphate (IP_3) (Figure **4.3.16**) and inositol tetraphosphate (IP_4) (Figure **4.3.17**), over a relatively short time course suggesting a possible direct PRL receptor mediated response. But it is more likely that it is not a direct PRL receptor mediated response, because increases in total inositol phosphate (IP) levels could be seen as function of PRL concentration over 30 mins (Figure **4.3.18**). Stimulation with an optimal concentration of PRL still displayed increases in IP levels after three hours (Figure **4.3.19**).

Figure 4.3.16

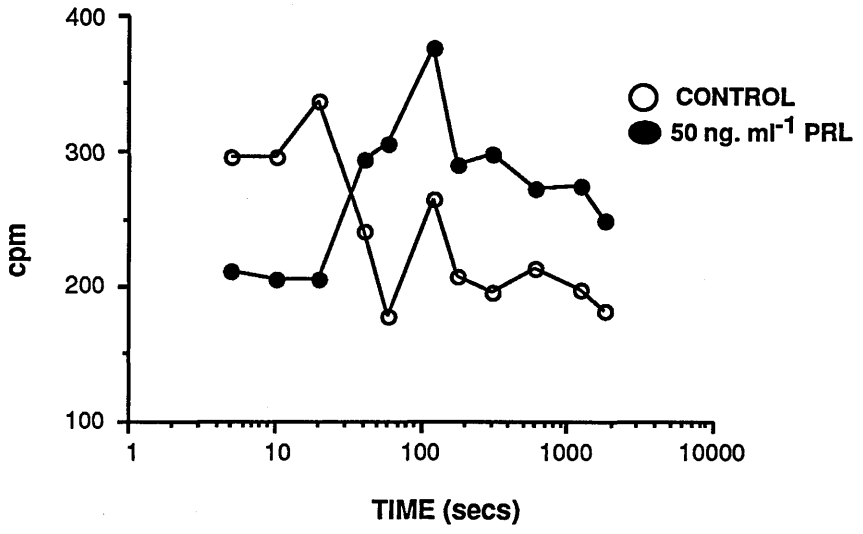
Effect of 50 ng. ml^{-1} human Prolactin on IP_3 levels



n = 4 experiments, Each experimental determination is the mean of triplicate wells. (SD < 7.5%.)
IP_3 levels were estimated by the method outlined in **3.3.9.**

Figure 4.3.17

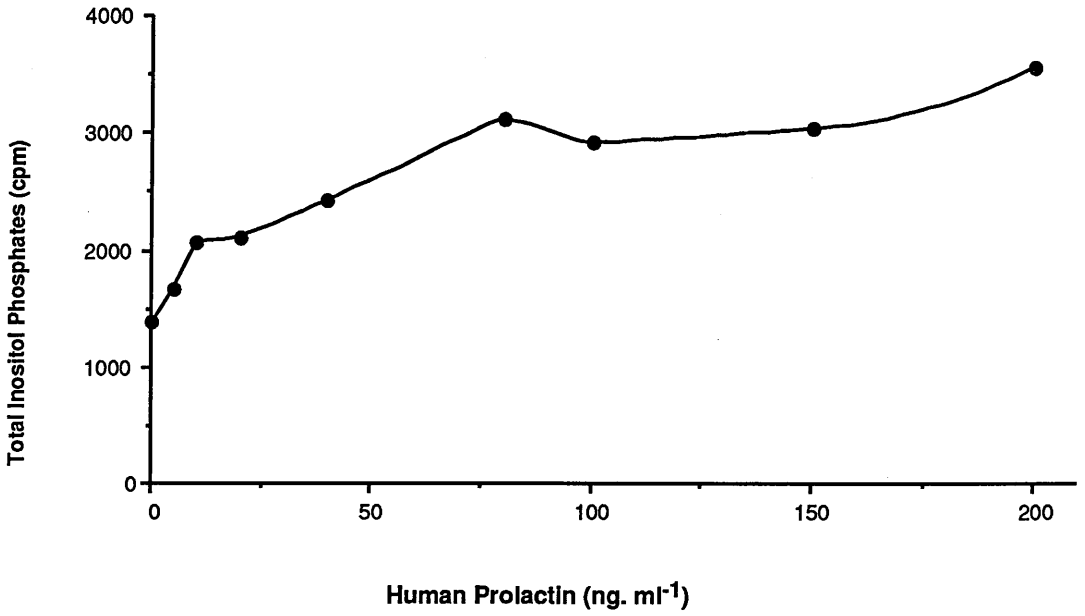
Effect of 50 ng. ml^{-1} human Prolactin on IP_4 levels



n = 4 experiments, Each experimental determination is the mean of triplicate wells. (SD < 6.3%.)
IP_4 levels were estimated by the method outlined in **3.3.9.**

Figure 4.3.18

Effect of increasing concentrations of hPRL on Phosphatidylinositol pathway turnover

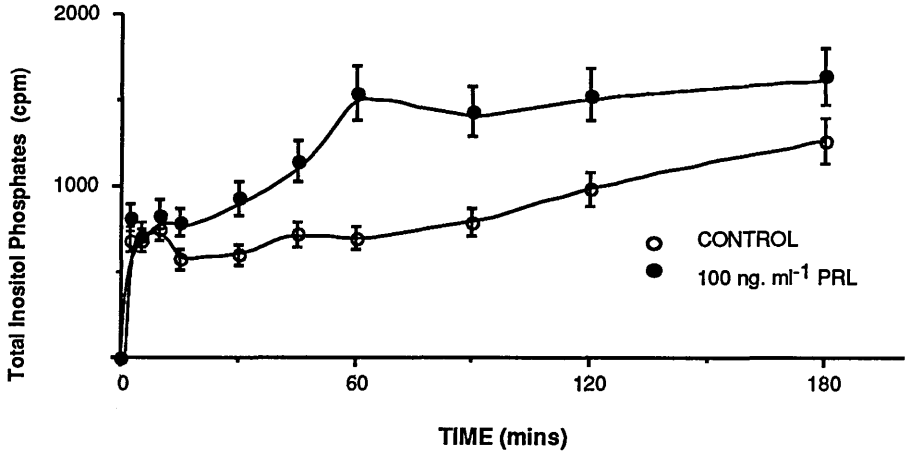


n = 3 experiments, Each experimental determination is the mean of triplicate wells. (SD < 10.5%.)

PI pathway turnover was estimated by the method outlined in **3.3.9.**

Figure 4.3.19

Effect of 100 ng. ml^{-1} human Prolactin on Phosphatdylinositol pathway turnover over a 3 hour time course.



n = 6 experiments, Each experimental determination is the mean of triplicate wells. (SD < 7.0%.)
PI pathway turnover was estimated by the method outlined in **3.3.9.**

4.3.10 Result Conclusion/ Discussion

Two major conclusions can be drawn from this study with regard to prolactin action.

(a) *De novo* production of the EGF receptor can be induced by both pharmacological levels of ovine PRL and physiological levels of human PRL in the ZR-75-1 cell line .
(b) The mechanism of receptor induction is mediated at least in part by a heat labile polypeptide autocrine/paracrine signal.

A few compounds are known to regulate the EGF receptor at the ligand binding , receptor protein, and mRNA levels in breast cancer cell lines *in vitro*. 1, 25 Dihydroxy-vitamin D (Koga *et al.*, 1988), progestins (Murphy *et al.*, 1986,1988) and neoplastic agents (Hanuske *et al.*, 1987) are examples.

PRL has not to our knowledge been studied in terms of a possible role in EGF receptor regulation in breast cancer cell models. It has however been shown to stimulate the flux of the Phosphatidylinositol pathway (PI) in mouse mammary explants (Etindi & Rillema, 1988). Buckley *et al.* (1988) have also reported that prolactin stimulates protein kinase C (PKC) activity in isolated rat liver nuclei through the lactogenic component of its action.

Earlier studies have shown that EGF and 12-o-phorbol-13-acetate (TPA) increase EGF receptor protein synthesis in the MDA-468 human breast carcinoma cell line (Kudlow *et al.*, 1986; Bjorge & Kudlow, 1987). More recently Shelton - Earp *et al.*, (1988) reported that EGF, Epinephrine, Angiotensin II and TPA stimulate PI hydrolysis and increase EGF receptor mRNA levels in rat liver epithelial cells.

These finding suggest that at least part of the mechanism regulating EGF receptor protein synthesis lies within the PI/PKC signal transduction system, which from the data contained in this thesis and from Etindi & Rillema (1988) has the capacity to be stimulated by PRL.

In contrast to this proposed mechanism of EGF receptor stimulation, earlier studies by Roos *et al.* (1986) presented findings to suggest that TPA treatment of MCF-7 and ZR-75-1 cell lines resulted in a decrease not only of receptor affinity (Receptor modulation), but also evidence of a reduction the number of receptor sites.

A more recent study by Kosano & Takatani (1988) which states that the alkylysophospholipid, ET-18-OCH_3 reduces EGF receptor levels probably by the inhibition of PKC activity is supportive of our view. It is hoped that further studies will help elucidate this dual regulatory function of PKC in these *in vitro* breast cancer cell models.

Studies on individual cell lines have given rise to conflicting results, such variations are assumed to arise from differences in laboratory techniques, cell passage number, plating density etc. Equally different breast epithelial cell lines although displaying similar receptor complements give in some cases surprising variations in results obtained. Therefore these results obtained in this section of the thesis should be extended to other cell lines. This report however is the first occasion that PRL has been observed to regulate a growth factor receptor in an oestrogen dependent human breast cancer cell line.

Studies by Vonderhaar (1989) have shown that the addition of PRL alone is sufficient for mitogenesis in the MCF-7 cell line suggesting a direct role for PRL in mammary growth. Whether this is mediated through the EGF receptor is still to be ascertained in their experimental system.

A recent report by Manni *et al.* (1990) has demonstrated that blockade of EGF binding to its receptor by two monoclonal antibodies markedly inhibited both oestrogen(E_2) and progesterone (P_2) conditioned medium(CM) mediated

cell mitogenesis in MCF-7 cells grown in soft agar in serum free clonogenic assay conditions. Ovine PRL mediated mitogenesis however was unaffected. It is therefore possible that PRL in addition to increasing EGF induced mitogenesis, also stimulates a number of other growth promoting substances. From this report and data contained within this thesis, E_2 CM may be acting by increasing TGFα production (Bates *et al.*, 1988) and as would appear from our data and that of Berthois *et al* (1989) may upregulate the EGF receptor. In contrast to Manni *et al.*, Arteaga *et al.* (1988) using one of the same monoclonal antibody (Mab 528) and two other antibodies found that antibody blockade of the EGF receptor in the MCF-7 cell line in serum free culture did not inhibit E_2 induced growth.

It is clear that much more research is needed to elucidate E_2 action within these cell lines. It is unlikely that E_2 mediated mitogenesis occurs solely through the EGF receptor signalling system, as it has been shown that E_2 can stimulate other mitogenic factors such as IGF-I (Huff *et al.*, 1986), IGF-II (Coranado *et al.*, 1987) and cathepsin D (Vignon *et al.*, 1986).

Chapter 5. Overall Discussion

The work contained in this thesis can be overviewed in three sections.

(a) We have described a ligand binding assay system for EGFR estimation which correlates well with the "common methodology" assay system proposed later by the EORTC study group (Benraad & Foekens, 1990). This assay can be used on relatively small amounts of tissue, yet gain full information on the number of binding sites and their relative affinities for ligand.

This permits amalgamation of present data with that of other workers who are currently using the EORTC protocol. This is very important if one considers that the sample pool often used for study of EGFR levels is only around 100 - 200 patients. Amalgamation of data allows for more accurate statistical analysis. The levels and incidence of EGFR reported here fall within the range reported by most groups (Sainsbury *et al.*, 1985; Nicholson *et al.*, 1988; Fekete *et al.*, 1989).

In the present study 38% of tumours were EGFR positive, 57% were ER positive (a relatively low value, but it must be noted that the presence of both soluble and nuclear ER were required before a tissue was designated ER positive) and 13% contained neither receptor. Only 14 of the 199 tumours studied contained both receptors and no correlation was noted with respect to receptor concentrations, suggesting that possibly these tumours were polyclonal. However ligand binding assay (LBA) techniques may not be sensitive enough to detect low levels of receptor which may still be of relevance in determining tumour growth. The harsh methods of iodination such as chloramine T used in the production of iodinated EGF and TGFα appear to decrease sensitivity of receptor detection (Leake & Benraad, 1991). More gentle methods such as iodine monochloride and enzymobeads display horizontal Holleman plot kinetics, indicating biological equivalence of labelled and unlabelled ligand. It is possible that ligands labelled by the these latter methods

will significantly increase receptor detection and identify levels which are low but physiologically relevant. Enhanced sensitivity of EGFR detection may help to explain the contrast between breast tumour tissue in which, the presence of EGFR is associated with the absence of ER levels and *vice versa*, whereas some breast cell lines show an interdependence of steroids and growth factors. It should be noted that the early studies of ER levels in breast cancer tissue were around the 40% region, and have gradually increased with improvements and changes in technology until receptor incidence is now as high as 80-95%. Further, the relevant portion of the tumour with respect to disease progression may be diluted in the preparation of the sample.

As more markers of patient prognosis are being proposed, the methods of detection must be modified so that there are both common methods and adequate material for satisfactory screening of appropriate markers in even small samples. LBA systems for receptor assessment allow for the estimation of functional receptor, but use relatively large amounts of tumour and involve homogenisation. Other markers may not be receptors i.e. oncogenes and their products (Guerin *et al.*, 1989), secreted proteins such as Cathepsin D (Rochefort *et al.*, 1988) etc. Therefore it is likely other methods of detection will overtake LBA. Ideally assays should be quantitative, give a measure of binding affinity yet permit a measure of heterogeneity of the marker both across tumour tissue and among different tissue types.

Immunocytochemistry with the appropriate antibodies is one avenue. The obvious advantage of this method is that it allows the precise assessment of markers with respect to their localisation within the tumour. However it does not test functionality (if the ability to bind ligand is taken as a marker of function) and is not readily quantifiable. Other methods such as *in situ* hybridisation, western, northern and southern blotting may also become more widely used.

(b) The demonstration that there may be a subclass of EGFR in the nuclear fraction of breast tumours bearing the appropriate receptors in the membrane fraction has some interesting implications. It opens up the possibility that growth factor receptors may act through the DNA machinery by direct interaction with the DNA:chromatin complex. Further, the possibility that related oncogenes may also bind is an intriguing possibility. Whether the nuclear localisation of EGFR (Rakowicz-Szulcynska *et al.*, 1989) and other growth factor receptors is either an essential component of their action or is a more accurate index of tumour prognosis is an area which we hope will be pursued.

The presence of nuclear localised receptor offers intriguing possibilities. Ligand, EGF or TGFα, may be taken up by EGFR and in addition to signal transduction events, a portion is targeted towards the chromatin matrix. The demonstration that some monoclonal antibodies (MAbs) directed against cell surface EGFR are taken up by the cell as a Mab/receptor complex and appear to translocate to the nucleus instead of the ligand/receptor complex, may account for their action at the DNA level. It has been shown that activation of EGFR tyrosine kinase by some MAbs does not stimulate DNA synthesis (DeFize *et al.*, 1986) . Inhibition of the A431 cell line by MAb 423 occurs without affecting tyrosine kinase activity. It seems likely from these studies that tyrosine kinase activity does not represent the early critical point in proliferation of this cell line. Studies by Jiang & Schindley (1990) using a technique developed by Bishayee *et al.*, (1982) in which EGFR was transferred in a biologically active state from donor hepatic membranes to EGFR deficient fibroblasts (3T3-NR), resulted in cells that were physically reconstituted with EGFR and which displayed ligand induced cell mitogenesis, have been shown to accumulate [^{125}I] - EGF within the nucleus. An interesting report which may help in the elucidation of the nuclear localisation phenomenon was that of DeFize *et al.*, (1989), who found that MAb 2E9 blocks EGF binding to the low affinity

component. No effect was noted with regard to EGFR tyrosine kinase activity. Addition of EGF resulted in the early responses associated with ligand activation, such as, inositol phosphate production, release of Ca^{++} from intracellular stores, rise in intracellular pH, phosphorylation of EGF on threonine residue 654, induction of c-fos gene expression, and altered cell morphology. It was disappointing that DeFize never assessed or reported MAb 2E9 with regard to EGFR nuclear localisation or cell growth which he himself had noted three years earlier.

Given more time and funding the nature of EGFR localisation in breast cancer cell line models would have been pursued. Preliminary studies using the MDA-MB-231 cell line have given indications that EGFR is present in the chromatin matrix as assessed by ligand binding assay and western blotting. It was also interesting to note that both the MCF-7 and ZR-75-1 cell lines also appear to contain immunologically recognisable EGFR with nuclear preparations despite the low concentrations of receptor available (results not shown).

A research scheme along the following lines is not impossible to imagine

(1) Nuclear localisation of [^{125}I] - EGF and [^{125}I] - TGFα over a concentration range and time course. The choice of using both ligands (whilst displaying similar kinetics in terms of competition etc.) is due to the differential effects reported in the literature.

(2) Regulation of nuclear localisation by EGFR specific tyrosine kinase inhibitors such as those described by Schlessinger's group, in particular Tyrphostin (Lyall *et al.*, 1989) which was found to inhibit EGFR tyrosine kinase activity and EGFR induced cell mitogenesis. Low affinity site blocking antibodies such as the MAb 2E9, or B4G7-IgG (Behzadian & Shimuzu, 1985) or

EGR/G49 (Gregoriou & Rees, 1984) could answer some questions as to the role of the low affinity receptor with respect to nuclear localisation.

(3) The effect of phorbol esters which have been shown to eliminate the high affinity EGFR binding component (see introduction **1.8.8**), may help provide an idea as to the role of the Protein Kinase C and inositol phosphates in the localisation of nuclear receptor.

(4) The interaction of other known growth factors such as PDGF, TGF β, IGF-I and IGF-II may give information on localisation with regard to receptor "cross talk". In particular PDGF is an interesting area, it has been demonstrated that PDGF in addition to phorbol esters also eliminates high affinity EGF binding (see Introduction **1.5.3** and **1.5.5**). An exciting scenario could be that the loss of high affinity EGF binding induced by PDGF (Olson & Pledger, 1990) would result in reduced nuclear localisation of EGFR and possibly corresponding increased localisation of the PDGF receptor. Many breast cancer cell lines have been noted to secrete PDGF-like activity (Rozengurt, 1986). To our knowledge there are little data available as to levels of PDGF receptor within breast cancer cell lines. The criteria for a study of this nature must be a breast cancer cell line which contains receptors for both EGF and PDGF and displays mitogenesis to the addition of either ligand. Further, nuclear localisation of the PDGF receptor must be assessed by ligand binding assay and immunological techniques. The ability to block PDGF binding/activity either chemically or immunologically would be required.

The role of hormones in nuclear localisation is another interesting area. The studies outlined in results chapter **4.3** point to a role for prolactin (PRL) in stimulating EGFR levels and EGF induced mitogenesis. It is possible that increases in membrane associated EGFR also leads to increases in nuclear localised EGFR The role of Oestrogen either acting directly, or by TGFα

stimulation, or by the induction of PDGF like activity is another worthwhile avenue.

(5) For nuclear localisation to proceed, transport through the cytoskeleton is probable. Electron microscope studies have shown cytoskeletal association of high affinity EGFR in A431 cells (Weigant *et al.*, 1986). Treatment with cholchicine which disrupts microtubules, and has been shown to inhibit cytoplasmic transport of TSH hormone, Growth hormone and PRL (Gautvik & Tashjian, 1973: Hall, 1984). Cytochalasin B and anti-actin antibodies which disrupt the function of actin (Mrotek & Hall, 1975) may resolve the role of microtubules and actin in the transport of EGFR to the nucleus. The cytoskeleton couples intracellularly with the nuclear matrix and provides the three dimensional organisation of DNA (for a review see Getzenberg *et al.*, 1990). The precise function of EGFR nuclear matrix interaction is difficult to ascertain. Nuclear matrix proteins play a key role in the regulation of gene expression and have been found to be altered by a number of post translational modifications. These include protein phosphorylation, methylation, and ADP ribosylation (Henry & Hodge, 1983: Moy & Tew, 1986). Teraoke *et al.* (1989) have reported a protein component of liver nuclear matrix which possesses tyrosine kinase activity. It is certainly possible that EGFR tyrosine kinase and indeed other tyrosine kinase growth factor receptors have a role in the tyrosine phosphorylation of matrix proteins. The precise function of tyrosine kinase phosphorylation of nuclear matrix proteins has still to be elucidated. Other aspects of research could include, site directed mutagenic studies to assess the domains that are essential for nuclear binding. Some of the techniques used in the elucidation of steroid hormone receptors with respect to the nucleus could prove useful (See Parker, 1991).

(c) The observation that PRL can positively regulate measurable levels of EGFR in an oestrogen receptor positive breast cancer cell line is novel. However it is important to realise that this study was carried out in a single cell line and should be extended to other lines containing PRLR (with or without ER). Further experiments given more time and funding would initially have involved extending these observations to other cell lines in particular the MCF-7 and T47-D lines. In principle the blockade of PRL binding by appropriate antibodies to the receptor would be advantageous in determining if the induction is a direct function of PRL and its receptor. Such an antibody however has not to our knowledge been described. Inhibition of Protein Kinase C (by commercially available enzyme inhibitors such as (+) - 1 - (5-Isoquinolinesulponyl) - 2 - methylpiperazine hydrochloride, H-7 (Ratz, 1990)) when under PRL stimulation may give an indication as to the role of the phosphatidylinositol pathway in PRL mediated EGFR induction. The physiological relevance of this study is difficult to assess. Whilst a role for PRL in rodent tumourigenesis is well established, its role within human breast cancer in terms of maintaining tumour growth is still unclear, though the relationship between pregnancy reduced plasma PRL and pregnancy protection against breast cancer remains intriguing. (Wang *et al.*, 1987). However the demonstration of variant forms of PRL and more accurate methods of estimation with regard to diurnal variation may reveal a clearer picture. The possibility still remains that PRL regulation of the EGFR system might stimulate breast epithelial growth.

If PRL has a role to play in breast cancer, the time point at which its action might influence either the induction or promotion of the disease is unknown. Certainly anti-prolactin therapies have never been successful in established disease (Bruning, 1987). It has been demonstrated that tumours containing ER usually contain PRLR and have a relatively good prognosis.

It is possible that E_2 and PRL maintain rigid control of the EGFR/TGFα mitogenic system. Loss of ER and PRLR may result in loss of that control resulting in a more autonomous highly proliferative tumour. These experimental observations could well be a vestige of PRL action with regard to the normal development of the breast. As with all observations contained within this thesis only time and more study will tell.

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