Mutagenesis studies on the membrane anchoring properties of human CD2

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The research in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.

Gertrudes Corcad

To my parents and my sister Gracieta,

for their encouragement to come here and support

over my long staying in Glasgow

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CHAPTER 2 : MATERIAL AND METHODS

SUMMARY

There is a lack of information about structural requirements for a functional transmembrane domain in eukaryotic membrane proteins. This led me to design a eukaryotic system to bring more information about this structure and the role of the positively charged residues situated at the cytoplasmic side of a transmembrane region. The CD2 molecule was chosen as a model for an integral membrane protein. I deleted the transmembrane domain (26 amino acids) by oligonucleotide site-directed mutagenesis and overlap extension using PCR mutagenesis. Truncated forms with transmembrane regions 14, 12, 10 and 8 amino acids long were created. The common positive cluster (Lys-Arg-Lys-Lys) at the cytoplasmic domain was disrupted by substituting it for polar residues (Gln-Gln-Gln-Gln).

The effects of such mutagenesis was examined after expression of the mutant proteins in eukaryotic cells (COS-7 and CHO). Cellular localization was determined by panning and immunostaining experiments. The functional state of the mutant proteins expressed on the cell surface was verified by rosetting experiments and immunostaining with antibodies against different epitopes of the CD2 extracellular domain. The interaction of the molecule with the lipid bilayer was determined by lateral diffusion studies, using photobleaching analysis.

It was demonstrated that a transmembrane domain at least 12 amino acids long is necessary for the CD2 protein to be

expressed on the cell surface as an integral membrane protein. Proteins with shorter transmembrane domains (10 and 8 amino acids respectively) were localized intracellularly as verified by immunostaining. This finding is in agreement with previous work on the VSV G protein (Adams and Rose, 1985b). Lateral diffusion studies revealed that even large deletions in the transmembrane domain of CD2 do not interfere with its lateral mobility in the lipid bilayer. Although the fraction of the free molecules for diffusion was higher in the CD2 protein with transmembrane region 12 amino acids long. This implies that deletions in the transmembrane domain can interfere with the stability of the protein within the lipid bilayer.

The disruption of the positively charged cluster of the CD2 cytoplasmic domain did not alter the orientation and stability of the molecule within the cell membrane. This suggests that the topology of a membrane protein with only one span domain is not influenced by this positively charged sequence. The orientation within the membrane should be determined in the early stages of translocation by interaction of the signal sequence and the hydrophobic domain with the protein-conducting pore in the endoplasmic reticulum membrane.

CHAPTER 1

INTRODUCTION

1. Insertion of membrane protein into the Endoplasmic Reticulum

The cell has specific transport mechanisms for the targeting of newly synthesized proteins to their site of function. The amino acid sequence of nascent polypeptides have the necessary information for intracellular protein targeting, to control the timing and location of protein folding and to determine the final three dimensional structure of the protein. Newly synthesized proteins can reach the cell membrane (integral membrane proteins), the lumen (secreted proteins) or enter other intracellular organelles. The first two have to be transported by the secretory pathway before they reach their targets. In eukaryotic cells, nascent polypeptides enter the secretory pathway at the rough endoplasmic reticulum membrane (RERM) and they travel through the Golgi complex either to cell surface or to secretory granules, as lysosomes or vacuoles, in order to be secreted from the cell or anchored to the cell surface.

1.1 Membrane proteins

Blobel (1980) proposed that discrete segments of the polypeptide chain act as topogenic determinants of protein structure and he called them topogenic sequences. These sequences can either start or stop the translocation of the polypeptide chain through the ER membrane. Stop-transfer

sequences can interrupt the chain translocation that was initiated by a start transfer sequence and they are effective means for asymmetric integration of membrane proteins.

Integral membrane proteins anchor to the cell surface and span the lipid bilayer at least once. They were classified on the basis of the number of times the polypeptide chain crosses the membrane (Blobel, 1980) and of their orientation in the membrane (von Heijne and Gavel, 1988)(Fig 1.1). Class I proteins present a cleavable signal peptide, their terminus is oriented towards the ER lumen and their C-terminus to cytoplasmic side. Human glycophorin A (Siebert and Fukuda, 1986), HLA antigens of human cells (Lopez de Castro et al., 1983), viral spike glycoprotein (Wiley and Skehel, 1987; Rose et al., 1980) are some examples of class I membrane proteins. In class II membrane proteins, the signal peptide is not cleaved, it can be internal and may overlap with the membrane spanning domain: as observed in human liver transferrin receptor (Zerial et al., 1986). They exhibit the C-terminus in the ER lumen and the N-terminus in the cytoplasm, for example, like influenza A neuraminidase (Block and Air, 1982), human HLA-DR invariant gamma chain (Strubin et al., 1984), human liver transferrin receptor (Schneider et al., 1984; Zerial et al., 1986). In class III membrane proteins the signal peptide may remain in the mature protein functioning as a start transfer sequence and it was also postulated that these polypeptides contain an internal stop sequence that interrupts the cotranslocation through the ER membrane, leaving the C-terminus exposed on the cytoplasmic surface, assuming an orientation as class I proteins. Rat cytochrome P450 (DeLemos-Chiarandini et al., 1987), human cytochrome P450 (Beaune et al., 1986), influenza A M2 protein (Lamb et al,1985) can be included in class III of membrane proteins. Some proteins can span the membrane several times, they are classified as polytopic membrane proteins. They contain multiple hydrophilic domains on both sides of the membrane, such topology could be because a succession of

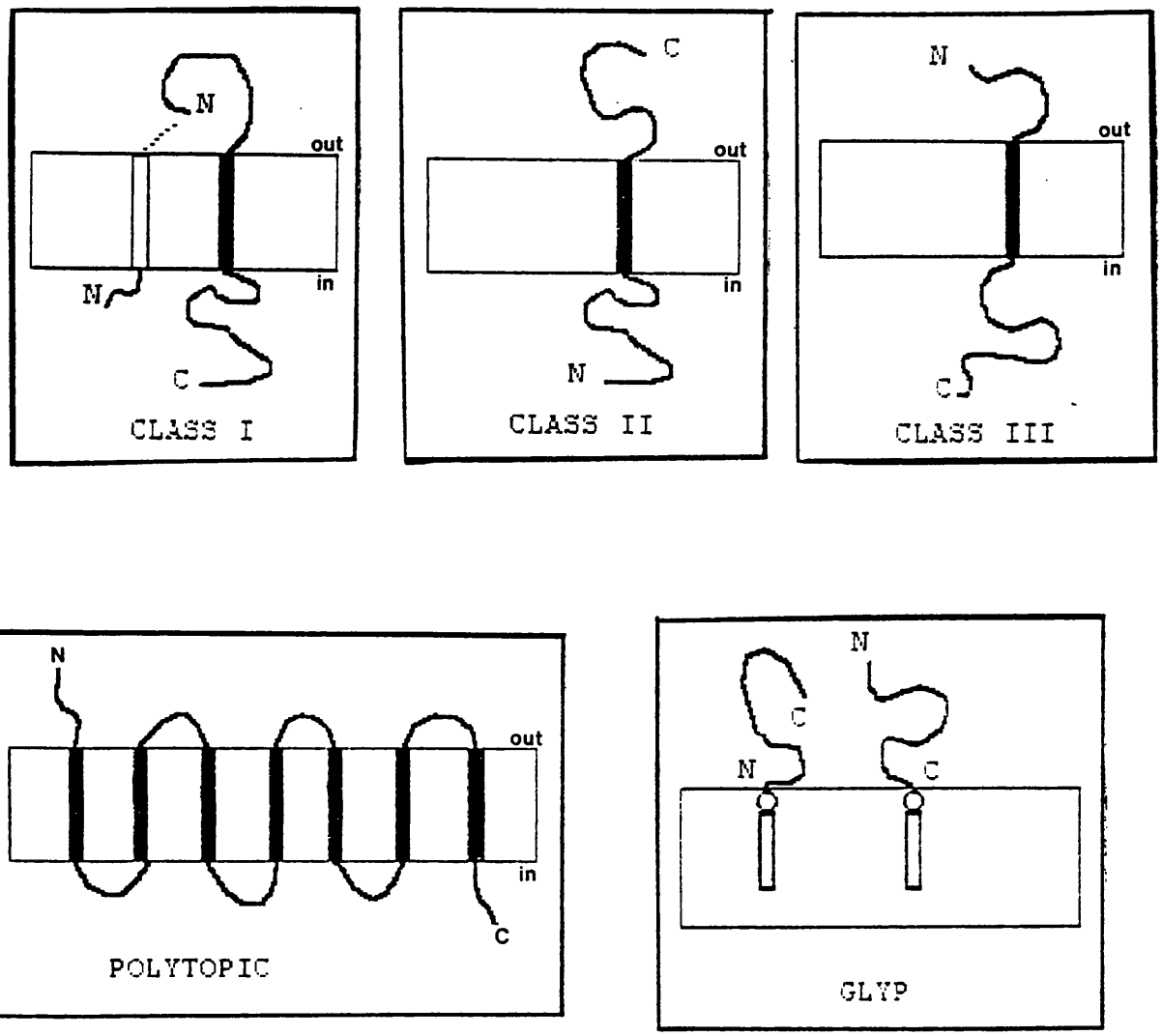


Figure 1.1 - Diagram showing the different classes of membrane proteins. The horizontal shaded lines represents the cell membrane; the black vertical rectangles represent transmembrane region of proteins. Classes I, II, III and IV are integral membrane proteins. GLYP represents those membrane proteins that are anchored by glypiation tails. For class I integral membrane protein, the N-terminal leader sequence (open box) is shown cleaved (dotted line) from the N terminus of the mature protein. The number of transmembrane seqeunces of class IV proteins need not be seven, and the orientation of their N and C termini not be as shown.

alternating internal signal and stop transfer sequences{eg human asialoglycoprotein (Wessel and Spiess, 1988), glucose transporter (Mueckler et al.,1985) and rhodopsins (Schobert et al.,1988)}.

An alternative mechanism to anchor proteins to the plasma membrane was first found in the Thy-1 antigen of rodent thymocytes and neurons (Williams and Gagnon, 1982) and in the variant surface glycoprotein (VSG) of *Trypanosoma brucei* (Allen et al.., 1982). Both molecules have properties of a membrane protein that bind the lipid bilayer but no hydrophobic properties or sequences were observed in their structure. This alternative anchoring mechanism involves the covalent attachment of a complex structure of phosphatidyl inositol, carbohydrate and ethanolamine to the C-terminus of a protein. Structurally, these proteins are glycoproteins composed of a single domain which is anchored to the membrane via a covalent linkage to a glycophospholipid-glycosyl phosphatidyl inositol (GPI)(Fig 1.1). The C-terminal amino acid of the extracellular protein moiety is linked via an ethanolamine residue to an oligosaccharide composed of mannose, a variable number of galactose residues and glucosamine (non N-acylated). This oligosaccharide is in turn attached to the inositol portion of PI (phosphatidyl inositol). The diacylglycerol portion of PI provided is the only site for membrane attachment in these proteins (Ferguson and Williams, 1988). Translocation mechanism of these proteins into the lumen of the ER is initiated through a conventional N-terminal signal sequence. A second cleavable hydrophobic sequence at the C-terminal end of the protein is also present and it serves as a signal for GPI attachment. This sequence is cleaved cotranslationally in the lumen of the ER, and GPI is added as a block by a set of enzymes (Caras et al., 1987, Caras et al.., 1989).

The ras family of proteins are members of a small guanine nucleotide binding proteins which were initially detected by their ability to transform cells, to function they have to bind GTP and be localized at the plasma membrane, as mature forms of polypeptide chain. Their localization involves two posttranslational modifications, polyisoprenylation and palmitoylation at the Cys186-AAX motif and 181/4 Cys, respectively, localized at the C-terminus (Hancock et al. , 1989;1991). The heterotrimeric G proteins, signal transducers from the superfamily of GTPases, are localized to the inner surface of the plasma membrane. They are composed of three subunits and none of them have hydrophobic membrane spanning domains, two types of posttranslational modifications, myristoylation and isoprenylation, play a role in their membrane association (Spiegel et al. , 1991).

Signal sequences

The signal sequence or signal peptide is the first topogenic sequence involved in protein targeting . Gething and Sambrook (1982) showed that the signal peptide act as a targeting signal, by deleting the entire signal peptide region of influenza virus hemagglutin, after expressing the mutants in Cos cells, it was found that instead of it being glycosylated and transported to cell surface, the mutant form was unglycosylated and located intracellularly. Lingappa and cols (1984), studied a fusion protein of coding sequence for chimpanzee α-globin (a cytoplasmic protein) and portion of β-lactamase gene coding for the N-terminal 28 aa . , including the 25 aa signal peptide. The protein was translocated across the ER and cleaved at normal β lactamase signal peptide.

Signal sequences do not exhibit primary sequence homology, their function depends more on general physical properties, like the hydrophobic character, than on specific residue

recognition (Landry and Gierasch, 1991). Some properties differ between prokaryotes and eukaryotes (von Heijne, 1985; Duffaud et al., 1985). The amino terminal region in eukaryotes carry a net positive charge, where in prokaryotes one to three positively charged amino acid residues will be present. Akita and colleagues (1990) suggested that basic residues in the signal sequence possibly interact with acidic residues in components of the secretion apparatus. The hydrophobic core in prokaryotes is longer than eukaryotes, and the presence of a serine and /or threonine in the former one, divides this hydrophobic domain into two regions of different degrees of hydrophobicity. The cleavage site seems to be preferentially negatively charged and it follows the motif AXA in prokaryotes and in eukaryotes it is preceded by six to eight residues.

Transmembrane domains

Most integral membrane proteins share a similar structural motif, a hydrophobic transmembrane stretch of 17-30 amino acids (Sabatini et al., 1982) that functions as a stop transfer sequence. Several experiments show that the hydrophobic character in such segments is important to membrane anchor properties as this hydrophobic segment may act as a anchor (von Heijne and Blomberg, 1979; Engelman and Steitz, 1981). von Heijne (1981) compared the overall amino acid composition of 10 transmembrane segments. The residues seemed to be selected only for their hydrophobicity, thus the amino acid composition of those segments are conserved. Davis and Hunter (1987) observed that this hydrophobic character is important not only to anchor the protein to the cell membrane, but also for intracellular transport. By introducing two point mutations into the transmembrane region of the Rous Sarcoma virus, they converted a hydrophobic leucine to an arginine. The mutant exhibited reduction on cell surface expression because it appeared to be transported normally through the cell until the trans-Golgi compartment,

where instead of being transported to the cell surface, it was transported to the lysosome and degraded. Adams and Rose (1985a) did a similar experiment with vesicular stomatitis virus G protein, and the mutant could be seen localizing in the Golgi. Davis and Model (1985), in order to prove that the hydrophobicity itself is responsible to membrane anchoring process, created an artificial anchor domain by inserting repeats of hydrophobic amino acids into the genes encoding a secreted version of coliphage f1 gene III protein (Davis et al.., 1985); the mutant spanned the membrane assuming a membrane topology.

Effect of positively charged amino acids

Another important feature of integral transmembrane protein is the location of positively charged amino acids stretches, in the cytoplasmic domain adjacent to transmembrane segments. By analysing the sequences of known membrane proteins from prokaryotes and eukaryotes (von Heijne, 1986; von Heijne and Gavel, 1988) it was found that lysine and arginine residues are four times more prevalent in the cytoplasmic domain than in periplasmic loops and this proportion is more than would be expected at random. The "positive inside" rule suggested that membrane proteins orientate themselves with the most positively charged end in the cytoplasm and the function of the charged residues is more related to orientation of the protein in the lipid bilayer than to insertion (von Heijne, 1986).

There is some experimental support for the idea that positively charged residues can act as topological determinants. The membrane topology of leader peptidase, which spans the membrane twice with a N_{out}-C_{out} pattern, can be inverted to a N_{in}-C_{in} pattern, by changing the charge distribution of the hydrophilic domains (von Heijne, 1989). Positively

charged amino acids were found to be important in anchoring cytoplasmic domains of MalF, when this was fused to alkaline phosphatase (Boyd and Beckwith, 1989) and various domains of the fusion proteins were deleted (McGovern et al.., 1991). Studies on the topology of hemaglutinin-neuraminidase proteins of paramyxovirus simian virus 5 (HN), a class II protein, support the positive inside rule, since removal of positively charged residues on the N-terminus side of the transmembrane region, causes an inverted topology, from N^{in}-C^{out} to N^{out}-C^{in} (Parks and Lamb,1991).

Nilsson and von Heijne (1990) demonstrated that addition or removal of a single positively charged lysine residue in the N-terminal side of the first spanning domain of leader peptidase, can be sufficient to reverse the topology of a polytopic membrane protein, these results provide a good support for the "positive inside" rule. It was also observed that a sufficient number of negatively charged residues can affect the topology.

McGovern and colleagues (1991) compared the charged residue composition of MalF/alkaline phosphatase fusion deleted mutants and found that the strength of cytoplasmic domains as topogenic signals varies and is correlated with the density of positively charged amino acids within them. This difference may be related to the potential to form stable ion pairs between charged residues, making the domain more readily exportable (Traxler et al., 1993). This MalF deletion system suggested that in the case of polytopic membrane proteins with various cytoplasmic domains, the rule for the effect of basic amino acids does not follow a sequence of events initiated by topological signals at the N-terminal of the protein. A more C-terminal cytoplasmic signal can compete with other signals that occur amino terminal to it and establish the protein orientation in the membrane.

Hartmann and cols (1989) proposed the charge difference hypothesis, in which the sum of the flanking charges of the signal/anchor domain is important for directing the topology of the protein, and it is not the absolute number of positive or negative charges surrounding this segment. This hypothesis is based on a comparison of sequences from eukaryotic type II and III membrane proteins, and a strong correlation between the sum of the charges surrounding the signal/anchor domain and its membrane topology was identified.

The way this interaction occurs is still unclear. One possibility is that the basic residues adjacent to the apolar segment interact with the acidic lipid head groups exposed on the cytoplasmic surface of the membrane, this is according to the loop model (Inouye and Halegoua, 1980). Another possibility is concerned with the eletrochemical gradient across the bacterial membrane, which is more negative on the cytoplasmic side, that seems to be suitable for hydrophobic sequences to insert with their positively charged ends on the inner face of the membrane (Li et al,1988; Yamane and Mizushima,1988). Although in eukaryotes no general membrane potential exists across the rough endoplasmic reticulum membrane, where protein translocation initiates, the possibility of local membrane potential has not been excluded (Boyd and Beckwith, 1990).

The positively charged segment is also required to localize $p21^{ras}$ to the inner face of the plasma membrane. The *ras* proteins, $p21^{H\text{-}ras}$, $p21^{N\text{-}ras}$ and $p21^{k\text{-}ras(A)}$ have sites of palmitoylation (C-terminal CAAX motifs), that target this protein to cell membrane (Hancock et al, 1989). It was demonstrated that $p21^{K\text{-}ras(B)}$, which is not palmitoylated, requires the combination of the C-terminal motif and a

polybasic domain for plasma membrane localization (Hancock et al, 1990; Hancock et al, 1991).

1.2 Insertion mechanisms

The endoplasmic reticulum (ER) is the place of topogenesis of most newly synthesized membrane and secreted proteins, it is also where many posttranslational modifications and assembly events occur. The orientation of a membrane protein into the phospholipid bilayer is likely to be determined by the mechanism of its insertion, for which there are several models. The main difference between the models is the participation or not of membrane receptors of the ER in the translocation process.

von Heijne and Blomberg (1979) proposed that the transfer of a nascent chain is directly through the lipophilic core of the ER membrane, and the driving force is provided by direct interaction between ribosome and membrane, this hypothesis was denominated "The direct tranfer model". The signal peptide assume an α-helical conformation and binds to the lipophilic core of any membrane, resulting in the binding of the ribosome to the ER membrane, forcing even strongly hydrophobic residues into the lipid bilayer.

Wickner (1979, 1980) proposed the membrane triggered folding hypothesis, which emphasizes the self assembly and folding properties of protein as they pass from cytoplasm to membrane. It is based on the assumption that assembly of proteins can be initiated during or shortly after the polypeptide synthesis and that it is an integral part of the folding pathway, as protein encounter an amphipathic surface. Larger proteins may assemble during synthesis, others after synthesis is

completed. Once they encounter a membrane, they may triggered to refold by the availability of the hydrocarbon core of the bilayer. During this assembly event, polar portions of the protein are protected from the fatty acyl chains by more apolar residues. The hypothesis is supported by examples of proteins that can exist in alternate conformation, compatible with aqueous or apolar environments like fumarase (Clarke, 1975), diphteria toxin (Bouquet et al,1976), adenyl cyclase (Homcy et al, 1978) and protein II of the *E.coli* (Schweizer et al, 1978).

Engelman and Steitz (1981) proposed the helical hairpin hypothesis, in which the translocation and insertion of membrane proteins are spontaneous processes, driven by hydrophobic forces. There is no need of additional specific membrane receptors or transport proteins, because of the distribution of polar and nonpolar sequences in the polypeptide chain. It was postulated that the nascent polypeptide chain will fold to form an antiparallel pair of helices, the hairpin structure, which should contain the signal peptide. This helical hairpin structure will insert spontaneously into the hydrophobic region of the bilayer. If the second helix is polar, secretion will initiate and it will continue until a hydrophobic segment is encountered. In the case of secreted proteins, after insertion of the helical hairpin structure, the synthesis will continue. When the translocation is completed and the C-terminus passes through the membrane, the signal sequence is cleaved and the protein released into the ER lumen. However, the insertion and orientation of membrane proteins will depend on the presence of a hydrophobic domain, which will halt the translocation. The insertion of membrane proteins from class II, where the C-terminus is found on the exterior side of the membrane, like sucrase-isomaltase dimer (Frank et al.,1978; Brunner et al.,1979) and Cytochrome b_5 (Jagow and Sebol, 1980) which is anchored to the ER and synthesized without a leader sequence

on free cytoplasmic ribosomes (Krieter and Shires, 1980), the insertion might be posttranslationally, either by a helical hairpin or a single hydrophobic helix. In the case of proteins with several transmembrane segments, like bacteriorhodopsin, several hairpin insertions may be involved.

Blobel and Dobberstein (1975) in studies on the presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma cells, observed that the processing of nascent chains *in vitro* by rough microsome is dependent on ribosome binding to the membrane and on the basis of these results the signal hypothesis idea was described. The essence of this hypothesis is that specific factors, membrane or cytoplasmic proteins, bind to the growing polypeptide chain and conduct it into the bilayer during its synthesis. According to this hypothesis the insertion of the nascent polypeptide chain is directed by a unique sequence of codons, the signal sequence , present immediately to the right of the initiation codon only in those mRNAs whose translational product are to be transferred across the cytoplasmic membrane (in prokaryotes) or the ER membrane (in eukaryotes), this sequence will collaborate with ribosome attachment to the membrane, that facilitates the translocation. The whole process consists of a series of events, involving distinct complex components. First, the signal recognition particle (SRP) binds the signal sequence of a nascent polypeptide chain, regulates translational elongation and initiates translocation across the membrane. An SRP receptor, found in the ER membrane, associates the SRP/nascent polypeptide complex with the membrane. The signal sequence receptor, also an integral ER membrane protein, interacts with the signal sequence once it has reached the membrane. The ribosome binds to the ribosome receptor, also located in the ER membrane, and after the translocation of the protein across the membrane, the signal peptidase cleaves the signal sequence, producing the mature form of the protein.

Class I and II of membrane protein orientation in the cell membrane is, in a way, easy to explain according to this hypothesis. On the other hand, class III membrane protein orientation is contradictory with the signal sequence/membrane interaction, crucial to the signal hypothesis. Sabatini and colleagues (1982), postulated the existence of a N-terminus interior uncleavable cotranslational signal peptide to explain the orientation of these membrane proteins . It interacts with the membrane, in a loop conformation, in order to promote cotranslation of the nascent polypeptide, until it is interrupted by a stop transfer sequence near the C-terminus region. This loop model shares similarities with the helical hairpin one, the difference is that in the former polypeptides are synthesized on bound ribosomes. The insertion mechanism of membrane proteins with multiple spanning domains (polytopic membrane protein), could be explained by a sucession of alternating internal signal and stop transfer sequences. Wessel and Spiess (1988) constructed artificial polytopic proteins spanning the membrane up to four-fold by tandemly repeating portions of the cDNA encoding the human asialoglycoprotein (ASGP) receptor H1. By *in vitro* translation in the presence of microsomes, it was observed that the first hydrophobic domain functioned as a signal peptide and together with SRP initiates a first translocation process. The second domain acts as a stop transfer, while the third initiates a second translocation process up to the next apolar domain that halts the translocation.

The three first hypotheses are quite related in the way that membrane receptors are not necessary for translocation, and existing evidence for posttranslationally insertion of membrane proteins provide support for them. Mueckler and Lodish (1986) described a cotranslational and

posttranslational mechanism of membrane integration of a multispanning membrane transport protein, the human facilitated diffusion glucose transporter (GT) (Fig 1.4C). RNA transcripts encoding the complete GT or fragments corresponding to the N-terminus (GT-N) and C-terminus (GT-C) were synthesized in reticulocyte or wheat germ cell-free systems and their insertion into pancreatic microsomes were assayed by endoglycosidase H and trypsin digestion or alkaline extraction of membranes. It was assumed, for illustrative purposes that GT possesses at least two distinct signal sequences, positioned arbitrary in the two halves of the protein and that the ribosomes engaged in the synthesis never interact directly with the ER membrane which functions during the cotranslational insertion. Elongation of the polypeptide chain continues until the first signal sequence emerges from the ribosome. SRP binds to this sequence facilitating the insertion of the N-terminal fragment into the membrane as a loop structure through interaction of the complex with the SRP receptor, without the ribosome being brought in direct contact with the membrane. The polypeptide chain elongation continues until the other signal sequences emerges and the cycle repeats as before. This mechanism can be considered as cotranslational insertion in a way that the polypeptide chain is still being synthesized while the N-terminus is inserted into the membrane via SRP/SRP receptor integration, where SRP may be required for targetting and initiation of the insertion process. On the other hand, it can be a posttranslational event because the polypetide chain folds into the membrane may be driven by the free energy change resulting from hydrophobic interactions with the lipid bilayer and the ribosome is not in contact with the ER membrane, driving the nascent polypeptide chain through an aqueous pore.

The cytochrome b_5, a class II membrane protein located on the outer surface of the ER, is considered to be synthesized on free ribosomes and to be inserted into the ER membrane

posttranslationaly[1]. Kutay and colleagues (1993) point out that because the hydrophobic segment of cytochrome b_5 (and other class II membrane proteins) is to close to the C-terminal fragment, it could not have emerged from the ribosome before termination of translation, which corroborates the idea of a SRP independent insertion pahtway. Although it follows a SRP/SRP receptor independent pathway, Mitoma and Ito (1992), by doing deletions in the C-terminal domain and fusions with 106 amino acids fragment of the mature adrenoxin and to artificial hydrophobic peptides 20 amino acids long, observed that the last 10 amino acids from the C-terminus contain the necessary information to target the cytochrome b_5 specifically into the ER membrane, so the mode of insertion was classified as receptor mediated pathway.

1.3 Function and components as the membrane supporter signal sequence hypothesis

Signal peptidase

The signal sequence or signal peptide is the only segment that keeps the translational complex bound to membrane after translocation. This hydrophobic segment can be either cleaved off and the protein released into the lumen of the ER, or it

can act as a stop transfer signal, as described before. In the first case, the signal peptidase will be responsible by the cleavage of the signal peptide. It is a special class of endopeptidases with a well-defined substrate specificities. The eukaryotic signal peptidase has been isolated from canine pancreatic rough microsomes (Evans et al, 1986). It was found to contain six polypeptides (25, 23,22,21,18 and 12 KDa), these polypeptides exhibited sensitivity to endoglycosidase H and ability to bind concanavalin A, so they were considered glycoproteins. It seems that only one subunit of the complex is responsible for signal peptide cleavage, maybe the other subunits are involved in other enzymatic processes. Each of the polypeptide chains is more abundant than either SRP or SRP receptor and is roughly equivalent in amount to the membrane bound ribosomes (Evans et al, 1986). The catalytic site is located on the noncytoplasmic face of the membrane.

Signal recognition particle

In order that translocation of nascent polypeptide chain occurs, it is necessary that the polysome binds to the ER membrane. This process is mediated by a ribonucleoprotein complex, the signal recognition particle, SRP (Walter and Blobel, 1981).It is a cytoplasmic ribonucleoprotein composed of six non identical polypeptide chains and a 300 nucleotide RNA molecule, 7SL RNA (Walter and Blobel, 1982). The complex functions as an adapter between protein synthesis in the cytoplasm and protein translocation in the ER membrane. It was first recognized by its ability to restore the translocation activity of salt-extracted microsomes *in vitro* (Warren and Dobberstein, 1978). The four polypeptide chains are noncovalently bound, these subunits are two monomers, a 19 plus 14 KDa and a 68 plus 72 KDa.

Signal recognition starts when SRP binds an inactive ribosome with relative low affinity, which increases with synthesis and exposure on the ribosomal surface of the signal sequence as part of the nascent polypeptide. This signal recognition gives rise to translational arrest and the SRP-ribosome-nascent chain complex will target the ER membrane via interaction with SRP receptor or docking protein, while the signal sequence interacts with signal sequence receptor. This last event is accompanied by SRP/SRP receptor complex detachment from the ribosome, ribosome/nascent chain complex associates with ER membrane, possibly involving a ribosome receptor and the translocation mechanism proceeds (Walter and Lingappa, 1986).

The different functions of SRP in the targetting process are specific to the different structural units of the particle (Siegel and Walter, 1988). Crosslinking experiments demonstrated that there is an interaction of signal sequence and SRP, and the 54 KDa subunit was responsible for this (Krieg et al, 1986; Kurzchalia et al,1986 and Wiedmann et al, 1987). Analysis of its amino acid sequence shows that it has two domains, a N-terminal one, with typical features of a GTP binding protein (G domain), and a C-terminal domain, methionine rich (M domain). This last one has been implicated in signal sequence binding (Bernstein et al, 1989; Romisch et al, 1989). The G domain plays a essential role in SRP-mediated targeting of nascent chain-ribosome complexes to the ER membrane and also influences signal sequence recognition, possibly by promoting a tighter association between signal sequences and M domain (Zopf et al., 1993). The 19 KDa* binds directly to the RNA (Lingelbach et al, 1986). The 9/14 KDa dimer is necessary for elongation arrest (Siegel and Walter,1988; Scoulica et al,1987). The 68/72 KDa* serves to bind the complex to the SRP receptor and promote transmembrane translocation (Siegel and Walter, 1988). Walter and Blobel (1982) pointed out that 7SLRNA is a constitutive and indispensable part of the SRP, it is thought to have a

* polypeptide

structural role, serving as a "core" or "matrix" on which the SRP proteins assemble.

Signal recognition particle receptor

As mentioned before, the SRP/ribosome complex interacts with an integral membrane protein, the SRP receptor. It is a heterodimeric protein with an α-subunit, the 69 KDa polypeptide, that comprises a 18 KDa anchor and a 52 KDa cytoplasmic domain, and a β, the 30 KDa popypeptide (Hortsch, et al 1988; Lauffer et al, 1985). The α-subunit it is also call docking protein (Meyer et al,1982) and it resembles nucleic acid binding proteins, which suggests that SRP receptor may interact with 7SLRNA in SRP, and this interaction could be mediated by protein-nucleic acid interaction.

The ER has on its membrane some integral membrane proteins that interact in order to form a multicomponent translocation apparatus, the translocon. Its function is to mediate the binding of ribosomes engaged in the synthesis of secretory or membrane proteins, that have been targeted to the ER membrane by SRP/SRP receptor complex. The translocon components can be also responsible by protection of the aqueous pore, needed for translocation of the nascent polypeptide through the hydrophobic core of the membrane. It also can promote integration of signal anchor sequences in a proper orientation to give rise to the different types of membrane protein (Walter, 1992).

Translocon (signal sequence receptor and ribosome receptor)

The signal sequence receptor is one of the components of the translocon. Wiedman and cols (1987) used a crosslinking approach to identify an integral glycosylated protein (35

KDa), called SSR, that interacts with the signal sequence during the process of protein translocation. However, Krieg and cols (1989), observed that not only the signal sequence could be crosslinked to the membrane glycoprotein but also to distant parts of the translocation nascent chains, suggesting that SSR can interact with various components of membrane proteins and not only with signal sequences. Migliaccio and cols(1992) also demonstrated that reconstituted ER vesicles, immunodepleted of SSR and associated subunits, were still able to translocate secretory and membrane proteins substrates. Gorlich and cols (1992) purified, cloned and sequenced a different ER membrane glycoprotein that participates in protein translocation. It was called TRAM (for translocating chain-associating membrane protein), its amino acid sequence indicates that it probably crosses the membrane eight times and it is as abundant as ER membrane bound ribosomes. In order to prove its functional significance, TRAM was depleted from ER vesicles. Translocation of two secretory proteins β-lactamase and prepro-α-factor was blocked in TRAM depleted vesicles, on the other hand, translocation of preprolactin was still observed, but with low efficiency, perhaps due to residual TRAM probably present in the vesicles. Readdition of purified TRAM to the extract restore the translocation activity of the two secretory proteins.

The other component of the translocon is the ribosome receptor. The large number of ribosomes attached to the RER, gives to it this rough appearance. It seems to be such a tight attachment that persists even during fragmentation of the ER and sucrose gradient centrifugation. Ribophorins I and II were the first membrane proteins described as characteristic of the rough endoplasmic reticulum. Although they copurify with the ribosomes and other components of the translocon, they do not show protease sensivity, exhibited by the ribosome binding activity of ER membranes (Hortsch et al, 1986). Also when a fraction enriched in ribophorins I and II was incorporated

into liposomes and binding assays were performed, only a marginal capacity for ribosomal binding was shown (Savitz and Meyer, 1990). Another candidate for a possible binding factor was identified by Savitz and Meyer (1990,), who isolated, purified and characterized a large integral membrane protein (180 KDa), with a large cytosolic domain (160 KDa). When this protein is proteolytically detached from the membrane, it can competitively inhibit the binding of ribosomes to intact membranes. This ribosome receptor was denominated p180. It was showed to be essential for both ribosome binding and protein translocation through the depletion of p180 with monoclonal antibodies from detergent extracts of the RER used in the preparation of translocation-competent proteoliposomes. Proteoliposomes from p180-depleted extracts were unable to bind ribosomes and lost the ability to cotranslationally translocate two secretory protein precursors. Re-addition of purified p180 to depleted extracts, restored ribosome binding and translocation activity (Savitz and Meyer, 1993). Yoshida and cols (1987) isolated a protein fraction from rat liver microsomes, and separated it into two fractions on a Con-A sepharose column. A glycoprotein fraction, including major part of ribophorins, and a non glycoprotein fraction were obtained. Both were reconstituted into liposomes and had their ribosome binding activities compared. The non glycoprotein fraction showed 90% of recovered binding capacity. Tazawa and cols (1991) separated the non-glycoprotein fraction, by affinity chromatography on a chelating-Sepharose column, into two fractions. Ribosome binding activity was observed at the flow-through fraction (chelate FT fraction),which one was treated with trypsin, and a parallel decrease in ribosome binding activity and the amount of a 34 KDa band was observed. By photocrosslinking studies, it was found that the 34 KDa component crosslinked with the 60S ribosome subunit and it was in proportion to the amount of 60S subunit added to the system. This 34 KDa polypeptide was called p34, and it appears to be another candidate for a ribosome receptor.

The signal hypothesis emphasizes that the newly synthesized polypeptide has to be conducted through an aqueous proteinaceous channel, and interactions that will determine topology will occur between the translocating protein and the components of the channel (Fig 1.2). Simon and Blobel (1991) described and characterized a protein conducting channel in the ER membrane by electrophysiological techniques, using puromycin to release nascent chain from ribosomes, membranes to induce protein conducting channel of 220 pS and high salt concentrations to detach ribossomes from the membrane. The results suggest a role for the attached ribosome in keeping an open channel.

Either the signal sequence itself opens the channel and binds to a stop tranfer receptor in the protein-conducting channel, or the SRP receptor, or the ribosome, serves as the ligand. In order to translocate secreted proteins the protein-conducting channel would need to open only perpendicularly to the bilayer. However, in the case of integral transmembrane proteins, it would need to open in two dimensions, perpendicularly to the membrane, so the domain of the protein can pass through and into the plane of the membrane, to allow another domain, the possible transmembrane one, to move laterally into the lipid bilayer (Simon and Blobel,1991). Blobel(1980) suggested that the protein conducting channel is formed by subunits that dissociate after translocation of the polypeptide, leaving the transmembrane segment embedded in the bilayer. Another possibility is suggested by Singer and colleagues (1987), where the channel structure remains intact and allows the transmembrane segment to slip between two of its subunits.

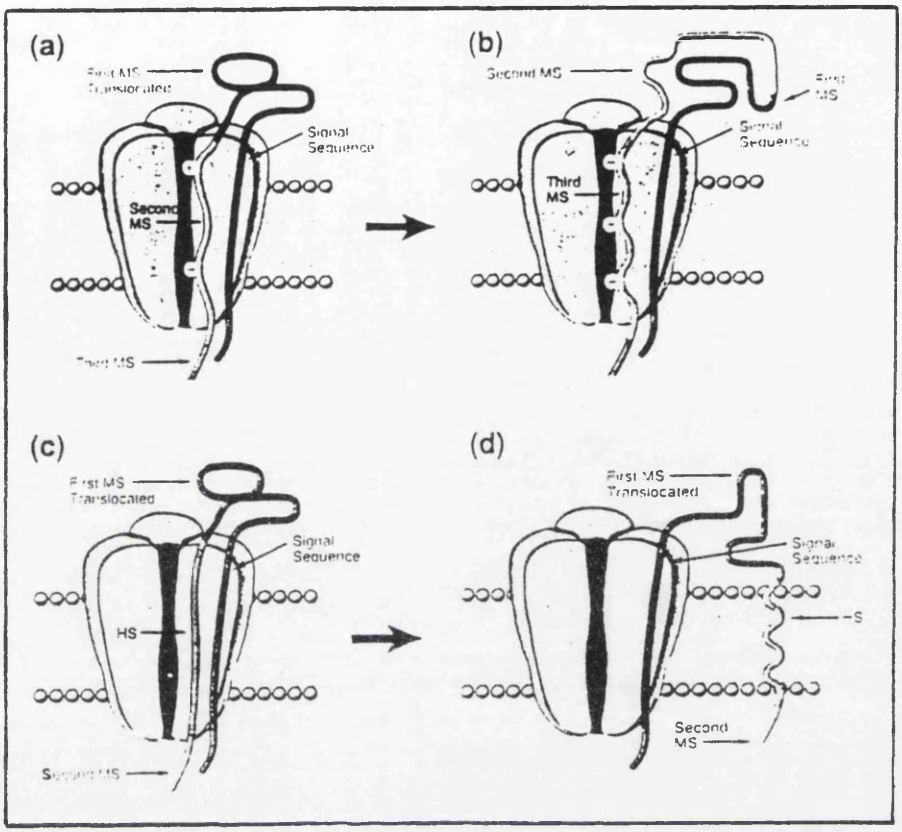


Figure 1.2 - Schematic representation of a mechanism for translocation of a soluble polypeptide across the protein-conducting pore in the ER membrane. The transition from **a** to **b** represents the translocation of a nonhydrophobic sequence vertically across the pore to the opposite side of the membrane (secreted proteins). The transition from **c** to **d** represents the translocation of a hydrophobic sequence horizontally into the lipid bilayer (integral membrane proteins)(From Singer and Yaffe, 1990).

1.4 The length of the transmembrane region

As mentioned before the transmembrane region is one of the stop transfer sequences of a polypeptide chain; the orientation of a membrane protein within the membrane would depend on a direct interaction of the protein with the lipid bilayer. Blobel (1980) characterized these segments as at least 20 uncharged amino acids with a largely hydrophobic character. Tanford (1978) and von Heijne (1981) pointed out that a 20 residue long segment in an α-helix gives a peptide 30 nm long, which is a sufficient length to penetrate the hydrophobic core of a membrane 3nm thickness. However, when transmembrane sequences of several membrane proteins are examined this length varies from 17 amino acids like HLA-B7 (Sood et al., 1985) and the β subunit of the T cell receptor (Hedrick et al., 1984), to 26 amino acids, like CD2 (Sayre and Reinherz, 1988).

Truncate forms of membrane proteins with short TM segments, showed different anchoring properties related to the different lengths. The bacteriophage f1 gene III protein (pIII) has a TM 23 amino acids long, deletions in this segment produced mutants with TM sequences of 4,6,11,12,13,16,17,20 and 22 amino acids long (Davis et al., 1985; Davis and Model, 1985). A loss of anchor function was observed for the mutant 4 amino acids long, mutants 6 to 13 showed a residual membrane association , and mutants 16 to 22 were still able to anchor the protein as the wild type. Adams and Rose (1985b) in a similar experiment, shortened the TM region of the VSV glycoprotein from 20 amino acids long (wild type) to 18, 16, 14, 12 and 8 amino acids. By indirect immunofluorescence it was observed that the mutants 18 to 14 were transported to cell surface and the others (12 and 8) seemed to span intracellular membranes, because they showed a Golgi-like region stainning pattern. From these two deletion experiments it was concluded that a TM consisting of 14 residues is able

to stabilize a protein membrane interaction and it is also possible to postulate that if the membrane has fixed dimensions, the shorter TM domains have a more extended structure than a α-helix (Adams and Rose, 1985b), on the other hand, the membrane could be locally deformed to accomodate a shortened hydrophobic core (Davis et al., 1985).

2. Processing of polypeptide chain into the ER

2.1 Protein folding

To be transported and to become functional, most of the proteins have to acquire a mature form, and a series of events are responsible for cleavage, folding, oligomerization and covalent modifications. Most of the oligomeric proteins have to oligomerize to become "transportation competent" to be correctly transported through the secretory pathway. Anfinsen (1973) demonstrated the spontaneous refolding of ribonuclease *in vitro*, and established that amino acid sequence dictates the final conformation of a protein. Several studies of refolding *in vitro* suggests that this process is initiated by collapse of hydrophobic regions into the interior of the molecule, forming the prefolded state, formation of stable secondary structure, and formation of covalent interactions, the disulphide bonds, which stabilize the polypeptide in particular conformation. These events result in an intermediate state, with significant secondary structure, but it lacking the well defined tertiary structure. Although, there is some evidence to support this mechanism, it is important to notice that *in vitro* experiments do not reflect the real folding process of a nascent polypeptide chain in a cell. In comparison to folding *in vivo*, refolding *in vitro* is very inefficient, it requires different protein concentrations and physicochemical conditions involving the whole polypeptide chain.

As discussed before, the translation of most proteins of the secretory pathway in mammalian cells occurs on membrane bound ribosomes. According to the signal hypothesis, the folding process, in most of the proteins, should occur posttranslationally. It is postulated that the folding of a protein can not occur sequentially as the amino acid residues emerge from the ribosome, but only when the complete protein is available. This applies to some proteins such as in rat serum albumin and mouse light chain, where intrachain or interchain disulfide bonds were shown to be formed when folding is near completion (Bergman and Kuehl, 1979; Peter and Davidson, 1982). However, in the case of larger proteins, when the carboxy-terminal is still being synthesized, the amino terminal domain may have already start the folding process (Brackman et al, 1991; Wettlaufer and Ristow, 1973).

An efficient folding process requires an antifolding activity, that prevents folding during synthesis and before translocation. There are a series of polypeptide chain binding proteins, also called molecular chaperones (Ellis, 1987; Ellis et al, 1989) that have a wide range of functions like stabilization of unfolded polypeptide chains before translocation, rearragement of protein oligomers, dissolution of protein aggregates and degradation of rapidly turned-over cytosolic proteins. They can be divided into three families, hsp70, hsp90 and chaperonin (groEL/hsp60), all of them are found both in eukaryotes and prokaryotes (Gething and Sambrook,1992)

The hsp70 family, in mammalian cells, consists of cytosolic proteins hsp70, hsc70 (p73, clathrin uncoating ATPase), prp73 (peptide recognition protein 73) and the ER protein BiP (grp 78). They are strongly inducible by heat shock and they contain typical amino-terminal targeting sequences that

directs their sorting to the correct membrane compartment. They exhibit a conserved mechanism of action, in the way that they present a differential recognition of targeting polypeptides, modulating either their conformation or state of assembly, they imply an ATP binding and/or hydrolysis interaction, when it is the case, they require other heat shock proteins or cellular factors and depending on the cellular compartment, the accumulation of unfolded proteins can cause induction of synthesis.

The Hsp70 proteins present in the lumen of the ER, interact with nascent polypeptide chain, maintaining them in a loose conformation. This was confirmed by Beckman and colleagues (1990) who found , using antibodies direct against hsp70, that newly synthesized chains associated with expressed hsp70 in the cytosol of HeLa cells. That it also maintains protein precursors in a translocating-competent conformation, destined for translocation across the ER membrane (Chirico et al, 1988; Deshaies et al, 1988; Zimmerman et al, 1988).

The lumen of the ER contains an hsp70 protein type which was firstly identified by its association with immunoglobulin heavy chain in pre-B cells. It is known as BiP, for binding protein (Haas and Wahl, 1983) and as grp78, because it is induced by glucose starvation (Munro and Pelham, 1986). It is retained in the ER against secretion because of the KDEL motif in the C-terminal sequence (Munro and Pelham, 1987). It has an important function in oligomeric assembly of proteins in the ER, proteins incompletely folded, modified or nonassembled in the ER are found as complexes with BiP. The accumulation of misfolded or mutants proteins in the ER induces synthesis of BiP (Kozutsumi et al, 1988, Normington et al, 1989; Rose et al, 1989). As a rule in the secretory pathway, proteins must fold and assemble before leaving the ER. So, the main function

of BiP could be either to retain normal proteins until assembly with other subunits is complete, as is the case of immunoglobulin heavy chains which remain bound to BiP awaiting the synthesis of light chain necessary for assembly (Bole et al, 1986) or to prevent abnormal proteins from leaving the ER by channeling them into the ER degradation system. There is also another protein that can act like a assembly factor, i.e., it is found associated with partially assembled oligomeric proteins in the ER and not in the final oligomeric structure. It is a 28 Kd polypeptide, TRAP, which interacts transiently with assembly intermediates of the T-cell receptor (Bonifacino et al, 1988).

Most of the members of Hsp70 family do not interact with defined sequences motifs, they present a broad pattern of polypeptide recognition, which allows them to participate in many cellular processes. Rothman (1989) suggested that Hsp70 proteins interact with chain segments only if they are part of unfolded structures, or if they extended as loops from folded proteins; they do not associate with native protein structures. Pelham (1986, 1988) proposed an interaction of Hsp70 with hydrophobic residues exposed by unfolded or partially denatured polypeptides, Flynn and colleagues (1991) point out that charged residues and prolines were excluded from the binding site. So the cytosolic hsp70 should bind to segments of nascent chain, preventing or delaying misfolding and aggregation during synthesis and maintaining the emerging sequences from the ribosome in an extended conformation, in order to produce after synthesis a stable loose conformation equivalent of the translocation-competent state.

Hsp 70 family members do not require ATP for binding, but their release from unfolded proteins or peptides is dependent on ATP hydrolysis (Flynn et al, 1989, 1991). Addition of ATP

to cell extracts causes dissolution of complexes between hsp70 proteins and polypeptide substrates, as demonstrated in hsp70 and heat-shocked nuclei (Lewis and Pelham, 1985), hsc70 and mutant forms of the cellular p53 protein (Clarke et al., 1988), and BiP and immunoglobulin heavy chain (Munro and Pelham, 1986).

Once the polypeptide chain has translocated the ER membrane, the folding process occurs quite immediately, it proceeds vectorially domain by domain from the amino terminal to the C-terminal. Assembly of most of the protein is essential to their exit from the ER, incorrectly folded proteins tend to aggregate. Sorting signals, that are involved with right transport of the proteins through the secretory pathway, maybe encoded in sequences that are only recognized in the final dimensional structure of the molecule. A sequence of events involving enzymes and protein factors, will take place in the ER lumen.

First of all, removal of the signal sequence by signal peptidase, as it was discussed before, may help in a correct and rapid folding (Randall and Hardy, 1986). Protein disulphide isomerase (PDI) is also an important enzyme in the folding process, because it facilitates formation of disulphide bounds (Freedman, 1987). This was observed by Bulleid and Freedman (1988) that restored cotranslational formation of disulphide bonds, in synthesized proteins using a cell free system, by reintroducing purified PDI into microsomes that have been evacuated of their lumen content by alkali or detergent treatment. The highly abundant and widely distributed proteins with peptidyl prolyl *cis-trans* isomerase (PPIase) activity, acts as "conformases", catalysing slow isomerization of X-P peptide bonds and can accelerate the refolding of proline containing polypeptides (Fischer and

Schmid, 1990). Addition of asparagine-linked (N-linked) carbohydrate side chain by oligosacharide transferase, a process known by glycosylation, allows correct folding of many proteins (Schlesinger and Schlesinger, 1987). The acceptor peptide (Asn-X-Ser) is glycosylated by addition of a $(Glucose)_3$-$(Mannose)_9$-$(N\text{-}acetylglucosamine)_2$ complex onto Asn residues. It seems to occur while the polypeptide is in an unfolded state, once the acceptor sites can be inacessible with the folding process. The glucose and mannose residues can be trimmed later on in the Golgi, by glucosidases I and II, and α-mannosidase, respectively (Kornfeld and Kornefeld, 1985).

2.2 Protein oligomerization

Many secretory and membrane proteins consist of multiple subunits,i.e., they are oligomeric proteins. The efficiency of subunit assembly is an essential feature of the oligomerization reaction and subunit polypeptides must fold correctly to bind specifically and stably. VSV G protein is composed of three subunits and mutations in the external and transmembrane domains of it prevent correct folding of the monomers, resulting in accumulation of large aggregates in the ER (Adams and Rose, 1985b; Doms et al, 1988). Other mutant G-proteins show that trimerization is necessary, but not sufficient for exit from the ER. Amino acid substituitions in the cytoplasmic domain, result in subunit assembly in trimers. However, the trimers were transported slowly and inefficiently from the ER, indicating that both domains must be correctly structured for efficient transport.

The T-cell receptor complex is composed by seven subunits, consisting of the disulphide-linked α–β heterodimer, the homodimer ζ–ζ, and the three noncovalently associated chains, CD3–γ, –δ, and –ε, all transmembrane polypeptides. In mature T

cells, these chains are expressed on cell surface as a complex with a minimum stoichiometry of $\alpha\beta\gamma\delta\varepsilon\zeta_2$. Prior assembly is required for its expression on the cell surface and it occurs through a series of intermediates in the ER, which are associated with TRAP, an assembly factor, prior to assembly (Samelson et al, 1985; Clevers et al, 1988). Only heptameric TCR complexes are correctly transported efficiently from the ER to the medial Golgi of T cells and targeted to the cell surface , the other partial complexes are degraded before reaching the cell surface (Sussman et al, 1988; Minami et al, 1987).

Class I MHC antigens are dimers, formed by a transmembrane heavy chain and a peripheral subunit, β2-microglobulin. The secretion of the heavy chain depends on its assembly with β2-M (Krangel et al, 1979; Owen et al, 1980). On the other hand, soluble β2-M can be secreted in the absence of heavy chain expression. An example supporting this observation was the constructed mutants of heavy chain, without cytoplasmic tail and transmembrane domain, (Zuniga and colleagues, 1983). These could assemble with β2-M and be secreted, indicating that the transmembrane domain is not important for the conformation of the extracellular domain. Miyazaki (1986a,b) analysed mutants of class I without heavy chain glycosylation sites or an internal disulphide bond. The assembly with β2-M intracellularly was observed in both cases, but secretion was inhibited. So assembly is required, but not sufficient for the transport of the heavy chains. A subunit can act as an assembly factor, but not be part of the final complex. This is the case of class II MHC antigens, that form a trimer in the ER consisting of α, β and invariant chains. The invariant chain is produced in excess and retained in the ER until assembly of α and β are completed, after transport to the trans-Golgi, it will be removed from the αβ complex (Owen et al, 1981).

In membrane proteins with a single membrane spanning sequence, extracellular and cytoplasmic domains fold independently of each other and remain structurally independent. Although a notable diversity is observed in the primary and secondary structures of extracellular and cytoplasmic domains, transmembrane segments show some similarity in length and hydrophobicity. Transmembrane proteins are folded in a stable state independent of the mechanism of insertion into the bilayer, and has an important role in mediating oligomerization, as it was demonstrated by Manolios and colleagues (1990) in studies with T cell receptor. It was observed that the association of the CD3 δ-chain subunit of the T-cell receptor with the α-chain is influenced by the transmembrane sequence of the latter one. In the case of multihelix transmembrane proteins, first each α-helix is folded in a stable conformation, influenced by hydrophobic effects and hydrogen bonding, then they assemble in a tertiary structure, which requires other factors like polar interactions, links between helices and to extra-membrane domains or other cytoskeletal proteins, interaction with prosthetic groups and packing effects (Bormann and Engelman, 1992).

2.3 Degradation system of the ER

Misfolded and misassembled proteins are produced continuously; the more complex the structure, the larger the fraction of misfolded and incompletely assembled side products. Cells have developed mechanisms that prevent mistaken structures from reaching the cell surface or surviving within the cells. These mechanisms are regulated either by retention of newly synthesized proteins within the ER or selective degradation of abnormal proteins. From these observations, the presence of a degradation system in connection with the ER was postulated.

The α-chain of the membrane protein nicotinic acetylcholine receptor was one of the earliest ER degradation system reported (Merlie et al., 1982). The degradation of the subunits of the TCR is much affected by their state of assembly, it seems that a selective degradation of unassembled or incompletely assembled TCR complexes ensures that only correct complexes reach the cell surface of T cells. Whereas the γ chain is rapidly degraded when expressed alone, the CD3 ε seems to be not suscetible to such degradation. However, when they are expressed together in fibroblasts, in an assembled way, they are quite stable and are retained in or close to the ER before their transport to the cell surface.(Bonifacino et al., 1989). On the other hand, when α , β and CD3 δ chains of human TCR receptor are expressed in fibroblasts, they are rapidly degraded by a pre Golgi degradative pathway, but when these subunits assemble with the ε and γ chains, their degradation is inhibited (Wileman et al., 1990). These observations suggests that two mechanisms exist to prevent transport of incomplete complexes to the cell surface: delivery of incomplete complexes to lysosomes and retention in the ER.

Lippincot-Schwartz and colleagues (1988) demonstrated the existence of an independent proteolytic system for rapid degradation in studies with α and β chains of T cell antigen receptor (TCR), which is insensitive to drugs that block lysosomal proteolysis. It was denominated "nonlysosomal degradative pathway", and serves as a more direct route for disposing of newly synthesized TCR chains; in this pathway proteins are degraded from the ER without transit through the Golgi. The site of this degradation system is uncertain, but should be either within or closely related to the ER, in a pre- Golgi compartment. Other systems of ER degradation were described, like H2 subunit of the asialoglycoprotein (Amara et al, 1989) and CD3-δ subunit in a cell hybridoma lacking the β

chain (Chen et al., 1988), that share the same characteristics of this pre-Golgi, nonlysosomal degradation pathway.

HMG-CoA reductase is a example of a ER membrane whose degradation can be regulated. Because it is the rate limiting enzyme for cholesterol biosynthesis, when the cholesterol levels are high, the protein is rapidly degraded. When the cholesterol levels fall, the stability of this protein is recovered (Jingami et al., 1987). Apoliprotein B, also degraded in the ER in a regulated way, depending on the nutrients conditions, is either secreted as a lumenal protein assembled into very low density lipoprotein particles or retained and degraded in the ER (Davis et al, 1990).

2.4 Retention of proteins in the ER

The mechanism of retention of proteins in the ER has been shown to be mediated by a linear stretch of amino acids which acts by binding to a specific receptor. This was first demonstrated by Poruchynsky and colleagues (1985) in one of the two SA11 rotavirus glycoproteins, it was assumed that the second hydrophobic domain should contain a retention signal. Although later Stirzaker and colleagues (1987) found out that the retention signal was in the hydrophilic portion of the N-terminal sequence. The transmembrane domain also can contain retention signals, Machamer and Rose (1987) observed that the IBV E1 glycoprotein is targeted to the Golgi membranes. It has three transmembrane segments and it was found by deletion experiments that the first domain has a retention signal. The cytoplasmic domain of an adenovirus E19 glycoprotein, a transmembrane glycoprotein retained in the ER, had 9 amino acids deleted. The mutant form was shown to be expressed on the cell surface. So it was assumed that this protein also had a retention signal at its cytoplasmic domain, although it was not shown to be transferable to other protein

(Paabo et al.,1987). None of these examples mentioned above exhibit an amino acid sequence that can be potentially recognized as a retention signal sequence. Munro and Pelham (1986) identified a C-terminal sequence KDEL on three lumenal resident ER proteins, grp78 (BiP), grp94 and PDI. When this sequence was deleted from BiP, a slow secretion of the protein from COS cells was observed and when it was transferred to the C-terminus of lysozyme, a secretory protein, the retention of this mutant in the ER of COS cells was verified. Proteins carrying this signal leave the ER, but they are retrieved from the Golgi or a pre- Golgi compartment. This sequence is both necessary and sufficient for retention. In yeast, this signal is modified to HDEL, proteins containing this signal, leave the ER, receive Golgi-specific carbohydrate modifications and are retrieved to the ER (Pelham et al., 1988). A receptor, encoded by the ERD-2 gene , mediate this recycling pathway (Semenza et al.,1990; Lewis et al., 1990). A mammalian ERD-2 homologue was identified by PCR (Lewis and Pelham, 1990). Jackson and colleagues (1990, 1993) described an ER targeting motif in some ER type I transmembrane protein (adenoviral protein E3/19K). Mutational analyses of the cytoplasmic tail of other membrane bound proteins demonstrated that two lysines positioned three and four or five residues from the C-terminus represent the retention motif. Arginines and histidines cannot replace lysines, suggesting that simple charge interactions are not sufficient to explain the retention.

A less specific mechanism appears to contribute to the retention of proteins that lack this linear signal. It was demonstrated that conformation of newly synthesized proteins determine whether they are transported or not out of the ER. As it was described before in the ER degradation section, misfolded and misassembled proteins normally are retained in the ER prior to degradation associated with BiP (binding protein).

2.5 Transport of proteins from ER, through Golgi to the cell surface

At any given time, the ER exhibits a large variety of newly synthesized proteins, some will remain retained in the ER, but the most of them will move to the Golgi. The transport of proteins through the secretory pathway seems to be a nonselective event, no specific transport signal has yet been identified. Palade (1975) proposed two mechanisms to explain both the specificity of protein export from the ER and the concentration of protein along the pathway. The presence of receptors, in the regions where vesicles form, which recognize positive sorting signals in the proteins to be exported. The other is a regulation process via negative sorting signal, so proteins necessary to the ER are retained. There are evidences that both mechanisms direct the rate of protein exit from the ER. Probably, proteins that exit the ER at high rates are concetrated at sites of vesicle formation, while others may move at a slower, bulk flow rate.

Fitting and Kabat (1982) studying expression on the cell surface of two viral glycoproteins, Lodish and colleagues (1983) studying rates of secretion of five proteins by rat hepatoma cells and Scheele and Tartakoff (1985) studying the exit of 12 proteins from the ER in the exocrine pancreas, found that different proteins exit from the ER at different rates. This could be explained by different affinities for cisternal surfaces of the ER, and also because one or more "receptors" localized in the ER bind proteins and concentrate them in specialized regions where transport vesicles form.

Wiedland and colleagues (1987) measured the rate of bulk flow, by adding tripetides, ^{125}I-labeled containing the acceptor

sequence for Asn-linked glycosylation, to CHO and HepG2 cells. It was found out that the half time for secretion was faster then that of known proteins transported through the same pathway and no signal is necessary for rapid and efficient transport from the ER to the Golgi, or from the Golgi to the cell surface, once this transport occurs by default. This argument against retention signals was criticized by Rose and Doms (1988), first because the glycosylated tripeptide might not be neutral, as required for bulk flow measurements, once it can interact with glycosidases. Also, because glycosylation affects protein folding and enhances the movement of proteins from the ER.

Ectoplasmic, transmembrane and cytoplasmic domains of membrane proteins should contain transport signals, so their correct folding should allow correct transport from the ER. There are some studies that give good examples of this events. Cytoplasmic domains are important in promoting transport of some but not all proteins. Rose and Bergman (1983) deleted residues from cytoplasmic domain of the VSV G protein and observed a large reduction on half-time for exit from the ER, although this was not completely blocked. However, deletion of almost all cytoplasmic domain of the Semliki Forest Virus E2 (Garoff et al., 1983), of the cellular H-2 protein (Zuniga et al.,1983), of the influenza HA (Doyle et al ,1986) or the Rous sarcoma virus glycoprotein (Perez et al., 1987) did not affect their expression on the cell surface. So it seems that there is no transport signal at the cytoplasmic domain , but because the deletion can reduce the half-time of exit from the ER, it probably can accelerate exit. But when the cytoplasmic domain is deleted and substituted by other amino acid sequence, a reduction on the transport rate to the ER is observed (Wills et al., 1984; Doyle et al., 1985; Shida, 1986; Gething et al., 1986), this suggests that an alteration on the sequence can affect the folding more than a deletion, resulting in a reduced transport rate.

Once the protein has assumed its right conformation, it is packaged into vesicles and transported to the Golgi apparatus, where further maturation, recognition and sorting of the protein will occur. The rate of protein transport through the secretory pathway varies. It was found that most of the delay is between the ER and Golgi, maybe because proteins need to interact with specific receptors in the ER, and carbohydrates might form part of the recognition signal. Also because proteins can only be transported from the ER when they fold and oligomerize in a transport-competent conformation, different time of folding and oligomerization are reflected in different times of transport.

The Golgi apparatus is composed by membrane stacks, which are arranged in three distinct cisternae, designated cis, medial and trans, according to their orientation with respect to the ER. Secretory proteins migrate from one end of the cisternae to the other, and this intra-Golgi movement depends on a "competent state for transport". Vesicle movement within the cisternaes is vectorial. Receptors on the surface are present to recognize vesicles budding from the preceding cisternae in the chain and vesicle fusion with the membrane of the acceptor cisternae occurs in order to release the contents into the lumen of the receptor cisternae. The cis-Golgi cisternae are where recognition and recycling of escaped ER proteins occurs. The proteins reach the trans-Golgi cisternae, from where different branches (constitutive, lysosomal, regulated, apical and basolateral) of the secretory pathway are formed, and the sorting events take place. This sorting of proteins is dependent on sorting signals, that also do not exhibit consensus sequence shared by proteins sorted in the same pathway, so again, these signals are provided either by secondary structures or conformational motifs instead of amino acids sequences (Pryer et al., 1992).

Transport of proteins between membranes can be either by the biosynthetic pathway (regulated and constitutive secretion) or by the endocytic pathway (lysosomal secretion), in both the formation of vesicles is required. Vesicle formation can be mediated by a protein coat, as clathrin coat (Brodsky, 1988); by cargo molecules (Roman and Garoff,1985) and by changes in the membrane organization (Sheetz and Singer, 1974). Newly synthesized lysosomal proteins carrying lysosomal sorting signals, determined by mannose-6-phosphate (M6P) residues on N-linked core oligosaccharides, will be recognized by a specific receptor, packaged into clathrin-coated vesicles, diverted from the constitutive pathway and enter the endocytic pathway for further delivery to the lysosome (Kornfeld and Mellman, 1987). The constitutive pathway has default sorting and involves the secretion of proteins that lack sorting signals, accumulated in transport vesicles without protein coats (Guiard, 1985; Orci et al., 1986).

Membrane protein sorting into vesicles is also dependent on signals, which are usually found in the cytoplasmic domain and they can be recognized by specific receptors of the sorting machinery, which guarantee their inclusion in the membrane of a budding vesicle. The inclusion of single-spanning transmembrane receptors into clathrin coated endocytic vesicles is dependent on recognition of a sorting signal by the cytoplasmic domain receptor. This was confirmed by deletion experiments on the cytoplasmic domain of transmembrane receptors that affected their endocytic uptake, Examples are LDL receptor (Lehrman et al., 1985), polymeric IgG receptor (Mostov et al., 1986), transferrin receptor (Rothenberger et al., 1987) and Fc receptor (Mieteinen et al., 1989).

3. The focus of the project

As reviewed above, many properties of a polypeptide chain and mature form of a protein are involved in the mechanism in which an integral membrane protein is inserted into the membrane, translocated and transported to the cell surface. This project will focus on the two classes of topological determinants in membrane proteins, the transmembrane segment and the adjacent positively charged hydrophilic segment.

Previous work showed by immunostaining that viral membrane proteins with short TM segments are still able to span the membrane.(Davis et al, 1985; Adams and Rose, 1985b; Doyle et al., 1986). It was pointed out that either the α-helix would extend its structure or the lipid bilayer would deformed to accomodate the hydrophobic core. However, little is known about the membrane anchoring properties of eukaryotic membrane proteins with short transmembrane domains. In particular, the ability of the protein to diffuse laterally in the lipid bilayer. This should contribute for the knowledge about associations of the α-helix and the lipid bilayer.

A second area of uncertanty concerns the role, if any, of the positively charged amino acid segment adjacent to the transmembrane region at the begining of the cytoplasmic domain. Some claim that this charge influences in the orientation of membrane proteins into the cell membrane by directing their own cytoplasmic localizaton. In proteins with one spanning domain, the signal sequence and hydrophobic segment, acting as start and stop-transfer signals, could already determined the orientation of the molecule into the cell membrane. Thus the positively charged segment could only be involved in the stabilization of the protein into the membrane. Most of the data presented until now is based on deletions and insertions of positive charges in critical

locations. The substitution of this positive charged residues for something less positive, would help to clarify the importance of this charged segment in membrane protein orientation.

It was proposed for this project, that a series of mutants in the transmembrane and cytoplasmic region of an eukaryotic membrane protein would be created. Deletions on the transmembrane domain and substitution of the positively charged amino acids adjacent to the C-terminal of the transmembrane sequence for something less positive would be carried out. The purpose of such experiments would be to obtain more information about the minimal length of a transmembrane region and the role of the positive charged segment in a eukaryotic system.

As the CD2 system was already established in the laboratory where the project took place, the CD2 molecule was chosen as a model to study membrane protein structure. The strategy devised to develop this project was to create by mutagenesis truncate forms of CD2, shortening gradually its transmembrane region and substituting the positively charged segment. The localization of the mutant protein would be assayed by panning, to verify surface expression. Indirect immunostaining with a panel of antibodies against different regions of CD2 external domain would bring information about surface and intracellular localization. The interaction and mobility of the mutant protein in the lipid bilayer would be investigated by photobleaching analysis. To determine if these mutant proteins will reach the cell surface in a functional state, rosetting experiments and immunostaining using antibodies to different CD2 extracellular domain epitopes would be carried out to check the integrity of such domain.

4. CD2 as a model structure

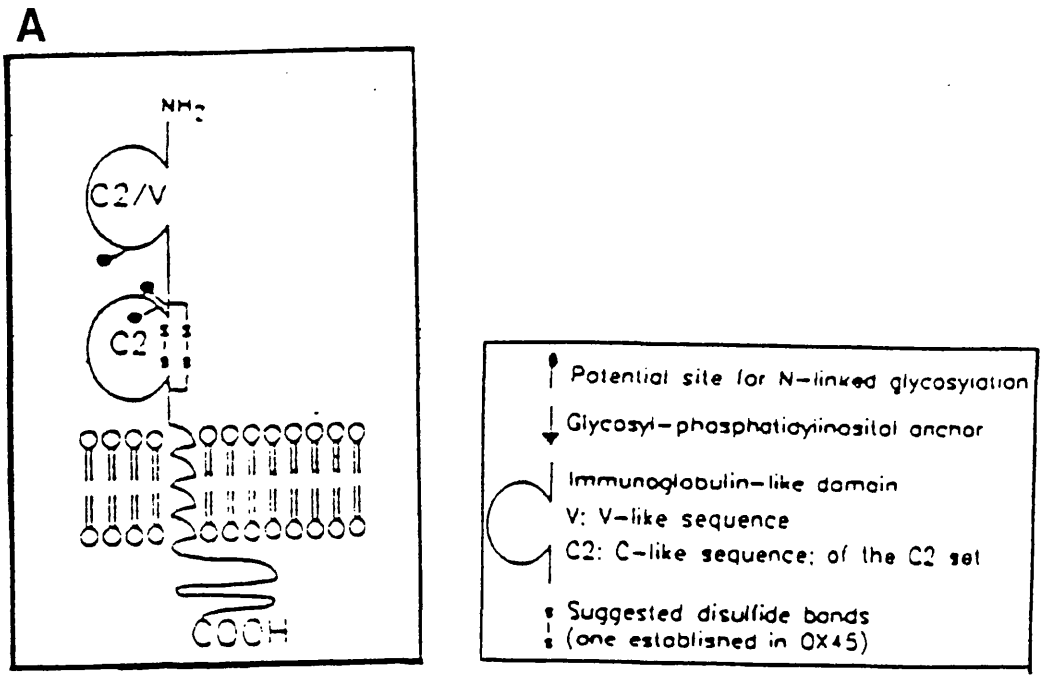
CD2, the T-lymphocyte adhesion receptor, is a non polymorphic glycoprotein, also known as T11, LFA-2 and E rosetting receptor. It was originally denominated the sheep red blood cell receptor by its ability to form spontaneous aggregates (rosettes) with sheep erythrocytes (Brain et al., 1970; Coombs et al., 1970; Lay et al., 1971). Human CD2 enconding cDNAs were isolated by screening a expression library with antiserum against the purified, denatured CD2 antigen (Sewell et al., 1986), by selection of CD2 epitope bearing COS cells transfected with pools from a plasmid expression library (Seed and Aruffo, 1987) and through the use of oligonucleotide probes based on NH_2-terminal protein sequence (Sayre et al.,1987). Murine (Sewell et al., 1987) and rat (Williams et al., 1987) CD2 have also been cloned and sequenced. After removal of the signal peptide that is 24 amino acids long, their sequences predict proteins of 327 amino acids for human CD2 and 322 amino acids for murine and rat CD2. The CD2 gene is located on human chromosome 1 and murine chromosome 3 (Richardson et al.,1988; Ruddle et al.,1988). It is composed of five exons separated by four introns, spanning 15 Kb of the genome (Lang et al.,1988; Diamond et al.,1988)

In human CD2, the 185 amino acids at the the N-terminus form the extracellular domain, 26 amino acids span the membrane as a transmembrane segment and the 117 amino acids at the C-terminus form the cytoplasmic domain, rich in proline and basic residues. Murine CD2 is 51% identical overall with the human CD2, it shows a higher degree of similarity with human CD2 cytoplasmic domain (59%) than with its extracellular (47%) or transmembrane (44%) domains. The histidine-proline cytoplasmic region is conserved within the 3 species of CD2, The unglycosylated protein is 36.9 KDa (Sayre and Reinherz, 1988), it contains three potential N-linked glycosylation sites in the mature protein (Bierer et al., 1989) which has a

relative molecular weight of approximately 45-55 KDa, as observed on SDS-PAGE.

Structurally, CD2 consists of two imunoglobulin superfamily domains (IgSF), the first one , denominated domain 1, includes residues 1 to 103 and domain 2, that exhibits two disulphide bonds,includes residues 107 to 180 (Fig 1.3A). A soluble two domain CD2 molecule ($T11_{ex2}$) of all 180 residues from the extracellular sequence and 2 residues of the transmembrane region has been produced in a baculovirus expression system (Sayre et al. 1989). Papain digestion of this segment produced a single-domain CD2 immunoadhesion molecule ($T11_{pap}$), which was purified and characterized (Recny et al., 1990). Circular dichroism analysis of both forms predicted the presence of α-helix in these segments, so a pattern of alternating α-helices and β-strands for the extracellular domains of CD2, suggesting that CD2 molecule should be included in an α-β protein folding class. Jones and colleagues (1992) reported the structure of the soluble extracellular region of CD2 (sCD2) determined at 2.8 Å resolution (Fig 1.3B). It consists of two antiparallel b-barrels with immunoglobulin folds, domain 1 has a similar topology to immunoglobulin variable (v) domain and domain 2 has both V-like and C-like features. These conclusion based on three dimensional data confirmed early comparisons based on primary sequence homologies of CD2 with immunoglobulin κ-chain variable region hypervariable sequences (Peterson and Seed, 1987), human CD4 domain I and IV (Williams et al.,1987) and other nonimmunoglobulin members of the immunoglobulin gene superfamily (William and Barclay, 1988). As is the case of LFA-3, BCM-1 (Kil .en et al., 1988) and CEA (Oikawa et al., 1987) the first domain lacks the disulphide bond.

Expression of CD2 is initiated in the thymus, and is found expressed on nearly all resting or activated human T



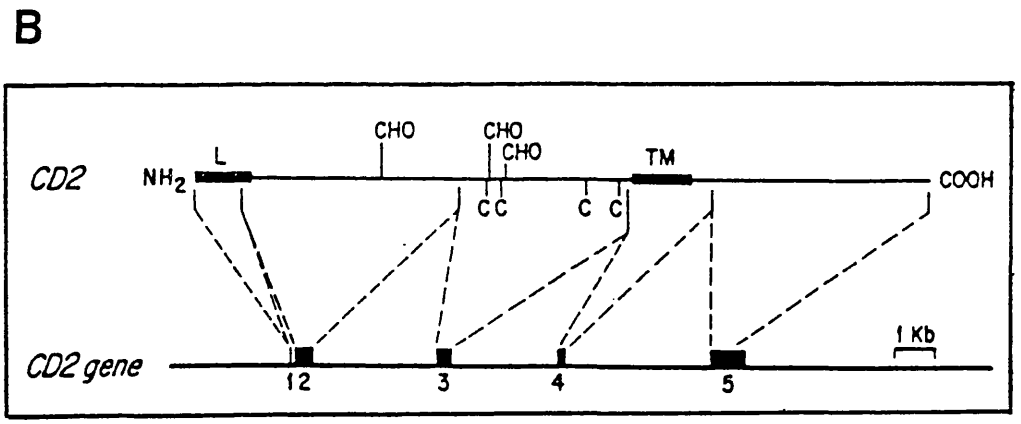
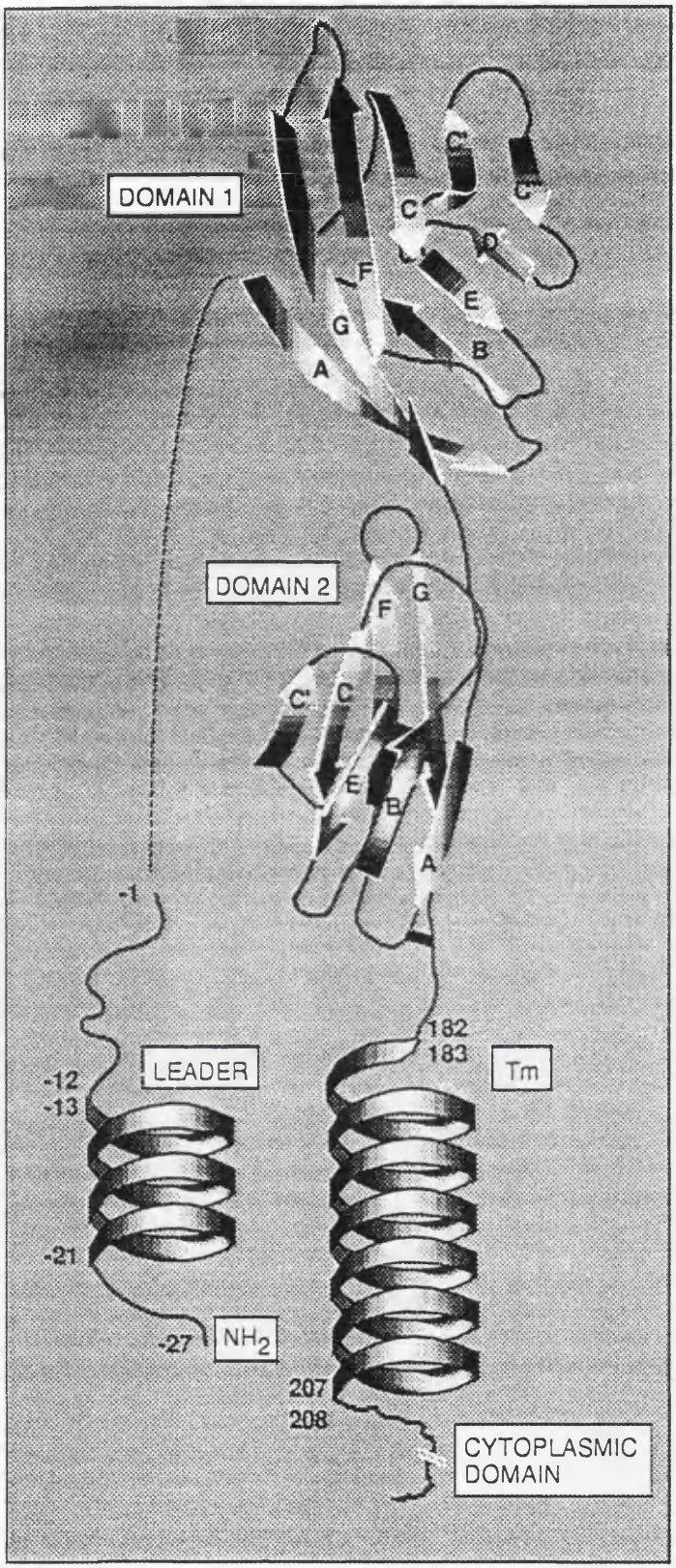


Figure 1.3A - A. Schematic structural diagram of the native CD2 molecule. B. Diagram of CD2 gene, exon 1 corresponds to CD2 amino acids -24 to -5, exon 2 to residues -4 to 103, exon 3 to residues 104 to 180, exon 4 to residues 181 to 221 and exon 5 to residues 222 to 327 (according to Sayre and Reinherz, 1988).

MOLECULAR STRUCTURE of HUMAN CD2



Structure of domains 1 & 2 is based on 2.8 angstrom structure of rat CD2 (Jones et al 1992, Nature 360: 232-239). The representation of the leader, Tm and cytoplasmic domains is notional. Residue numbers count from the start of the mature polypeptide (TNAL..).

Domain 1	
A:	3 - 9
B:	13 - 17
C:	27 - 34
C':	39 - 45
C'':	50 - 53
D:	57 - 60
E:	64 - 67
F:	76 - 84
G:	89 - 100

Domain 2	
A:	107 - 111
B:	116 - 120
C:	128 - 133
C':	136 - 140
E:	144 - 148
F:	157 - 163
G:	166 - 172

cys-cys bonds
119-159 (B-F)
112-176 (A/B-G/Tm

Figure 1.3B - Schematic diagram of CD2 external and transmembrane domain according to structure of the soluble, extracellular domain of rat CD2 (sCD2) described in Jones et al., 1992).

lymphocytes. One of its function is to allow thymocytes and mature T cells to adhere to thymic epithelium targetting cells through another cell surface protein, CD58 or LFA-3, a ligand for CD2 on human cells (Selvaraj et al., 1987: Dustin et al., 1989) that is widely distributed on endothelial cells, thymic epithelial cells and most of blood cells including erythrocytes (Krensky et al., 1983; Seed, 1987; Volger et al., 1987). This interaction, CD2/LFA-3, activated the thymocytes, promoting their differentiation. A second physiological ligand for CD2 was identified, CD59, a glycoprotein with complement-inhibitory function by monoclonal antibody inhibition studies (Deckert et al., 1992; Hahn et al., 1992a). By single amino acid substitution in region I and II, it was demonstrated that the binding site on CD2 for LFA-3 and CD59 are overlapping but not identical (Hahn et al, 1992a). The immunoadhesion functions of the CD2 molecule are likely to reside within the domain 1. Peterson and Seed (1987) by saturation mutagenesis studies, SRBC rosetting assays and using 16 anti-CD2 monoclonal antibodies, identified the epitopes for this antibodies related with three distinct regions on the extracellular domain. In domain 1, region I is centred about Lys^{48} and it is involved in T-cell activation and adhesion function; region II is situated around Gly^{90} and it is involved in adhesion. In domain 2, region III is situated around Tyr^{135}-Gln^{136} and is related with T-cell activation. CD48 is a related cell surface glycoprotein with a similar tissue distribution in rats to that of LFA-3 in humans. van der Merwe and colleagues (1993) shown that the rat CD 48 is a ligand for rat CD2 and that the N-terminal domain of the latter one binds CD48 with a lower affinity than CD2-LFA-3 interaction. The binding is independent of glycosylation, once the fusion sCD48-CD4 (extracellular domain of CD48 and domains 3 and 4 of rat CD4) binds in solution soluble forms of rat CD2 (domain 1 of CD2) expressed in bacteria.

CD2 also plays a role in the T cell activation pathway. The interdependence of CD3-Ti and CD2 molecules in the activation pathway of human T lymphocytes was demonstrated by induction of IL-2 gene and measurement of cytosolic Ca^{2+} in mutants which selectively express either CD3-Ti or CD2 on cell surface (Alcover et al.,1988). Brown and colleagues (1989) observed that cell surface CD2 molecules can be specifically co-precipitated in association with the CD3-Ti complex, suggesting that they are physically associated. The histidine and proline rich, 117 amino acids long cytoplasmic region of CD2 is required for T cell activation, deletions in this segment (Chang et al., 1989; He et al., 1988; Hahn et al., 1992b) and chimaeric constructs from rat CD2 cytoplasmic domain and rat CD4 extracellular and transmembrane sequences (Beyers et al., 1991) support this idea.

CHAPTER 2

MATERIAL AND METHODS

2.1 General material and methods

Bacterial strains

The bacterial strains used in these studies were derivatives of *Escherichia coli* K-12

DS941 — supE44, recF143, LacZ M15, lacIq derived from strain AB 1157.

MC1061/P3 — F^-, araD139, (ara-leu)7679, (lac)Y74, GalY, GalK,hsr^-,hsm^+,Str^r (Casadaban and Cohen,1980)

P3 is a single copy stably maintained 57Kb epis ome. It is derived from the "P" group of plasmids (Windass et al, 1980). It specifies resitantce to Km and carries amber mutated Tet and Amp resistance genes.

JM109 — endA1, recA1, gyrA96, thi, hsdR17 (r_k-,m_k^+), relA1, supE44,λ^-, Δ(lac-proAB), [F',tradD36, proAB, lacqZΔM15] (Hanahan, 1985).

BMH71-18 mutS — thi, supE, Δ(lac-proAB), [mutS::Tn10] [F',$proA^+B^+$, laqlqZΔM15](Kramer et al.,1984)

NOVABLUE — endA1, hsdR17(r_k^-,m_k^+), supe44, thi-1, gyrA96, relA1, lac,[F',proAB, lacqZΔM15, relA1, Tn10(tet^r)]recA1

Plasmids

pCDM8 is derived from the cDNA expression vector pπH3, which is derived from pπSV (Little et al., 1983). It is a 4.4Kb vector and it was constructed by inserting a synthetic transcription unit between the supressor tRNA gene and the SV40 origin. The transcription unit is a chimeric promoter, composed of the human cytomegalovirus (CMV) AD169 immediate early enhancer sequence fused to the HIV long terminal repeat (LTR). Immediately downstream from the LTR, there is a polylinker, and further downstream from this, the SV40 small tumour antigen and the splice and early region polyadenylation signals derived from pSV2 are located. The pCDM8 has no selectable markers for growth in *E. coli*, but it carries a supressor tRNA gene, supF. So , when it is transformed into MC1061/P3, the supF complements the amber mutations and confers Tet and Amp resistance to the cell.

pcDNAINeo (INVITROGEN) is an eukaryotic expression vector, 7.0 Kb long, derived from pcDNAI, which in turn is derived from pCDM8. Because it carries the neomycin resistance gene, it can be used for transient or stable expression of recombinant proteins in mammalian cell. It also offer an enhancer/promoter sequence from the Rous sarcoma virus long terminal repeat (RSV LTR) for high level transcription.

pT7Blue (NOVAGEN) vector was designed for cloning of PCR products, based on two observations, that many thermostable DNA polymerases, such as Taq DNA polymerase, leave single 3' A-nucleotide overhangs on their reaction products (Clark,1988) and these molecules can be ligated to a vector containing compatible single T-nucleotide overhangs (Marchuk et al, 1990). The vector was digested by EcoRV, generating two blunted ends to which T-nucleotides were added creating T-nucleotide overhangs. This EcoRV belongs to a polylinker situated in the LacZ α-peptide, so disruption of this peptide by the insert will produce white colonies containing the recombinat plasmid.

pAT153 plasmid vector(Twigg and Sherrat, 1980) is 3.6Kb long, it is a high copy variant of pBR322, because a HaeII fragment from Col E1 was removed.

pSELECT-1 (PROMEGA) vector is a phagemid, defined as a chimeric plasmid containing the origin of a single-stranded DNA bacteriophage. It contains a multiple cloning site flanked by the SP6 and T7 RNA polymerase promoters and inserted into the lacZ α-peptide. Cloning of a DNA insert into this polylinker results in inactivation of the α-peptide, what facilitates the selection of recombinants. Although it carries gene sequences for both ampicilin and tetracycline resistance, it is ampicilin sensitive because a frameshift was introduced into this resistance gene by removing the Pst1 site.

Microbial culture media

L-broth	10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose and 20mg thiamine, made up to 1 litre in distilled water and adjusted to ph 7.0 with NaOH.
L-agar	As L-broth, but containing 15g/l and no glucose.
SOC medium	20g/l tryptone, 5g/l yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10mM $MgSO_4$ and 2mM glucose.

Antibiotics

	Stock solution	Solvent	working concentration
Ampicilin	100.0mg/ml	Water	100.0µg/ml
Kanamycin	25.0mg/ml	Water	50.0µg/ml
Tetracycline	12.5mg/ml	50% ETOH	12.5µg/ml
G418	80.0mg/ml	Water	400.0µg/ml

Buffer solutions

TE	10mM Tris/HCl, 1mM EDTA, pH 8.0
10x TBE	0.90M Tris/borate, 0.20M EDTA pH 8.3
10x TAE	0.40M Tris/acetate, 0.05M EDTA pH 8.3

Organic solvents

Phenol — Phenol was equilibrated to pH 7.6 by extracting with 1M Tris/HCl pH 9.5 for several times until the 1M Tris/HCl solution reaches pH 7.6. 8-hydroxyquinoline (0.1%) was added as an anti-oxidant and aliquots of phenol were stored at -20°C, under 0.1M Tris/HCl pH7.6.

Chloroform — Analytical grade chloroform was mixed with iso amyl alcohol (24:1 v/v) and stored protected from light at RT.

Ethanol — Analytical grade ethanol and 70% ethanol used for DNA precipitation, were stored at 4°C.

Other solvents like , methanol, acetic acid, dimethylformamide, DMSO and isopropanol were of analytical grade, so they were stored at RT temperature and used directly.

Sterilization conditions

All growth media were sterilized by autoclaving at 120°C for 15 min. Supplements, buffer solutions and calcium chloride were autoclaved at 108°C for 10 min. Heat-labile reagents were sterilized by filtration through disposable 0.2µm filter units (COSTAR). Plasticware was sterilized by autoclaving at 120°C for 90 min and glassware underwent dry sterilization.

Microbial growth conditions

Liquid culture of bacteria, for transformation and DNA isolation, were grown in L-broth at 37°C with vigorous shaking. Plates cultures were incubated overnight at 37°C, antibiotics, X-gal and IPTG were added as required. Stocks of the bacterial strains were made by making the liquid cultures (1 ml) 15% (v/v) with respect to glycerol and storing in a tightly sealed vial at -70°C.

2.2 Preparation of plasmid DNA

Mini preparations of plasmid DNA

This preparation was used for characterization and subcloning, it was performed according to the lysis alkaline method, a modification of that described by Birnboim and Doly (1979).An overnight culture of 1.5 ml with plasmid containing clone in selective L-broth, was harvested by centrifugation (bench microfuge, 13Krpm, 40 sec, RT). The pellet was re suspended in GTE buffer and the cells were lysed by a treatment with a NaOH/SDS solution followed by 3.5M KAc. Chromosomal DNA and cell debris were precipitated by centrifugation (bench microfuge, 13Krpm,15min, RT). Contaminating proteins were removed by phenol extraction, the DNA was recovered by ethanol precipitation and further centrifugation (bench centrifuge, 13Krpm, 30min, 4°C).

Solutions

GET	NaOH/SDS	3.5M KAc
25mM Tris/HCL, pH8.0	0.2M NaOH	60ml 5M KAc
50mM Glucose	1% SDS	11.5ml Glacial acetic acid
10mM EDTA pH8.0		28.8ml distilled water

Large scale preparations of plasmid DNA

The DNA from this preparation was used for subcloning, double stranded DNA sequencing, as template for PCR reactions and in transfection experiments. This procedure is based on the modified alkaline lysis (mentioned above) combined with the use of QIAGEN resin columns to purificate plasmid DNA (Joanne Crowe, 1992). An overnight culture of 100ml L-broth with the required plasmid and selection, was pelleted by centrifugation (Beckman centrifuge, 5Krpm, 5min, 4°C). The pellet was ressuspended in buffer P1 and the cells were lysed using buffers P2 and P3. After centrifugation, to remove cell debris and denatured protein, the supernatant was added to the column, which has been previously equilibrated with buffer QBT. The contaminants of the preparation were discarded by washing the column with buffer QC. The DNA was eluted with buffer QF, a phenol extraction was included at this step. The DNA was recovered by precipitation with 0.7 volumes of isopropanol and centrifugation at 4°C for 30min. The pellet was washed with 70% ethanol and re suspended in TE pH 8.0.

Buffers

P1	P2	P3
100µg/ml RNase A	200mM NaOH	3.0M KAc pH5.5
50mM Tris/HCl	1% SDS	
10mM EDTA pH 8.0		

QBT (pH 7.0)	QC (pH 7.0)	QF (pH 8.5)
750mM NaCl	1.0M NaCL	1.25M NaCl
50mM MOPS	50mM MOPS	50mM Tris/HCl
15% ethanol	15% ethanol	15% ethanol
0.15% Triton X-100		

2.3 Transformation of *Escherichia coli*

Preparation of competent cells using $CaCl_2$ method

50 ml of L-broth were inoculated with 1 ml of an overnight culture and incubated with vigorous shaking until the OD_{600} reached 0.7. The 50 ml culture was put on ice for 10 min. The cells were harvested by centrifugation (Beckman centrifuge, 4Krpm, 5 min, 4°C). The pellet was resuspended on 20 ml of 50 mM $CaCl_2$, left on ice for 20 min and centrifuged again at the same conditions. At this stage, the pellet was resuspended on 2 ml of 50 mM $CaCl_2$ and 1 ml of 50% glycerol/$CaCl_2$, it was aliquoted and freezed immediately on dry ice. An aliquot of 300 µl was used in each transformation.

Transformation of DNA into competent cells

Vector DNA (20ng in 20µl) was added to an aliquot of competent cells, gently mixed and maintained on ice for 30 min. Following this, the cells were heat shoked at 42°C for 2 min and placed on ice for another 2 min. L-broth (1 ml) was added to the cells and incubation continued at 37°C for 1 hour. The cells were plated on selective L-agar plates and incubated overnight at 37°C. A selection of transformants from these plates were picked for DNA isolation and analysys.

In the case of NOVABLUE competent cells there was some modifications on this transformation procedures because they are high efficiency competent cells. An aliquot of 20µl of cells was used per transformation, they were heat shocked for only 40 sec and 80µl of SOC buffer was added to each aliquot. After 1 hour incubation at 37°C, another 300µl of SOC buffer was added, the cells were pla ted on selective L-agar plates with IPTG and X-gal indicators and incubated overnight at 37°C.

2.4 DNA purification procedures

Phenol/chloroform extraction to remove proteins from DNA samples

Contaminating proteins were removed from DNA samples by sequential extraction with phenol/chloroform (1:1 v/v) and chloroform/isoamyl alcohol (24:1 v/v). The DNA samples were mixed with equal volumes of these solvents by vigorous vortexing. Phase separation was enhanced by centrifugation in a bench microfuge for 15 min. Protein separated into the phenol phase while the DNA remains in the aqueous phase. Residual phenol was reextracted by chloroform and residual organic solvents dissolved in the aqueous phase were removed during ethanol or isopropanol precipitation.

Ethanol precipitation

This procedure was used in order to remove salts from the DNA and to concentrated the samples after preparation of plasmid DNA. A 1/10th volume of 2.5M sodium acetate pH 5.2 was added to the DNA sample, followed by two volumes of absolute ethanol. The mixture was stored at -20°C for 30 min and centrifuged for 15 to 30 min at 4°C (bench microfuge, 13Krpm). The excess salt was removed by repeatedly washing the pellet with 70% ethanol. The DNA pellets were vacuum desiccated and resuspended in TE pH 8.0.

Isopropanol precipitation

In this procedure no salt is required to induce precipitation, provided the DNA concentration exceeds 70µg/ml. Because it has the advantage of precipitating the DNA in a smaller final volume (0.6 vol), it is commonly used to concentrate DNA after large scale preparation of plasmid DNA, although it also precipitates proteins with higher efficiency. Isopropanol was added, at the required volume, to the DNA sample. The mixture

was vortexed and allow to stand at RT for 10 min, followed by centrifugation (bench microfuge, 13Krpm, RT, 15min). The DNA pellet was washed with 70% ethanol, to remove any trace of isopropanol and facilitate rapid dessication of the pellet.

2.5 DNA gel electrophoresis

Agarose gel electrophoresis

Agarose gels electrophoresis was used to separate and analyse DNA fragments. Electrophoretic mobility of linear double-stranded DNA is inversely proportional to $\log_{10}$ of the number of base pairs. Because there is a relationship between the logarithm of the electrophoretic mobility of DNA and the gel concentration, it is possible to resolve a wide size range of DNA molecules by using gels of diferent concentrations. The molecular weights of DNA fragments were estimated by comparison with DNA standard of known molecular weight, electrophoresed on the same gel.

Depending on the size of the fragments being separated and the degree of resolution required, agarose gels of diferent sizes, concentrations and applied voltage were used. The agarose was dissolved by boiling in 1X running buffer and EtBr (10mg/ml) was added to a final concentration of 0.5µg/ml. The DNA samples were mixed with 1/10th vol of 6X loading buffer prior to loading and electrophoresis.

6X Loading dye

0.25% bromophenol blue
0.25% xylene cyanol FF
30% glycerol in water

Visualisation and photography of the gels

DNA fragments were visualised by UV-induced fluorescence of ethidium bromide on short (254nm), or medium (302nm) or long(366nm) wavelength transilluminators. Gels were photografed using a polaroid camera loaded with polaroid 545 or 667 type lan film and fitted with a Kodak Wratten filter n9.

2.6 Isolation of DNA fragments from agarose gel

Purified DNA fragments were used for enzymatic manipulations and in PCR reactions. The success of these reactions depend very much on the quality of these fragments and in order to minimize the presence of impurities, ultra pure agarose was used and all gels were formed and run in TAE buffers, as the presence of residual borate ions from gels run in TBE can inhibit the efficiency of enzymatic reactions.

Electroelution

The digested DNA was run on a TAE gel and the required fragment was sliced from the gel under long wavelength UV light (to reduce photo-nicking of the DNA). The gel slice was placed in a buffer-filled (200µl)dialysis tubing, clamped and subject to electrophoresis at 100V for 30 min. By using a hand-held long wavelength UV light, it was possible to follow thw DNA migration out of the gel slice. When the DNA was observed to have migrated to the buffer, the gel slice was removed from the dialysis tubing, which was resealed, placed back in the gel tank and electrophoresis carried out for 2 min under reverse polarity to detach the DNA from the dialysis tubing. The DNA solution was transfered to an Eppendorf tube, extracted with phenol chloroform to remove any residual agarose and ethanol precipitated to concentrate the sample. The pellet was resuspended in the appropriate volume

of sterile water and an aliquot was run on a gel to check its concentration.

Isolation of DNA fragments by glass wool method

This glass wool method (Gannon et al., 1990) is recomended for fragments in the range 0.1Kb to 3.0Kb but the recovery can be lower for fragments above this size. Initial amounts of DNA can be as low as 20ng and the recovery yields a small volume of eluate containing purified DNA . The DNA fragment required was sliced from the gel (TAE) and transferred to a punctured Eppendorf microcentrifuge tube that had been plugged with 2-3 mm of siliconized sterile glass wool. This is placed on the top of other Eppendorf tube in a two tiered system and centrifuged for 10 min at 6Krpm in a bench microfuge. The eluate containing the DNA fragment was collected in the bottom tube, the recovery of the fragment could be verified by examining its fluorescence under UV illumination. In order to purify the fragment from any possible agarose, the eluate was extracted by phenol/chloroform followed by an ethanol precipitation to concentrate the sample.

QIAEX agarose gel extraction

The DNA fragment was excised from the gel in a minimum size gel slice and its size was determined by weighing. The gel slice was solubilazed by adding buffer QX1 (300µl/100mg of gel). The QIAEX resin was added and mixture by vortexing. To solubilize the agarose, it was incubated at 50°C for 10 min, vortexing it briefly every 2 min. The sample was centrifuged and the pellet was washed two times with buffer QX2, and once with buffer QX3. The pellet was dried and he DNA was eluated from the pellet by resuspending it in TE. After centrifugation the supernatant was collected and ethanol precipitated to concentrate the sample.

2.7 DNA manipulations

Digestion of DNA with restriction enzymes

Single digestions were performed using 0.2-5µg of DNA, 5 units of enzyme per 1µg of DNA , compatible react buffer at 1/10th of the reaction final volume. Normally they were carried out in a final volume of 20 µl at 37°C for 2 hours, unless otherwise stated. Multiple enzymatic digestions were carried out in the react buffer in which each enzyme retained at least 50% of its activity. If no compatible react buffer was found, it was digested for one enzyme, followed by a ethanol precipitation prior to the second digestion. The volume of restriction endonuclease added should be kept to less than 1/10th of the volume of the final reaction mixture, because the glycerol present in the enzyme storage buffer might have an inhibitory effect on the enzymatic digestion of DNA.

Ligation of DNA fragments

T4 DNA ligase catalyzes the formation of phosphodiester bonds between adjacent 3'-hydroxil and 5'phosphate termini in DNA. It is able to join fragments with either blunt or compatible cohesive ends. Theoretically, maximal ligation efficiency should result when the reaction contains equimolar concentration of the ends. However, cohesive end ligations require a molar excess of the insert (3:1), and blunt end ligations, being less efficient require five-fold molar excess of insert. The molar ratios were adjusted according to the molecular size of the vector and insert. Ligation reactions were carried out in a final volume of 10 µl, the total DNA concentration was 0.2µg/µl, 1 unit of ligase (BRL system) , 2 µl of ligation buffer (BRL system) and 1µl of 10mM ATP were added. They were incubated at the appropriate time and temperature depending of the nature of the fragments. Although its activity is optimal at 30°C, ligations between cohesive ends were carried out at 16°C, which should allow adequate annealing of the DNA. Blunt ended ligations were carried out at 4°C and it is

considerably less efficient than cohesive end ligations. Afterwards 1/10th of the ligation mixture was transformed into the required cell strain and plated onto selective L-agar plates.

Dephosphorilation of vector fragments

The vector was digested to completion with an excess of the required restriction enzyme. The digested plasmid was run on a gel and isolated by glass wool method, in order to avoid any uncut DNA. The fragment was treated with CIP (Calf intestine phosphatase) to remove the phosphate groups and avoid religation of the vector. The sample (1µg) was incubated at 37°C for 30 min, with 1 unit of CIP and 2 µl of CIP react buffer, in a final volume of 20µl. Another 1µl of CIP was added, followed by another incubation at 37°C for 30 min. To terminate the reaction the mixture was extracted with phenol/chloroform, then ethanol precipitated and res uspended in an appropriated volume of sterile water.

Filling recessed 3'ends of double stranded DNA

Filling of 3' recessed ends following digestion of DNA with restriction enzymes does not require either the inactivation of the enzyme or altering the buffer. A completed digest of DNA was incubated with 1µl of 0.5mM dNTP mix and 1 unit of Klenow polymerase, for 30 min at RT. The reaction was terminated by addition of 1µl of 0.5M EDTA and followed by phenol/chloroform extraction and ethanol precipitation.

2.8 PCR procedures

Oligonucleotides

The oligonucleotides were synthesised on an Applied Biosystem PCR Mate.

Oligo C 297721 - CD2 18 TM

5' ATA TGT GGA GGA GGC AGC CTC GTT TTC TAT ATC 3'

Oligo C 298696 - CD2 14TM

5' ATT GGC ATA TGT GGA GGA TTC TAT ATC ACC 3'

Oligo G 259949 - CD2 12TM

5' ATT GGC ATA TGT GGA TAT ATC ACC AAA AGG 3'

Oligo A 276056 - CD2 8 TM - Primer b

5' ATA TAT CCG ATG AGA TAG ATG TCC A 3'

Oligo G 248334 - CD2 8TM - Primer c

5' ATC TCA TCG GAT ATA TCA CCA AAA G 3'

Oligo A 298772 - CD2 10 TM - Primer b

5' ATA TAT CCG CCA ATG ATG AGA TAG A 3'

Oligo G 260354 - CD2 10 TM -Primer c

5'TCA TTG GCG GAT ATA TCA CCA AAA G 3'

Oligo A 276166 - CD2 +VE - Primer b

5' ACT CCT CTG TTG CTG TTG CTG GGT GAT ATA 3'

Oligo T 237916 - CD2 +VE - Primer c

5' TAT ATC ACC CAG CAA CAG CAA CAG AGG AGT 3'

Oligo T 257155 Primer d (pcDNAINeo, 2380 - 2397)

5' AA ATC TCT GTA GGT AGT 3'

Oligo G 288024 Primer a (pcDNAINeo, 2070 - 2086)

5' ATT GAC GCA AAT GGG CG 3'

Oligo CD2B7 CD2 (556-551)

5' CCA CCA GCC TGA GTG 3'

Oligo 234920 CD2 (817-833)

5' TCA ACC CCT CAG AAT CC3'

Purification of oligonucleotides

This procedure gives high yields of de-salted oligonucleotide longer than 20 bases in a quantity greater than 0.1 OD. To 360µl oligonucleotide in ammonium hydroxyde, 40 µl of 3M sodium acetate solution was added, to give 400µl of 0.3M sodium acetate solution containing oligo and 1.2 ml of absolute ethanol. It was mixed by inverting the tube, left at -70°C for 30 min and centrifugated at 4°C for 30min (13Krpm). The supernatant was removed as much as possible and the pellet was washed three times with 70% ethanol. The pellet was vacuum dried, resuspended in 100µl of water and stored at -20°C.

PCR reaction conditions

The PCR reactions were carried out in 1X react buffer, 0.2 mM dNTPs, 1µg of template, 1µM of each primer and 0.05 units of Taq polymerase in a final volume of 50µl. Otherwise specified in the text, the conditions described below were used to obtain the PCR products. The PCR reactions were performed in a Hybaid "Omnigene".

Denaturing	94°C	1:30 min	1 cycle
Annealing	50°C	0:30 min	
Extension	72°C	1:30 min	30 cycles
Denaturing	91°C	1:00 min	
Annealing	50°C	0:30 min	1 cycle
Extension	72°C	15:00 min	

2.9 Mutagenesis experiments

A.Altered sites™ *in vitro* mutagenesis system (PROMEGA)

This system consists of a mutagenesis vector pSELECT™-1 and a procedure for the selection of oligonucleotide-directed mutants based on the use of a second mutagenic oligonucleotide that restores ampicilin resistance to the mutant DNA during the mutagenesis reaction. Both mutagenic oligos are annealed to the ssDNA template at the same time, followed by synthesis of the mutant strand with T4 DNA polymerase and its ligation, linking the two. Afterwards, the mutant DNA is transformed into a repair minus strain of *E. coli* (BMH71-18 mutS), the cells are grown in the presence of ampicillin and a second transformation in JM109 ensures proper segregation of the mutant and wild type plasmid (Fig 2.1)

Preparation of phagemid single-strand DNA

The DNA fragment to be mutated was ligated to pSELECT-1. The ligation mixture was transformed into JM109 and plated on blue/white selective L-agar plates containing 15µg/ml tetracycline, 0.5mM IPTG and 40µg/ml X-GAL. White colonies from the recombinant phagemid and the control vector (it had the lacZ gene disrupted by removing the Pst-1 site within the polylinker) were grown overnight in 2ml of L-broth, containing 15µg/ml tetracycline. The next morning 5 ml of L-broth (15µg/ml tetracycline) were inoculated with 100µl of the overnight culture. After 30 min of vigorously shaking at 37°C, the culture were infected with 40 µl of helper phage R408, the incubation with shaking continued for another 6 hours. The supernantant were harvested by centrifugation, twice at 12Krpm for 15 min, and precipitated with 0.25 volume of phage precipitation solution. The mixture was left on ice for 30 min and centrifuged for 15 min at 12Krpm. The supernatant was aspirated and the pellet was resuspended in 400µl of TE. To lyse the phage, a chloroform/isoamyl alcohol extraction was carried out, followed by several phenol/chloroform extractions

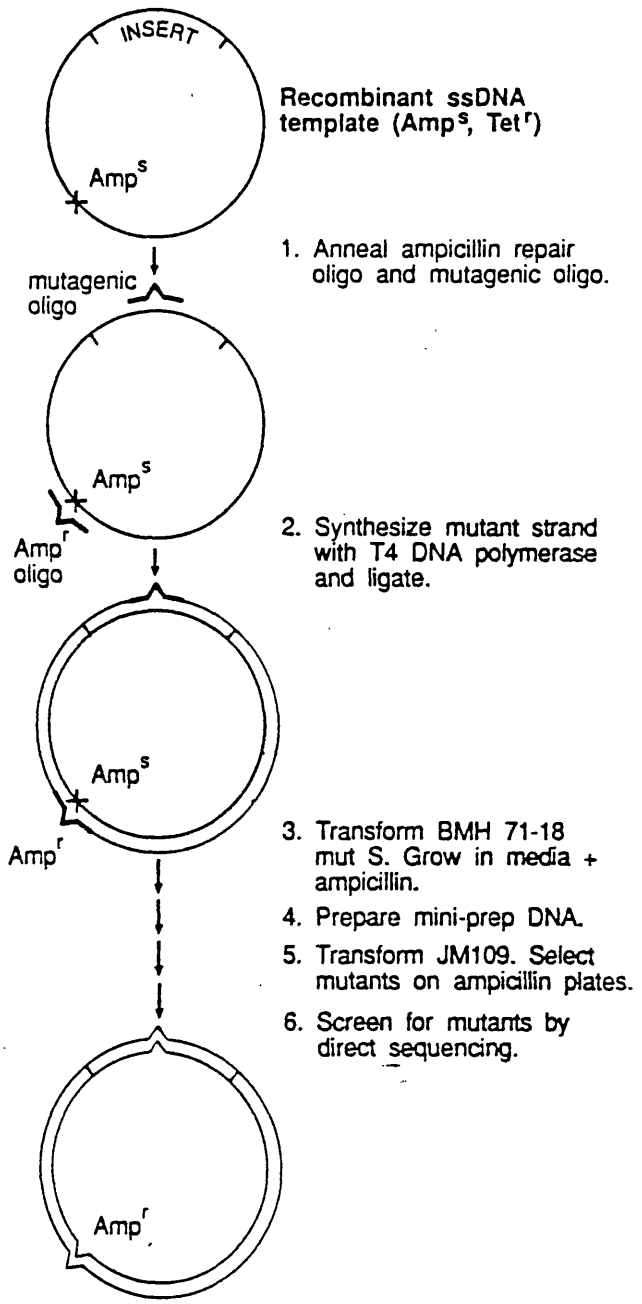


Figure 2.1 - Diagram of Altered sites *in vitro* mutagenesis (PROMEGA)

until the interface was clear. The upper aqueous phase was precipitated by adding 0.5 volume of ammonium acetate plus 2 volumes of absolute ethanol. The mixture was centrifuged at 12Krpm for 15 min, the pellet was washed with 70% ethanol and resuspended in 20µl of water.

5' Phosphorylation of oligonucleotides

The required oligonucleotide (100pmol), 1X kinase buffer and T4 polynucleotide kinase(0.2 units/µl) were mixed to a final volume of 25µl and incubated at 37°C for 30 min. To inactivate the kinase the reaction was incubated at 70°C for 30 min, the products were stored at -20°C.

Mutagenesis procedures

The mutagenesis annealing reaction were performed with 100ng of ssDNA(0.05pmol), 2.2ng(0.25pmol) of ampicilin repair oligonucleotide, 1.25pmol of phosphorilated mutagenic oligonucleotide and 1X annealing buffer in a final volume of 20µl. In the control reaction the mutagenic oligonucleotide was substituted by 10.8ng of lacZ control oligonucleotide, which was used to repair the lacZ gene function disrupted at the pSELECT-1 control vector. Both reactions were heated to 70°C for 5 min and allowed to cool slowly to RT. Subsequently, the annealing mixture was placed on ice and T4 DNA polymerase (10 units) and T4 DNA ligase (2 units) plus synthesis buffer was added to a final volume of 30µl. The reaction was incubated at 37°C for 90 min and transformed into BMH71-18 mutS, the cells recovered after 1 hour at 37°C, ampicillin to a final concentration of 125µg/ml was added followed by incubation at 37°C for another 14 hours.

An alkaline-lysis mini preparation of plasmid DNA was carried out with 2.0 ml of the overnight culture. The final pellet was resuspended in 50µl and half of this was transformed into JM109. The cells were plated on L-agar plates containing 125µg/ml ampicillin and incubated at 37°C for 16 hours.

Colonies were picked randomly, mini plasmid preparation and sequencing of mutants were carried out.

Phage precipitation solution
3.75M ammonium acetate, pH7.5
20% PEG-8,000

10X Kinase buffer
500mM Tris/HCl pH7.5
100mM $MgCl_2$
50mM DTT
1mM spermidine
10mM ATP

10X Annealing buffer
200mM Tris/HCl pH7.5
100mM $MgCl_2$
500mM NaCl

10X synthesis buffer
100mM Tris/HCl pH7.5
5mM dNTPs
10mM ATP
20mM DTT

B.Site-directed mutagenesis by overlap extension using the PCR

It consists in using complementary oligo primers that generate, by PCR, two fragments with overlapping ends (Ho et al., 1989). These fragments can be fused by denaturing and annealing, and reamplified by futher PCR reactions. As it is possible to generate mutations by incorporating changes into complementary oligos, the product from the second PCR reaction will be the mutated DNA fragment. The external primers (referred as primers a and d in the text) hybridizes at one end of the required sequence, and the internal primers (referred as primers b and c) hybridizes at the site of the mutated sequence and contains the mismatched bases (Fig 2.2).

The first PCR products AB (using primers a and b) and CD (using primers C and D) were obtained following the conditions described before for PCR reactions. The whole sample (50µl) was run on a gel and band purified by QIAEX method. The second round of PCR was carried out using 500ng of each first product as template and primers a and d, in order to purify the whole mutant fragment, the other reagents

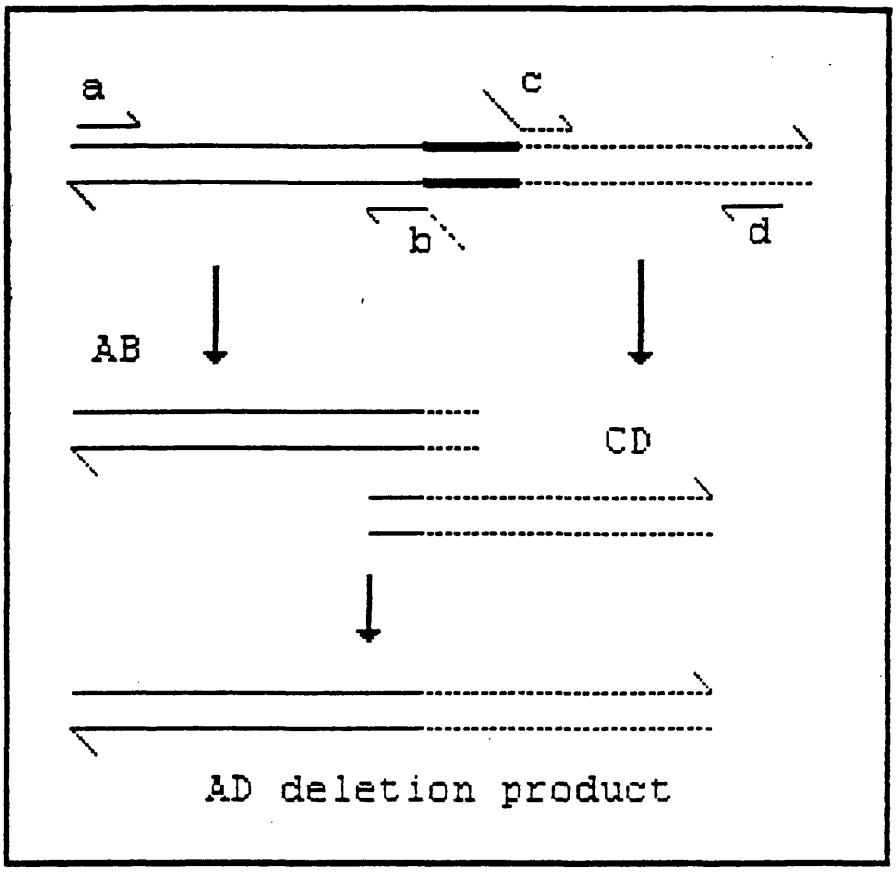


Figure 2.2 - Schematic representation of site- directed mutagenesis by overlap extension using PCR.

were kept at the same concentration. The AD product were band purified by QIAEX method and used for further ligations to the required plasmid.

Screening bacterial colonies by PCR

10µl from an overnight culture was diluted with 100µl of sterile water and boiled for 5 minutes to lyse the cells and denature DNases. The mixture was centrifuged for 1 min (microfuge, 12Krpm). An aliquot of 10µl from the supernatant was used as a template in 50µl PCR reaction, the conditions of the reaction were the same as described before.

2.10 Double strand DNA sequencing

This was carried out using the Sequenase Version 2.0 system (purchased from USB), described by Tabor and Richardson (1987). The system is based on the chain termination DNA sequencing method, which involves the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single stranded DNA template. The full method and reagents utilized are described in the Sequenase Version 2.0 protocols book.

Double stranded DNA (5µg) was denatured by incubating it with denaturing buffer (0.2M NaOH, 0.2mM EDTA) for 30 min at 37°C. The mixture was neutralized by adding 0.1 volumes of 3M sodium acetate followed by an ethanol precipitation. The pellet was resuspended in 7 µl of water, 2µl of reaction buffer and 1µl of required primer (1 pmol). The annealing step was done at 65°C for 2 min, the mixture was left to cool slowly to nearly 30°C. Once it has reached the right temperature, the labeling reaction was carried out by adding 1 µl of 0.1M DTT, 2 µl of diluted labeling mix (1:5), 0.5µl of [α^{35}S]dATP and 2µl of diluted Sequenase(1:8) to the annealing mixture and it was left at RT for 3 min. This labeling mixture was aliquoted to 4 tubes containing each 2.5µl of termination mix, a solution of diferent dideoxynucleotides (ddNTPs). The

reaction was stopped by adding 4µl of the stop solution to each termination reaction tube.

Analysis of the sequencing reactions was done on polyacrylamide gels (6%), run in 1X sequencing TBE at 1500V. The samples were heated to 100°C for 2 min before loading, and because of the two dyes(bromophenol blue and xylene cyanol) of the stop solution it was possible to monitor the electrophoresis. Usually the gel was run until the bromophenol blue band reached the bottom of the gel. The plates were dismantled, the gel fixed in 5% methanol/5% acetic acid for 15 min and dried under vacuum for 1 hour. The dried gel was placed in a cassete adjacent to a sheet of Fuji RX 100 film and exposed for 48 hours, at RT.

10X sequencing TBE buffer	1M Tris, 0.89M boric acid, 0.02M EDTA, pH8.3.
Acrylamide stock	40% (w/v) acrylamide/bisacrylamide (19:1)
Polyacrylamide gel 6%	15ml of 40% acrylamide, 50g of UREA, 10ml 10X sequencing TBE, 1ml of 10% APS, 20µl TEMED and 35 ml of water to complete 100 ml.

2.11 Mammalian cell culture

Cell lines

COS-7, derived from CV-1, transformed by an origin defective mutant of SV40, supports the growth of recombinant SV40 virus (Gluzman, 1981)

CHO.A2H, chinese hamster ovary cells

Cell culture media

For maintenance of the cells and transfections experiments, GMEM (Glasgow Modified Eagles Medium) medium was used. It was made from a 10X stock and the following supplements were added to 400ml of 1X media:

300.0ml	ddH_2O
40.0ml	10X GMEM
13.4ml	sodium bicarbonate (7.5% w/v)
4.0ml	sodium pyruvate (10mM)
4.0ml	non-essential amino acids (100X)
4.0ml	Penicillin/Streptomycin (10000 units/10000µg/ml)
4.0ml	L-Glutammine (200mM) (for COS-7 cells media)
	Proline (for A2H.CHO media)
40.0ml	FCS (Fetal Calf Serum) heat treated for 1 hour at 65°C

Non-essential amino acids (100X liquid)

	g/l
L-Alanine	0.89
L-Asparagine	1.50
L-Glutamic acid	1.33
Glycine	0.75
L-Proline	1.15
L-Serine	1.05

The media was always filter sterilized to remove large protein and lipid precipitates, prior to use.

Cell culture conditions

All the cells were grown as monolayers in plastic tissue culture flasks (base area 25cm^2) under 10ml of GMEM at 37°C in an atmosphere containing 5% carbon dioxide. When confluence was reached, the cells were diluted. The medium was aspirated, the monolayers were washed with 1XPBSA and the cells were detached by treating with 1X Trypsin/EDTA (Gibco BRL) for 1 min at 37°C. The cells were dislodged by tapping the flask, the trypsin was blocked by adding fresh medium to the flask.

Several dilutions (1:10, 1:20 and 1:50) were made from the cell suspension in 10ml of media and incubated at the usual grown conditions.

PBSA
8.0 gr of NaCl
0.2 gr of KCl
1.44 gr of Na_2PO_4
0.24 gr of KH_2PO_4
1000 ml of dH_2O

Transfection of mammalian cells using $CaPO_4$ method

This method is described by(Davis et al., 1986) and used only for stable transfection of A2H.CHO. Basically, the DNA is presented as a calcium-phosphate DNA coprecipitate to the cell. The cells were plated, 24 hours before transfection, at $3x10^5$ cells per dish (6mm), prior to transfection the media was changed. The coprecipitate is formed by mixing plasmid DNA (10µg), 0.125M $CaCl_2$ and 1X HBS in a final volume of 1ml, the mixture was left at RT for 30 min, to allow the precipitate to form. The precipitated was mixed by pippeting up and down once and added slowly to the cells. After 48 hours, the cells were trypsinized, plated in another two tissue culture dishes and G418 selection started.

2X HBS buffer
1.5 mM Na_2HPO_4
10mM KCL
280mM NaCl
12mM glucose
50mM HEPES, pH7.0

Transfection of mammalian cells using DOTAP method

This method consists in combine the plasmid DNA with the cationic lipid N- [1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimetyl-amoniummthylsulfate (DOTAP), in order to form a stable complex that will adhere to the cell surface, fuse with the cell membrane and release the DNA into the cytoplasm. The system used was purchased from Boehringer Mannheim Biochemica.

The cells were plated on 60mm tissue culture dish 24 hours before transfection at 10^5 cells/ml per dish (6mm). The DOTAP solution was diluted to 5µg/ml in HBS and added to 5µg of plasmid DNA resuspended in 100µl of HBS. The mixture was left at RT for 10 min and applied gently to the cells, which were incubated for 16 hours. Afterwards the medium was replaced by fresh medium and after 72 hours the cells were harvested (in the case of transient transfection) or put under G418 selection to obtain stable transfectants.

HBS buffer pH 7.4
20mM Hepes
150mM NaCl

Selection of stable transfectants

The mechanism of selection is based upon the cells sensitivity to the aminoglycoside antibiotic G418. The resistance genes are dominant and are located on both Transposons Tn601(903) and Tn5. The antibiotic affects the function of 80S ribosomes and protein synthesis. When the coding sequences for a resistance gene are arranged in a mammalian transcription unit to allow efficient expression and cointegrated with a desired gene, this chimeric gene confers resistance to G418 selecting for stable integration and expression of this desired gene, once it is stably integrated after transfection. The amount of G418 needed for selection was established by a cytotoxicity assay. Cells were plated in a multiwell plate (10^3 cells/well) and diferent concentrations of G418 (200µg/ml, 400µg/ml, 600µg/ml,

800µg/ml and 1000µg/ml) were tested. After 10 days, the cells were stained with Trypan Blue (0.4% solution) and the percentage of survival was calculated. For CHO the optimal concentration was 800µg/ml.

Stable transfection was performed on CHO cells, COS-7 cells are not appropriate for this purpose because they are transformed with an origen defective SV40 genome, which increases the efficiency of replication, overproducing foreign DNA sequences, that is poisonous to the cells, which can not tolerate high levels of extrachromosomally replication DNA. Because of this COS-7 cells were preferentially used in transient transfection, where they can produce high levels of foreign DNA in a short time. After 10 days of selection of transfected cells, clones were lifted using cloning rings and trypsin, and plated to multiwell plates were they were grown until they reached confluence. They were then passed to tissue culture flasks (25cm^2) and analysed. The frequency of transformed clone was calculated by the following formula:

$$\% \text{ Frequency of transformed clones} = \frac{\text{number of colonies after selection}}{\text{number of cells plated for transfection}} \times 100$$

2.12 Panning experiments

This method was based on the protocol described by Wysocki and Sato (1978).

Preparation of panning plates

Falcon bacteriological plates (100mm) were coated with affinity purified Goat anti mouse IgG (Seralab, 1mg/ml). The diluted antibody (10 ml of a solution of 3µg/ml in Tris/HCl, pH 9.5) was added to each plate and allowed to bind for 5 hours at RT. The plates were washed (3x) with 5ml of 0.15M NaCl, to remove the unbound antibody, and incubated overnight

at RT with 5ml of a 1mg/ml solution of BSA/PBS, to reduce non specific binding. Next day, the BSA/PBS solution was aspirated, the plates were sealed in plastic bags and stored at -20°C.

Panning cells

Transfected cells were harvested with PEA, the cell suspension was transferred to a falcon tube and pelleted (MSE minor S benchtop centrifuge, setting 3.5 for 4 min). The cells were resuspended in PEA/5%FCS in a concentration of $2x10^6$ cells/ml and antibody added (1:10 dilution for anti-CD2 and 1:50 for anti-W632). The tube was agitated gently and incubated on ice for 30 min, To prevent cells from settling, it was periodically agitated. In order to remove the unbound antibody, the cell suspension was submitted to a Ficoll density gradient. An equal volume of PEA was added to the cell suspension which was layered on an equal volume of a PEA/2%Ficoll solution. The cells were centrifuged at the same conditions and the supernatant containing the unbound antibody was removed by aspiration. The pellet was resuspended in 1ml of PEA and pipetted onto the panning plate containing 5ml of PEA/5%FCS. The plate was swirled gently to obtain an even spread of cells and left at RT for 3 hours. The unbound cells were washed with PBSA/5%FCS and the plates were analysed under a inverted microscope.

PEA 0.5mM EDTA
0.02% sodium azide
made up in PBSA

2.13 Rosetting experiments

Transfected cells were haversted with PEA, centrifuged and resuspended in 250 µl of PBSA/2% FCS. Sheep blood red cells (SBRC) were washed 2 times in PBS and incubated for one hour at 37°C with neuraminidase (0.2 unit/ml). The neuraminidase greatly enhances the SRBC specific binding and rosette

formation. After incubation the red cells were washed again 3 times with PBS and 250µl of a 0.5% suspension (v/v) was added to the cells. Firstly the mixture was incubated for 5 minutes at 37°C, centrifuged for 5 minutes (bench centrifuge, 1000rpm), so the cell membranes were tightly together, and incubated for one hour at 4°C. The pellet was gently re-suspended and fixed with 3% glutaraldehyde for 20 minutes on ice. The tubes were filled with water, inverted several times and centrifuged for 5 minutes (1000 rpm). The cells were stained with 0.4% Trypan Blue solution, mounted on slides and analysed on a inverted microscope.

2.14 Immunostaining of transfected cells

Preparation of the cells

Transfected cells were grown in slide-flasks. After 24 hours they were washed in PBS, the slide-flasks were dismantled and the cells fixed in PBS/acetone (3:2) for 5 minutes at 4°C, followed by several washings in PBS to remove any excess of acetone and left to dry at RT.

Immunofluorescent staining

The slides were placed in a humid slide box and wetted with TBS/BSA . The appropriate dilution of the respective primary antibody was made in TBS/BSA (listed below). The slides were drained and the diluted primary antibody was added (100µl/slide), followed by a incubation of 30 min at RT. Thereafter, the slides were washed three times in a circulating bath of TBS. The slides were drained and the second antibody conjugated to FITC, previously diluted in TBS/BSA , was added (100µl/slide). They were incubated for 15 minutes at RT, washed and dried as described before. A cover slip was put over the cells with a 50% glycerol/TBS(v/v),0.1% propidium iodide solution. The propidium iodide was added to help visualize the nucleus and damage cells. The slides were

analised under a fluorescent microscope (Leitz). Photomicrographs were obtained with a standard camera attachment and EKTAR 1000 ASA film. The transfection efficiency was calculated at this step, by counting the mean number of fluorescent green cells in a random field of view (minimum size of n=5) and expressing this as a percentage of total cell number, determined by the number of fluorescent red nuclei.

$$\% \text{ efficiency} = \frac{\text{number of FITC stained cells (green)}}{\text{total number of cells (red nuclei)}} \times 100$$

TBS
9.5g NaCl
50ml 1M Tris/HCl pH 7.5
q.s to 1 litre with distilled water

TBS/BSA
3mg/ml BSA
1XTBS

Primary antibodies used

Antibody	**Comments**	**Dilution**
W632 (Seralab)	monoclonal antibody against HLA-A, -B, -C. IgG2a (mouse)	1:50
CD2RFT11 (Brown et al., 1987)	linear antibody used for immuno-staining and Western Blotting	1:10
CD2 (Seralab)	IgG1 (mouse)	1:10
CD2GT2 (Huet et al., 1986)	IgG1 (mouse)	1:100

Secondary antibody used

Antibody	Comment	Dilution
Goat FITC (Seralab)	anti-mouse conjugated polyvalent	1:100

2.15 Western blotting analysis

SDS-polyacrilamide gel electrophoresis

SDS-PAGE gels were performed on discontinuos Laemli gels. The resolving gel (10%) was made up from 30% stock of acrylamide (acrylamide:bis ratio is 30:0.8) diluted in 0.575M TRIS pH 8.8/0.1% SDS and polymerised by the addition of 0.1% APS and 0.05% of TEMED. A solution of 0.1% SDS was overlaid to avoid entry of oxygen (inhibitor of polymerisation). Once this gel had polymerized, the SDS solution was removed from the surface, which was washed several times with water to remove any trace of SDS. The stacking gel (5%) was prepared by diluting acrylamide (30% stock solution) in 0.126M TRIS pH 6.8/0.1%SDS and also polymerized by addition of 0.1% APS and 0.5% TEMED, and poured on the top of the resolving gel. The samples were diluted in final sample buffer (1:2) and heated at 100°C for 10 minutes, prior to loading. In the case of the analysis of antibodies the non reducing-final sample buffer was used. The gels were always run on TRIS/Glycine buffer at 40mA for at least 30 minutes (depending on the molecular weight of the protein). A marker of known molecular weights (Prestained SDS-PAGE Standard Solution, Sigma) were loaded at the same gel.

Electroblotting

The electroblotting procedures were carried out according to Towbin et al.(1979), with some modifications. After electrophoresis, the gel aparatus was disassembled and the gel was placed in a tray with BEB (Blotting electrode buffer). The gel and nitrocellulose were sandwiched between 3MM Whatman

paper and placed in the electroblotting apparatus, with the nitrocellulose side facing the anode, as proteins from reducing gels are negatively charged and migrate towards the anode. The electroblotting reaction were carried out in BEB at 250mA with cooling and stirring for 1 hour. The sandwich was the disassembled, the nitrocellulose washed twice in TBS/Tween and the gel stained with comassie blue to determine the efficiency of transfer.

Blotting electrode buffer (BEB)
25mM Tris/HCL
192mM Glycine
20%(v/v) methanol

Comassie blue staining

After electrophoresis the gel were stained in a solution of 0.1% Coomassie blue (1 hour to overnight, depending on the concentration of the protein) and destained by washing the gel in a solution of 40% methanol/10% acetic acid, until the background had cleared.

Coomassie blue stain	Destain
0.1% Coomassie blue R-250	40% methanol
made up in destain	10% acetic acid

Immunodetection of the proteins

The nitrocellulose membrane was incubated for 15 minutes at RT in a solution of 5% dried milk/TBS-Tween to block the non-specific protein binding sites. It was rinsed in three changes of TBS/Tween. The primary antibody (CD2 RFT11) was diluted in TBS/Tween (1:10) and added to the membrane in a plastic bag. The bag was sealed and incubated with gentle shaking for 1 hour at RT. Following this, the membrane was washed as before, transferred to another plastic bag and incubated with the second antibody (alkaline phosphatase conjugated in 1:5000 dilution) for 15 minutes, shaking as before. After this incubation, the membrane was once more washed in TBS/Tween.In order to develop the blots, the blots the membrane was

incubated with AP (alkaline phosphatase) color development solution for 3-5 minutes, until the bands appeared. The membrane was immediately placed in the stop solution to avoid background.

AP color development solution
10ml AP buffer
66µl NBT (nitroblue tetrazolium) (50mg/ml, 8% methanol)
33µl BCIP (5-bromo-4-chloro-3-indolyl phosphate) (50mg/ml)

AP buffer	Stop solution	TBS/Tween
100mM Tris/HCl pH9.5	20mM Tris pH8.0	140 mM NaCl
100mM NaCl	5mM EDTA	10mM Tris
5mM $MgCl_2$	0.1% Tween 20	

2.16 Lateral mobility and fluorescence quantitation analysis

Preparation of F(ab)$_2$ fragment from CD2 MAb

This method is according to Harlow and Lane (1988), with some modifications because of the small quantity of antibody used. First the antibody was diluted with 20mM Na_2PO_4/10mM cysteine/10mM EDTA pH 6.2 buffer to a final concentration of 0.2mg/2ml and dialysed overnight against the same buffer. Next day, the dialysed antibody was incubated with immobilised papain (prewashed in the same buffer metioned before) for 5 hours at 37°C with rocking. After that, 10mM Tris/HCl pH 7.5 was added, the mixture was centrifuged and the supernatant was applied to a 5ml imobilised protein A column (previously equilibrated with 10 mM Tris/HCl pH 7.5). The column was washed with 1 ml of 10mM Tris/HCl pH 7.5, the eluted protein was the Fab fragment, To remove the Fc fragment and indigested IgG, the column was washed once more with 0.1M Glycine/HCl pH 2.8.

Labeling of F(ab)2 fragment with FITC

The eluate was concentrated by freeze drying it, until 1/10th of the volume (0.1ml), the concentration was calculated by measuring the absorbance, 1 OD_{280} of IgG is equal to 1.35mg/ml, and it was dialysed overnight in carbonate buffer pH 9.0. Fluorochrome isothiocyanate (FITC)/DMSO solution was added to a final concentration of 0.05mg/mg of antibody and incubated overnight at 4°C with rocking . The solution was dialysed for 2 days at 4°C in PBS, in order to remove the excess of unbound FITC.

Ammonium sulphate precipitation

This method is described by Harlow and Lane (1988), with some alterations. The antibody was diluted in saline, 1:2 (v/v) dilution. A saturated solution of ammonium sulfate pH 7.2 was added to bring the final concentration 45% saturation. The mixture was left stirring for 30 minutes and centrifuged for 15 minutes at 5000rpm, 4°C. The precipitate was washed with 0.5ml of ammonium sulphate 45% saturated solution and centrifuged again at the same conditions. The precipitate was re suspended in 0.5ml of PBSA and reprecipitated with 40% ammonium sulphate saturated solution. After centrifuged and washed the precipitate was re supended in 0.1ml of PBS and dialysed against 5 changes of PBS, overnight at 4°C. The integrity of the antibody was checked by running 10ml of the dialysed solution on a SDS-PAGE gel under non reducing conditions. The concentration of antibody recovered was given by absorbance measurements at 280nm using a uv spectrophotometer, 1 OD_{280} for mouse IgG is equivalent to 1.35mg/ml. The FITC labeling was carried out as described before.

Preparation of the cells and staining

Stable transfected CHO cells were transfered to slideflasks ($3X10^3$ cells/flask) 24 hours before the experiment and grown in media depleted of FCS. The slideflask was

dismantled, the cells were washed with warm media without FCS, the required antibody was diluted (1:10) in the same media, applied to the cells and left for 60 minutes. The slide was washed 4 times with warm media with FCS and mounted with 100ml of media.

For control measurements the slides were stained with succinyl concanavalin A FITC conjugated, that is a probe specific to mannose and glucose. The cells were treated with 1:10 dilution of 200µg/ml stock solution in PBS, and left for 30 minutes at 37°C. The cells were washed and the slide mounted as described before. Inhibitions assays with 0.2M Glucose and 0.2M Mannose solutions were performed to confirm the specificity of the probe. Cells were treated with these solutions and labeled with succinyl Concanavalin A as before. To monitor the damage due to handling of the cells during staining and washing processes, the cells were labeled with Hoescht probe and propidium iodide.

Fluorescence quantitation

The Leitz MPV compact consists of a photomultiplier tube and control panel which allows quantitation of fluorescence from a defined area, which was fixed at 400µm^2. For each group of readings an average background reading from a non fluorescence region of the slide was subtracted. The voltage across the photomultiplier was fixed at 500V and the mains current held constant by a Leitz steady current power pack.

Fluorescence Recovery After Photobleaching (FRAP) analysis

The biophysical technique of fluorescence recovery after photobleaching (FRAP) is used extensively in the study of lateral diffusion of plasma membrane components (Wolf, 1988). Fluorescence recovery after photobleaching (FRAP) denotes a method for measuring two-dimensional lateral mobility of fluorescently labeled molecules in regions of approximately 10µm^2 of a single cell. It is assumed that the molecules are in

constant movement within the lipid bilayer. A small spot (1.5μm radius) of the cell surface containing fluorescent molecules is photobleached, causing the movement of the molecules out from this spot. As the fluorophore is photobleached at a wavelength which does not destroy the molecule, the rate of recovery of fluorescence and lateral mobility which results from transport of the fluorophore into the bleached region, can be monitored and measured. In FRAP, the molecule whose diffusion is to be measured must be fluorescently labelled directly or indirectly in a non cross linking manner. A laser bean is focussed using a modified fluorescence microscope to a small spot (approximately 1.2μm in radius) on the sample. The fluorescent molecules within this spot were excited, the fluorescence was detected by a photomultiplier tube and the signal recorded. The laser bean was briefly unattenuated for approximately 70ms which served to increase the laser power by 10^4 fold and provide a bleach pulse. As a result of this pulse a proportion of the fluorophores (30-70%) in the spot were irreversibly bleached. This produced a reduced fluorescence intensity when examined, immediately after the bleach, with the attenuated laser bean. If there is no freedom of motion of molecules in and out of the spot, the fluorescence intensity will remain at this level ad infinitum, which is the condition of no diffusibility. If, however, there is complete freedom to diffuse in and out of the spot, the fluorescence intensity will return to the prebleach level. The diffusion coefficient is obtained by fitting the recovery data to diffusion theory (Axelrod et al., 1976). FRAP thus provides two measures of diffusibility: the fraction or percent of the molecules free to diffuse (%R or the percentage of recovery), and the diffusion coefficient of that fraction (D).

Instrumentation used in FRAP (Fig 2.3)

The laser (light amplification by stimulated emission of radiation)

A 1.0W LEXEL MODEL 85 Argon ion laser was used (Lexel Corp, Palo Alto, Calif.), with a tap water cooling system, and adjusted for maximal first order diffraction at 488 or 514.5 nm. Argon ion lasers produce the highest visible power levels and have up to ten lasing wavelengths in the blue and green portion of the spectrum. In addition to being monochromatic, laser light has a very defined mode structure. The fundamental mode, referred to as the TEM_{00} mode, is Gaussian in intensity profile.

The bean attenuator

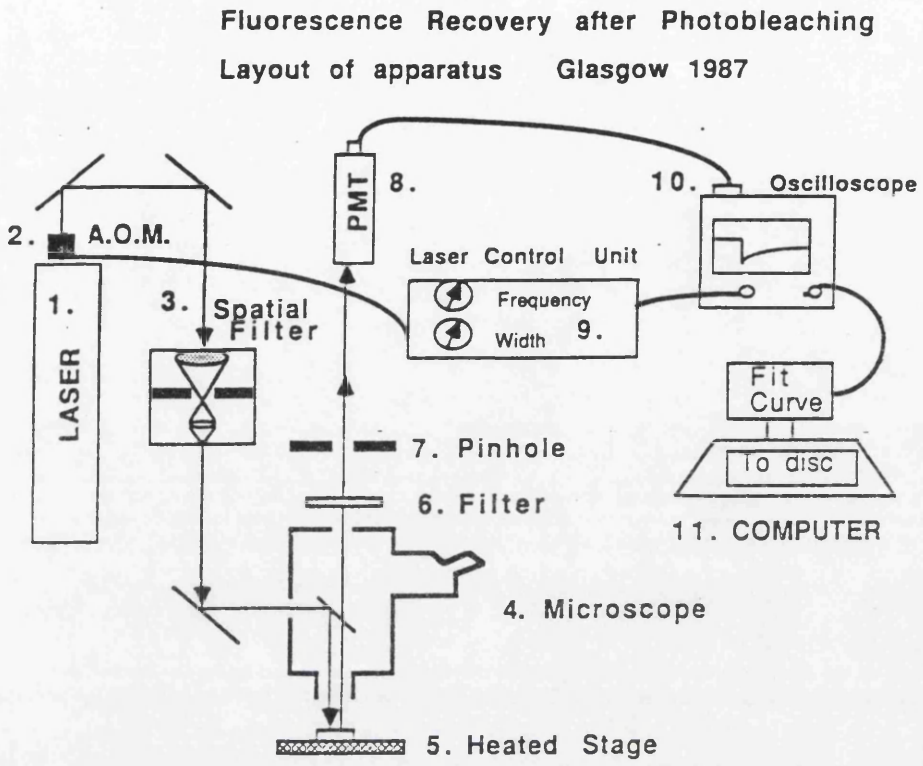
The bleaching and measuring beam intensities are controlled by a method which uses a digital acoust-optic modulator (Model 304D, Coherent Associates, Danbury, Connecticut) to attenuate the average intensity of the laser beam by variation of the on-off duty cicle. For example, to achieve an attenuation of 10^4 the measuring beam is delivered as 10 µs pulses at 10Hz, whereas bleaching is achieved by an uninterrupted pulse of 0.1-10s (Garland, 1981).

Spatial filter

The spatial filter was obtained from Photon control, Cambridge, U.K. Spatial filtering is simply an opto-mechanical method of separating the wanted beam from the undesirable noise, and then mechanically blocking the unwanted noise.

Fluorescence microscope

The Leitz microscope (Ortholux), contains two secondary image planes. The first of these being where the objective focusses the specimen to a real image. This is usually about 170 mm above the objective in an upright microscope. A second secondary image plane is located also about 170 mm beyond the objective, only along the imaginary path of a ray reflectd back by the dichroic mirror. If the laser beam is focussed



1. LEXEL MODEL 85 Argon ion laser HERTS. U.K.
2. Acousto Optic Modulator 304D COHERENT U.K.
3. Spatial Filter PHOTON CONTROL , CAMBRIDGE U.K.
4 Microscope LEITZ (Ortholux) , LUTON, BEDS. U.K
5. Heated Stage LEITZ.
6. Filters Leitz K530 , k590 .
7. Pinhole 0.5mm Dept Nat. Phil. Workshop Glasgow Univ.
8. PMT Photomultiplier tube Model 9924b THORN EMI U.K.
9. Control Unit Dept. Electronic engineering Glasgow Univ.
10 Oscilloscope DIGITAL STORAGE MODEL 4035 GOULD U.K.
11. Computer Hewlett Packard Model 82927A

Figure 2.3 - Schematic representation of the FRAP apparatus

onto this image plane, it will also be in focus on the object plane.

Optical components

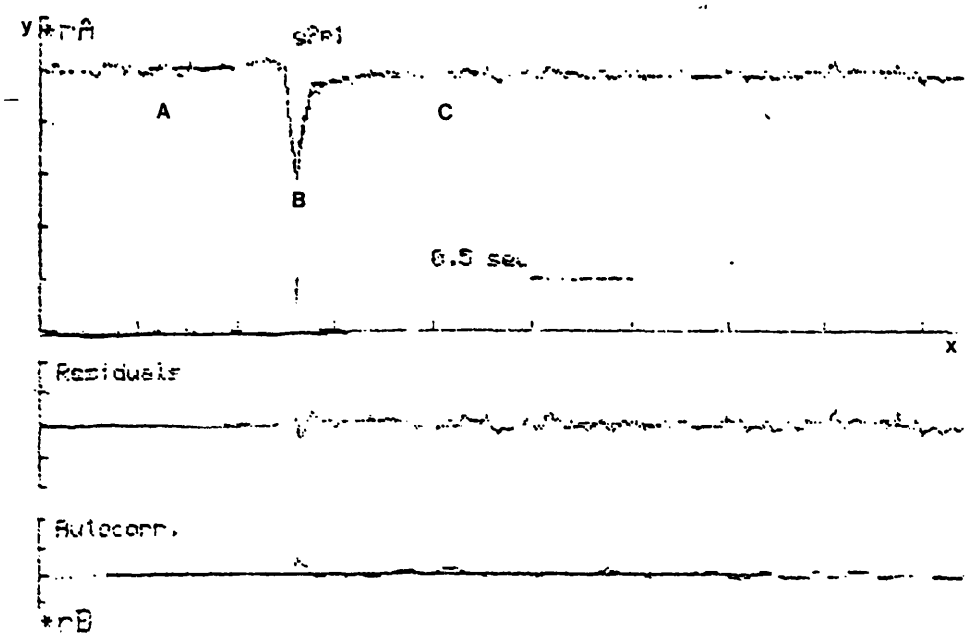
The complete FRAP apparatus is arranged on an optical table (Photon Control, Cambridge). Optical compnents are all mounted on optical rails (Oriel standard rails). Adjustable mirror mounts(Oriel model 1450) are used to align and direct the laser beam.

Control eletronics

The FRAP laser controller GUED 729 was designed and constructed by the Department of Eletronic Engineering, Glasgow University. The photomultiplier tube (P.M.T.) model 9924b was obtained from Thorn E.M.I., UK, and powered by a Brandenburg P.M.T. power supply. The signal from the P.M.T. was collected by a Coherent P.M.T. signal collector model 304. A Gould digital storage oscilloscope type 4035 was used to "capture" the signal which produces the FRAP curve. A Hewlett Packard Integral Personal computer model 82927A, and plotter model 7470A (supplied by Electroplan) were used to retreive the relevant information about the FRAP curve, using a Fortran program which was supplied by Dr John Birmingham.

Measuring the beam radius (spot size)

A variety of methods exists for performing these measurements, and the method of Thompson and colleagues (1980) was used. The photobleaching experiment is carried out on FITC-labelled BSA suspended in 95% glycerol at room temperature. A typical FRAP curve for FITC-BSA in 90% glycerol is shown in figure 2.4. The theoretical value for the diffusion of albumin in solution is 6×10^{-7} cm^2s^{-1} (Proudfoot, 1991). By taking this theoretical value and the viscosity of glycerol at 37°C, the predicted diffusion coeficient was estimated. This value was calculated to be $7.5 \times 10^{-9} cm^2s^{-1}$. This value was inserted into the diffusion equation:



X - time (ms)

Y - fluorescence intensity

A - Pre bleach fluorescence

B - Bleach pulse

C - Recovery of fluorescence

A random scatter of the residuals and their autocorrelation function around zero indicates a satisfactory fit of the calculated curve to the observations.

Figure 2.4 - FRAP curve for FITC-BSA in 90% glycerol.

$D_L = (w^2/4t_{0.5})\gamma$

Where γ and $t_{0.5}$ can be obtained from the recovery curve and w is the required beam radius.

Lateral diffusion coefficient (D) and mobile fraction (%R) measurements

Lateral diffusion and mobile fraction were measured by fluorescence photobleaching and percentage of recovery. The apparatus used, preparation of the cells and antibodies were described before. All measurements were made at room temperature within 120 minutes of staining and with the following conditions:

Pulse frequency : 384 Hz
Pulse width : 10 µs
Bleach pulse : 70 ms
Pulse time : 25 ms

The quality of the fluorescence staining of the cells was verified under UV microscope. The photobleaching experiment was carried out in a modified fluorescence microscope, where the fluorescence field was restricted to the laser spot. The cells and points in the membrane were chosen under normal light. After, the light source was covered and the spot could be visualized. Due to these limitations, the membrane points that would suffer bleaching had to be choosen randomly, without selection for intensity of fluorescence. An average of 4 measurements per cell were taken. The data was stored on a disk and subsequently transferred to a Dec 10 main-frame for running the curve-fitting programe in Fortran. The data that could not be fitted into a curve was discarded by the computer. Such data should be due to movement of the cell while receiving bleaching. For each mutant, the mean and standard deviation was calculated. As the values of D.L. and %R are not a typical normal distribution, the means were

compared by a non parametric Wilcoxon's rank signed test and a parametric student *t*-test.

CHAPTER 3

RESULTS

3.1 Introduction

Leading from the discussion in Chapter I, the experiments were to confirm observations on the minimal length of a Tm region is consistent with stable association with the cell membrane. Deletions in the CD2 TM region were therefore made, shortening it from 26 amino acids to 18, 14 , 12 , 10 and 8 aminoacids (Fig 3.1). The positively charged sequence, KRKKQR, at the beginning of the cytoplasmic domain of CD2, may play a role in anchoring the protein in the cell membrane. These amino acids were mutated to something less positive, QQQQQR, in order to demonstrate the importance of this segment in stabilizing the protein insertion into the cell membrane (Fig. 3.1). The construct CD2B7 (Hundal, 1991) was included in the experiments related with conformation of the external domain. It is an in-frame fusion of the external domain of CD2 to a foreign TM sequence (HLA-B7, 17 amino acids long), it has a "stalk" of 25 amino acids situated just before the TM region and a deletion of the last 8 amino acids from the CD2 external domain (Fig 3.1).

The mutagenesis was done by the altered sites^{tm} *in vitro* mutagenesis system from Promega and site-directed mutagenesis based on overlap extension using PCR. Cells were transfected with the plasmid constructs to verify the cellular expression, possible targeting, changes in conformation and

CD2 Transmembrane region 26 amino acids long (CD2 WT)

EPVSCPEKGLDIYLIIGICGGGSLLMVFVALLVFYITKRKKQRSRRNDEE

CD2 Transmembrane region 18 amino acids long (CD2 18TM)

EPVSCPEKGLDIYLIIGICGGGSLVFYITKRKKQRSRRNDEE

CD2 Transmembrane region 14 aminoacids long(CD2 14 TM)

EPVSCPEKGLDIYLIIGICGGFYITKRKKQRSRRNDEE

CD2 Transmembrane region 12 aminoacids long(CD2 12TM)

EPVSCPEKGLDIYLIIGICGYITKRKKQRSRRNDEE

CD2 Transmembrane region 10 aminoacids long(CD2 10 TM)

EPVSCPEKGLDIYLIIGGYITKRKKQRSRRNDEE

CD2 Transmembrane region 8 aminoacids long(CD2 8 TM)

EPVSCPEKGLDIYLIGYITKRKKQRSRRNDEE

CD2 with mutated positively charges (CD2+VE)

EPVSCPEKGLDIYLIIGICGGGSLLMVFVALLVFYITQQQQQRSRRNDEE

CD2B7 fusion

EPVREFPVVVPSGEEQRYTCHVQHEGLPKPLTLGWEPSSQGAVVAAVMGRRK

CD2 external domain — Stalk of 26 amino acids — TM

Figure 3.1 - Amino acid sequence of CD2 wild type and mutants with Tm region deleted from 26 amino acids to 18, 14, 12, 10 and 8 amino acids respectively, mutant with cytoplasmic positively charged stretch (KRKKQR) substituted (QQQQQR) and CD2B7 construct

mobility within the cell membrane of the CD2 mutant forms. The first approach was to verify surface expression of the mutants on transfected cells by panning experiments. Once this was established, the cellular localization of the mutant protein needed to be determined. Therefore, staining of the transfected cells with antibodies against CD2 and Western blotting were carried out. In order to show any alteration in the conformation of the external domain of CD2, the cells were stained with antibodies against different epitopes on the external domain. Also because this domain is related with the adhesion function of CD2, rosetting experiments were done to check the structure of the adhesion domain. The stability of the association of the mutants with the cell membrane was examined by photobleaching studies, where the lateral mobility and the mobile fraction of the molecule were analysed.

3.2 Construction of CD2 mutants

The CD2 mutants with Tm region 18, 14 and 12 aminoacids long were made by The Altered Sites ^{tm} *in vitro* mutagenesis system from Promega. CD2 full length cDNA fragment was obtained by digesting pCDM8CD2 (Hundal, 1991) with PstI and XbaI. The 1.5 fragment was band purified by electroelution and then ligated to pSelect digested with PstI and XbaI. The ligation was transformed into *E. coli* (JM109) by $CaCl_2$ method. The recombinants were selected on tetracyclin plates with white/blue selection. XbaI and PstI digestions were carried out to confirm the presence of insert (Fig 3.2). Single strand DNA from the recombinant pSelect plasmid were prepared, and oligonucleotide primers were used to synthesize the mutant double strand DNA (Fig 3.3).

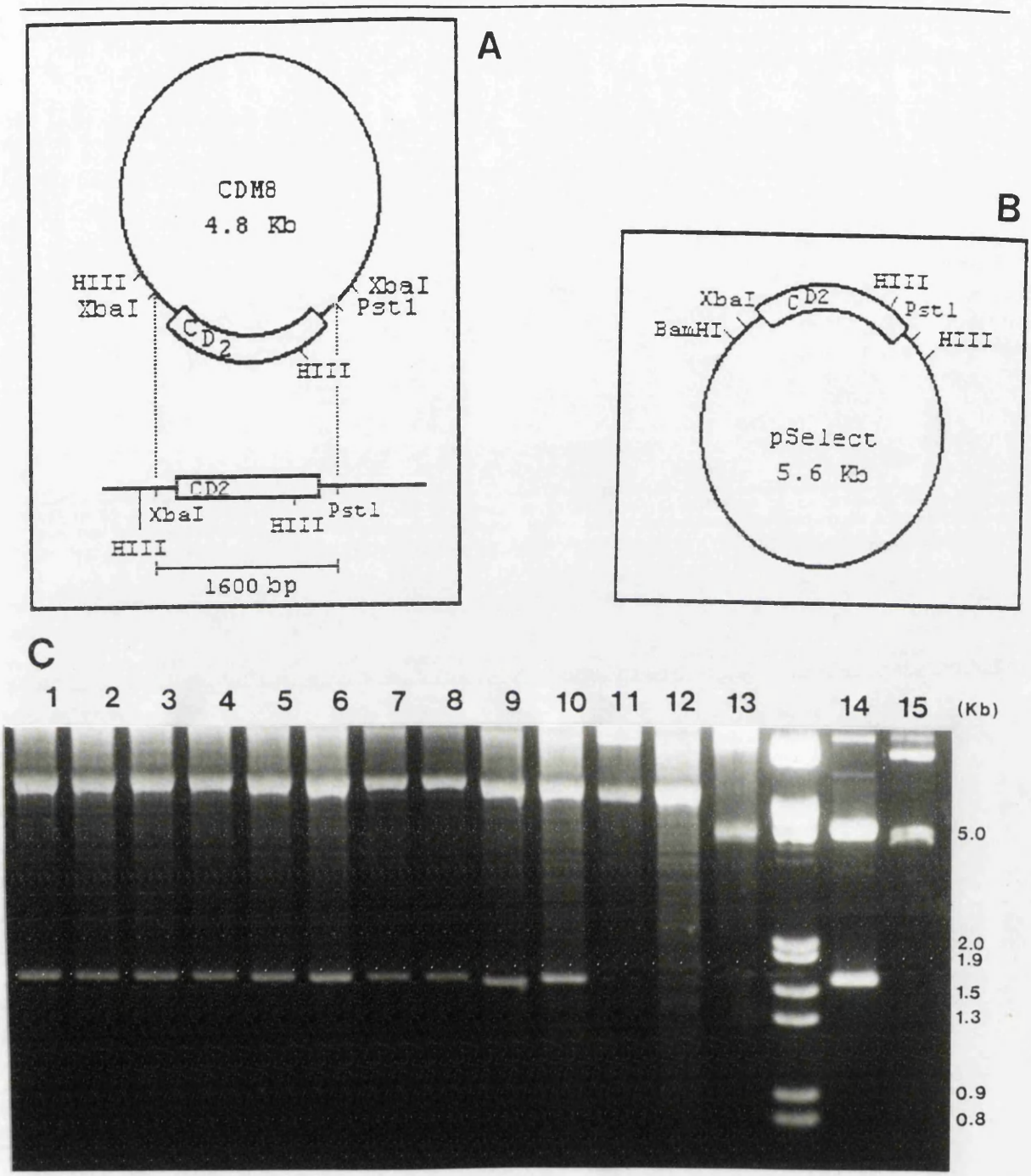
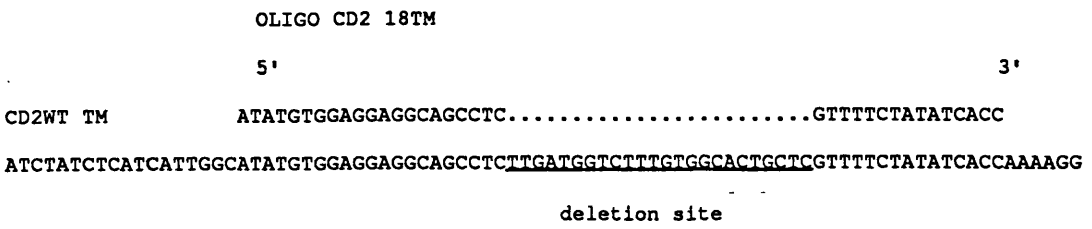
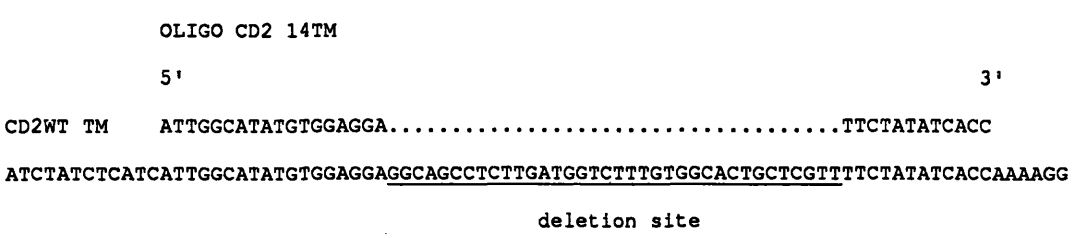


Figure 3.2 - Cloning of CD2 wild type fragment into pSelect. **A** - Schematic representation of CD2 fragment excised from the construction CD2CDM8. **B** - Schematic representation of CD2 wild type cloned into pSelect by Xba-1/Pst-1 digestion. **C** - Diagnostic digestion of pSelectCD2 wild type with Xba-1 and Pst-1 to confirm cloning. Lanes 1 to 10 showed 5.6 Kb and 1.6 Kb fragments that corresponds to pSelect and CD2 wild type respectively. Lanes 11 and 12 represents the linearized pSelect (5.6 Kb) and lane 13, the uncut pSelect plasmid. Lane 14 and 15 represents CD2CDM8 digested with Xba-1/Pst-1 and uncut CD2CDM8 plasmid respectively.

CD2 18TM



CD2 14TM



CD2 12TM

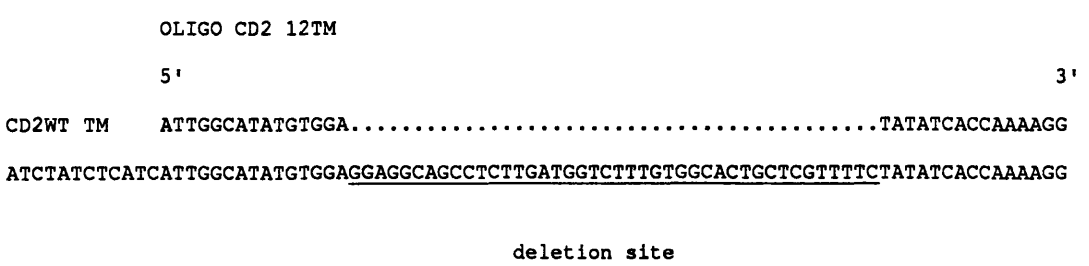


Figure 3.3 - Schematic representation of Altered Sites *in vitro* mutagenesis . The template used in the 3 mutagenesis reactions was ssDNA from the pSelectCD2 construct. The oligos described before in Material and Methods are positioned as in the annelling reaction. The deleted fragment for each mutant is underlined. The dotted line represents the continuity of the primer.

The mutagenesis annealing mixture was ligated and transformed in *E. coli* (BMH-71-18 mut S), a repair deficient strain. Plasmid DNA were prepared and transformed again into *E. coli* (JM109). Colonies were picked randomly and screened by sequencing using CD2B7 as a sequencing primer (Fig. 3.4). Further sequencing was carried out using primer C234920, to check if point mutations had occurred during the mutagenesis reaction, a fragment of 430bp (from PvuII to NcoI sites) was checked. The mutants were designated CD2 18TM, CD2 14TM and CD2 12TM, according to the deletions. The mutation frequency was respectively 0%, 33% and 9.09%. Due to the restoration of the ampicilin resistance to the mutant DNA, almost 90% of mutants were expected, but because factors such as incomplete *in vitro* polymerization, primer displacement by the DNA polymerase used and *in vivo* host-directed mismatch repair mechanisms which favor repair of the unmethylated newly synthesized DNA strand, this observed low frequency can be explained. The failing in CD2 18TM mutagenesis can be explained by a possible mismatch in the oligo that prevent the annealing , so the mutant double strand could not be synthesized.

The relative low CD2 14TM and CD2 12TM mutation frequency resulted in extensive sequencing to find the mutants. I therefore changed to a system of site-directed mutagenesis based on overlap extension using the PCR, another pair of oligos for each mutant was synthesized (Fig. 3.5). At the first PCR reaction, primers a/b and c/d were used to produce the fragments AB and CD, respectively, for each mutant (Fig 3.6a and 3.6b). The fragments were isolated from the gel by glass wool method and used as a template for the second PCR reaction, at least 500ng of each was used per reaction. The annealing temperature used in the second PCR reaction was

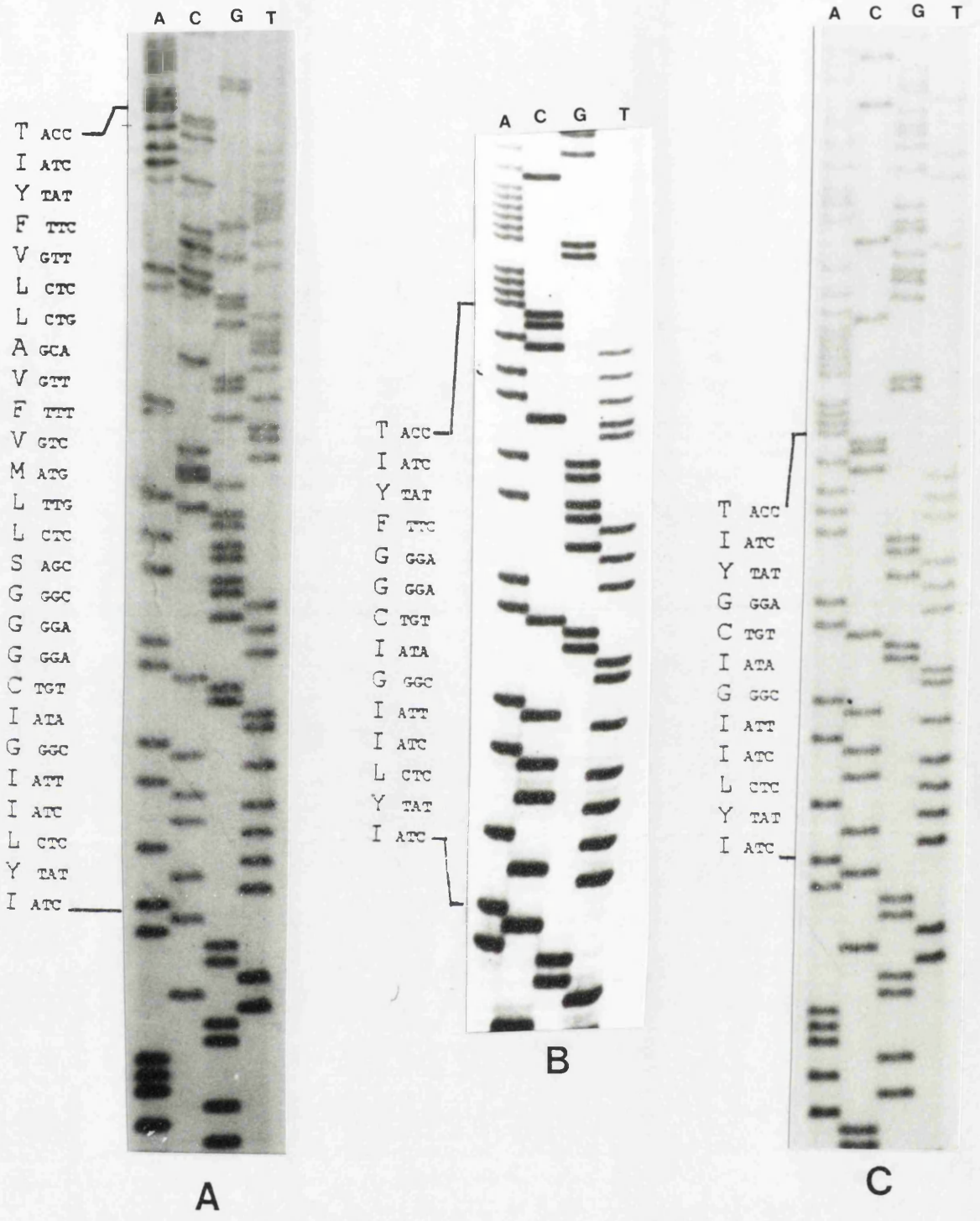
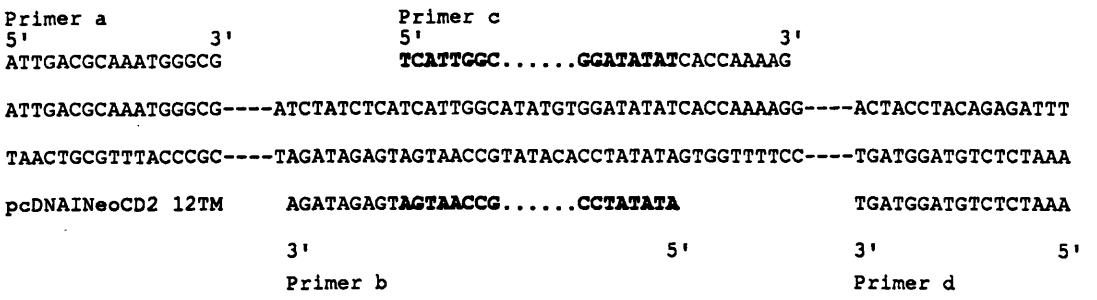
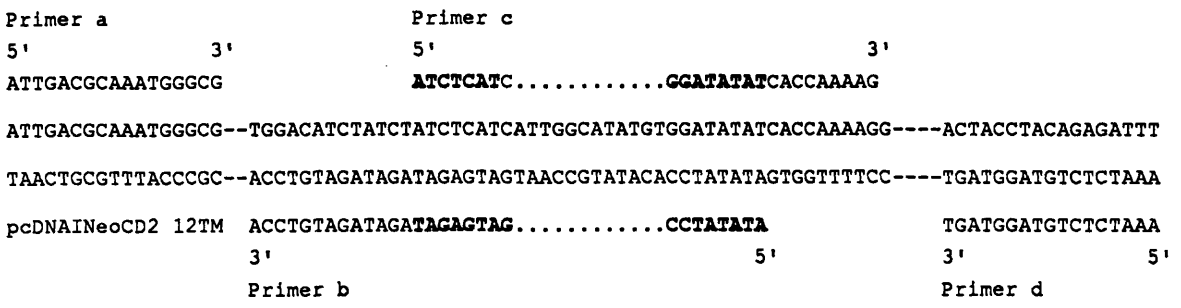


Figure 3.4 - Plasmid sequence of CD2 mutants in PSelect generated by Altered sites *in vitro* mutagenesis, usin CD2B7 as a sequencing primer. **A** - CD2 wild type (CD2 WT) Tm region. **B** - CD2 14TM mutant. **C** - CD2 12TM mutant.

CD2 10 TM



CD2 8 TM



CD2 +VE

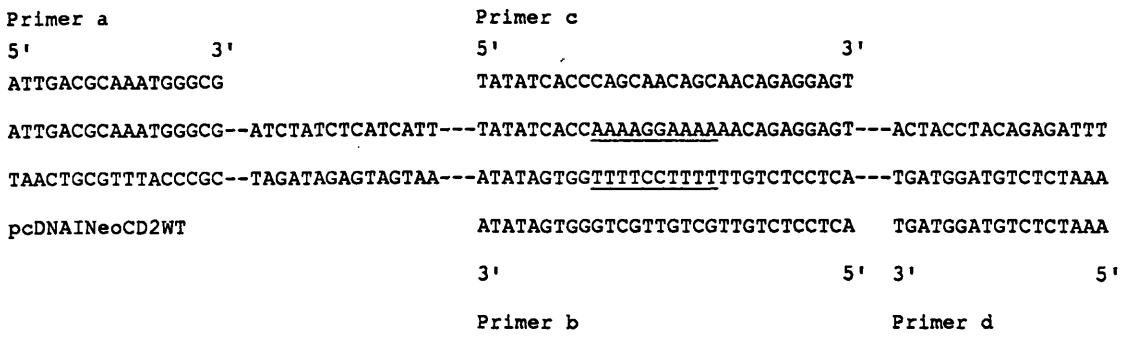


Figure 3.5 - Schematic representation of site directed mutagenesis by overlap extension using PCR for CD2 10TM, CD2 8TM and CD2+VE. The template DNA used for CD2 10TM and CD2 8TM mutagenesis reaction was pcDNAINeoCD2 12TM and for CD2+VE, it was pcDNAINeoCD2 WT. Primers a and d hybridises with pcDNAINeo, primers b and c hybridises with CD2 12TM sequence. The dotted line represents continuity of the primer and the line, continuity of the plasmid.

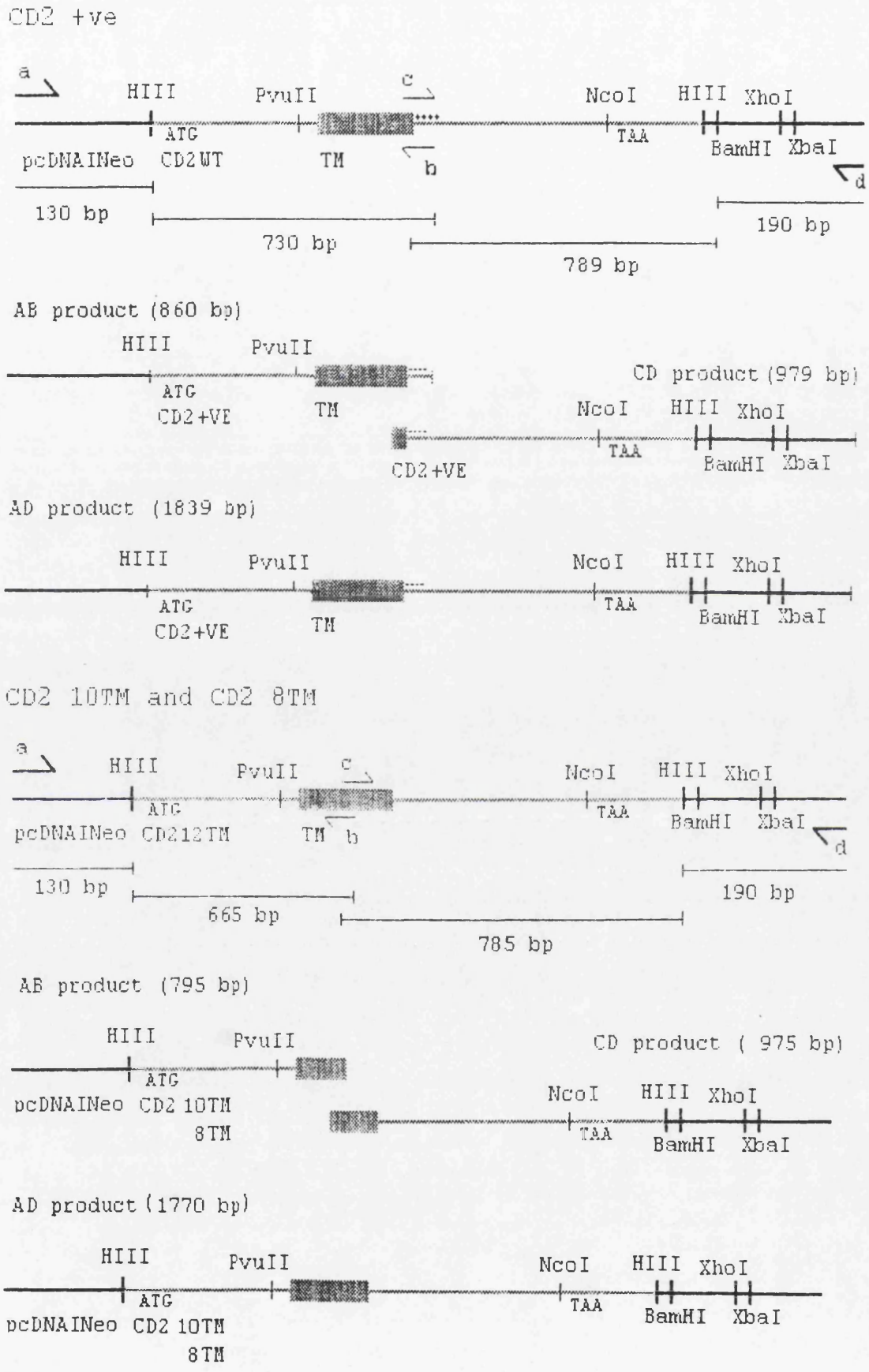


Figure 3.6a - Schematic representation of AB, CD and AD PCR products from the PCR mutagenesis reaction for CD2+VE, CD2 10TM and CD2 8TM.

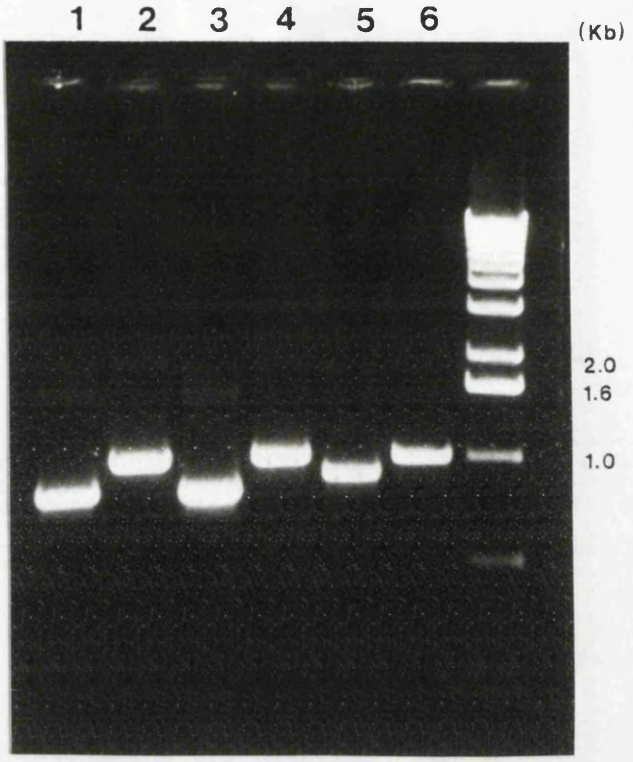


Figure 3.6b - AB and CD PCR products after glass wool extraction from agarose gels.
Lane 1 - CD2 8TM AB product
Lane 2 - CD2 8TM CD product
Lane 3 - CD2 10TM AB product
Lane 4 - CD2 10TM CD product
Lane 5 - CD2+VE AB product
Lane 6 - CD2+ve CD product

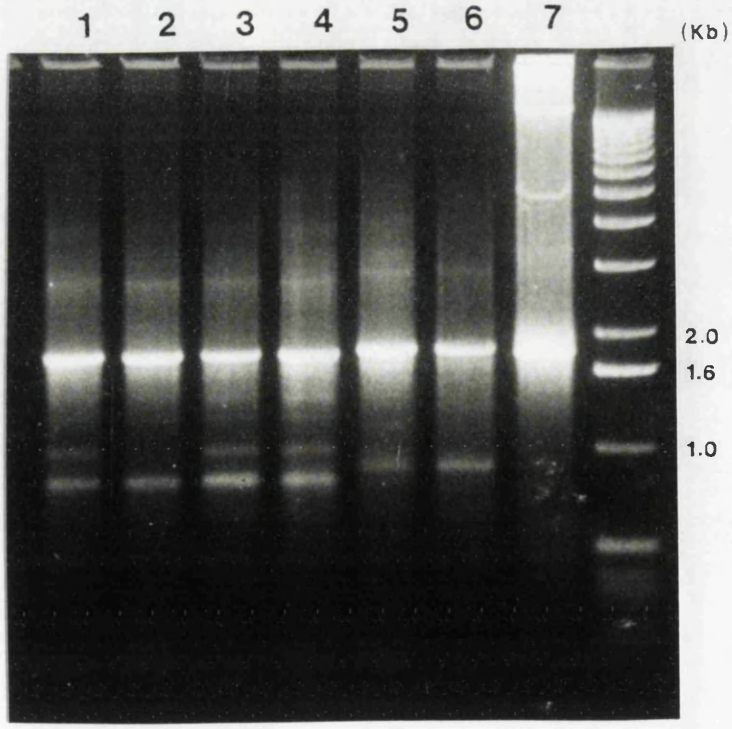


Figure 3.6c - AD products from second PCR reaction.
Lanes 1 and 2 - CD2 10TM AD
Lanes 3 and 4 - CD2 8TM AD
Lanes 5 and 6 - CD2+VE AD
Lane 7 - control CD2 WT AD

initially set at 50°C, but did not yield the AD PCR product, This product was obtained when the annealing temperature was changed to 45°C (Fig 3.6a and 3.6c),

The a d external primers were designed to yield an AD product with Hind III sites approximately 100bp away from both ends. The first idea was to digest this 1.8 kb fragment with Hind III, yielding two small fragments of 130 bp and 190bp, respectively, and a 1.5 kb fragment , which would correspond to CD2 mutant fragment (Fig 3.6a). This fragment would be cloned directly to pcDNAINeo digest with HindIII and treat with calf intestine alkaline phosphatase (CIP). However, the AD product could not be completely digested by HindIII, it always gave products of partial digestion (Fig 3.7). Even when the 1.5 Kb fragment was band purif by the QIAEX method, it was not possible to cloned it into pcDNAINeo/Hind III. So the AD PCR product, for the three mutants, was ligated to pT7Blue vector into the modified EcoRV site. The ligation mix was transformed into NOVABLUE cells. The transformants were select into ampicilin and tetracyclin plates, with white/blue selection. The white colonies were screened by PCR, using primers a and d (Fig. 3.8). Plasmid DNA were made from the positive clones, using the alkaline lysate method. The presence of insert and the orientation was checked by Hind III digestions (Fig. 3.9). The mutants were sequenced , using CD2B7 and C 234920 primers (Fig 3.10), once more the PvuII/NcoI fragment could be checked for possible point mutations created during PCR reactions.

In order to avoid mutations created during any of the mutagenesis process, fragments from all five mutants, CD2 14TM, CD2 12TM, CD2 10TM, CD2 8TM and CD2+VE were lifted by PvuII / NcoI digestions, 410bp, 410bp, 362 bp, 356 bp and

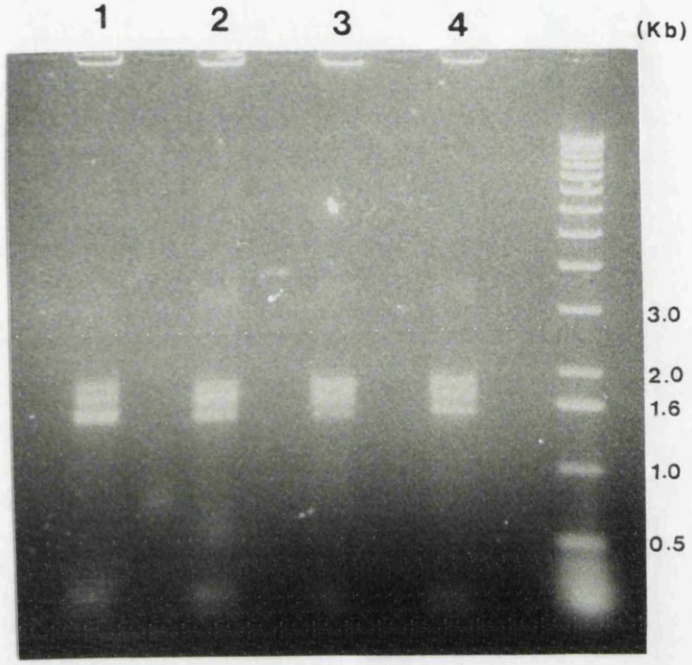


Figure 3.7 - Partial digestion of CD2 mutants AD PCR products by HindIII digestion.

1.8 Kb fragment - uncut PCR product
1.6 or 1.7 fragment - PCR product partially cut by HindIII
1.5 fragment - PCR product totally cut by HindIII

Lane 1 - CD2 8TM AD product
Lane 2 - CD2 10TM AD product
Lane 3 - CD2 +ve AD product
Lane 4 - CD2WT AD product

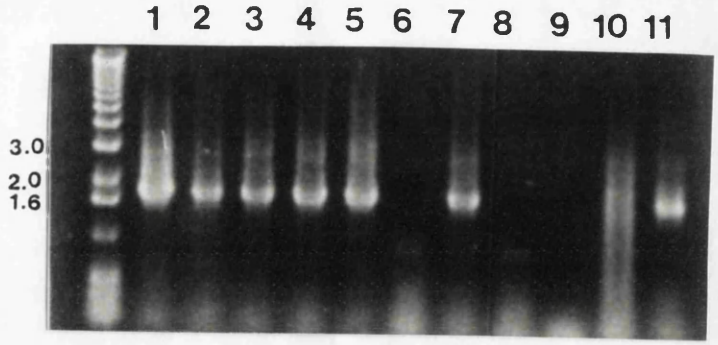
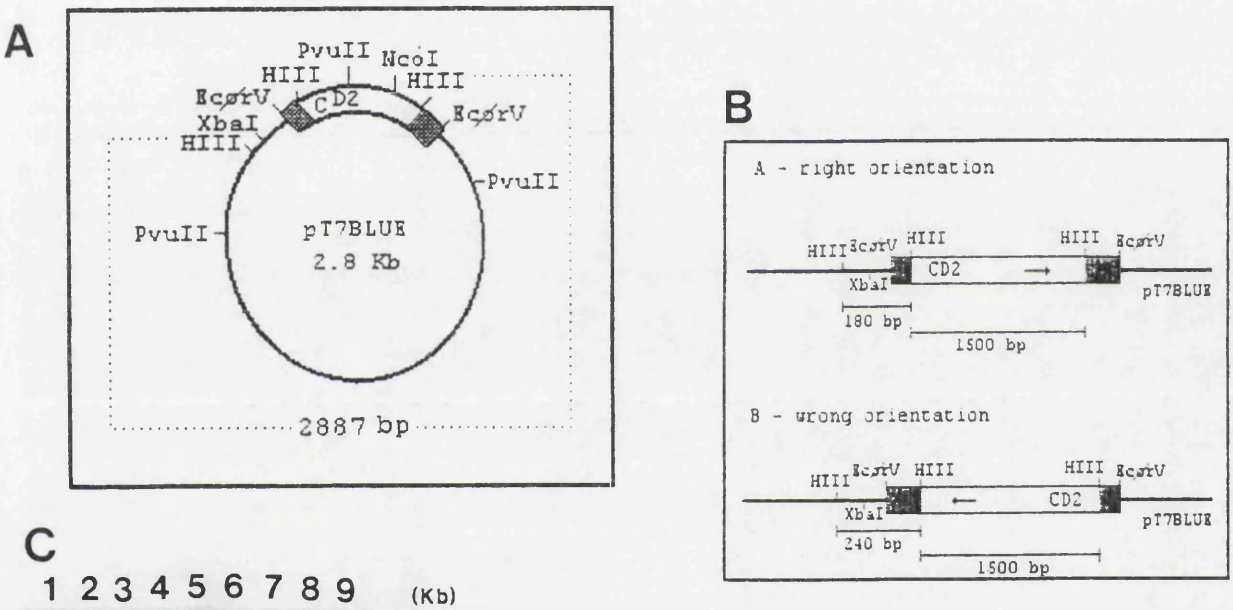


Figure 3.8 - PCR screening of bacterial colonies containing AD fragment cloned into pT7Blue. The 1.8 fragment amplified with primers a and d corresponds to AD fragment of CD2 10TM (lanes 1 to 3), CD2 8TM (lanes 4 to 7) and CD2+VE (lanes 8 to 11).



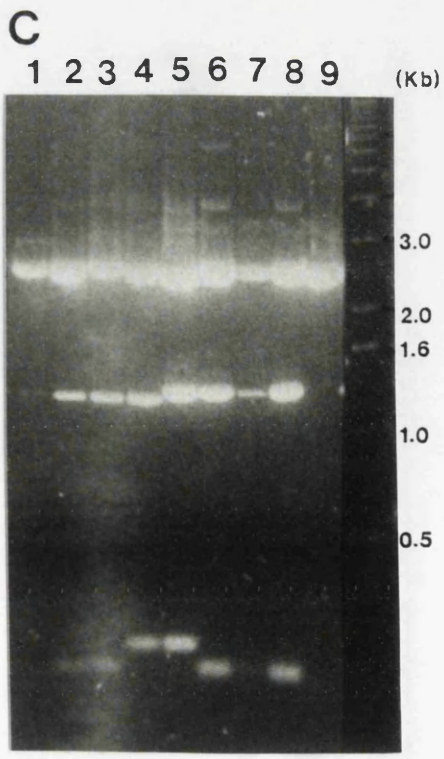


Figure 3.9 - **A** -Schematic representation of AD PCR product cloned into pT7Blue by modified EcoRV site. **B** - Diagram of the two possible orientations of the insert (AD fragment) into the vector (pT7Blue) with the diagnostic HindIII sites. **C** - Diagnostic HindIII digestion of pT7Blue/AD fragment to check orientation of insert. Lanes 1 to 3 CD2 10TM, lanes 4 to 6 CD2 8TM and lanes 7 to 9 CD2+VE. The 180bp fragment observed in lanes 2,3,6 and 8 corresponds to the right orientation of the insert.

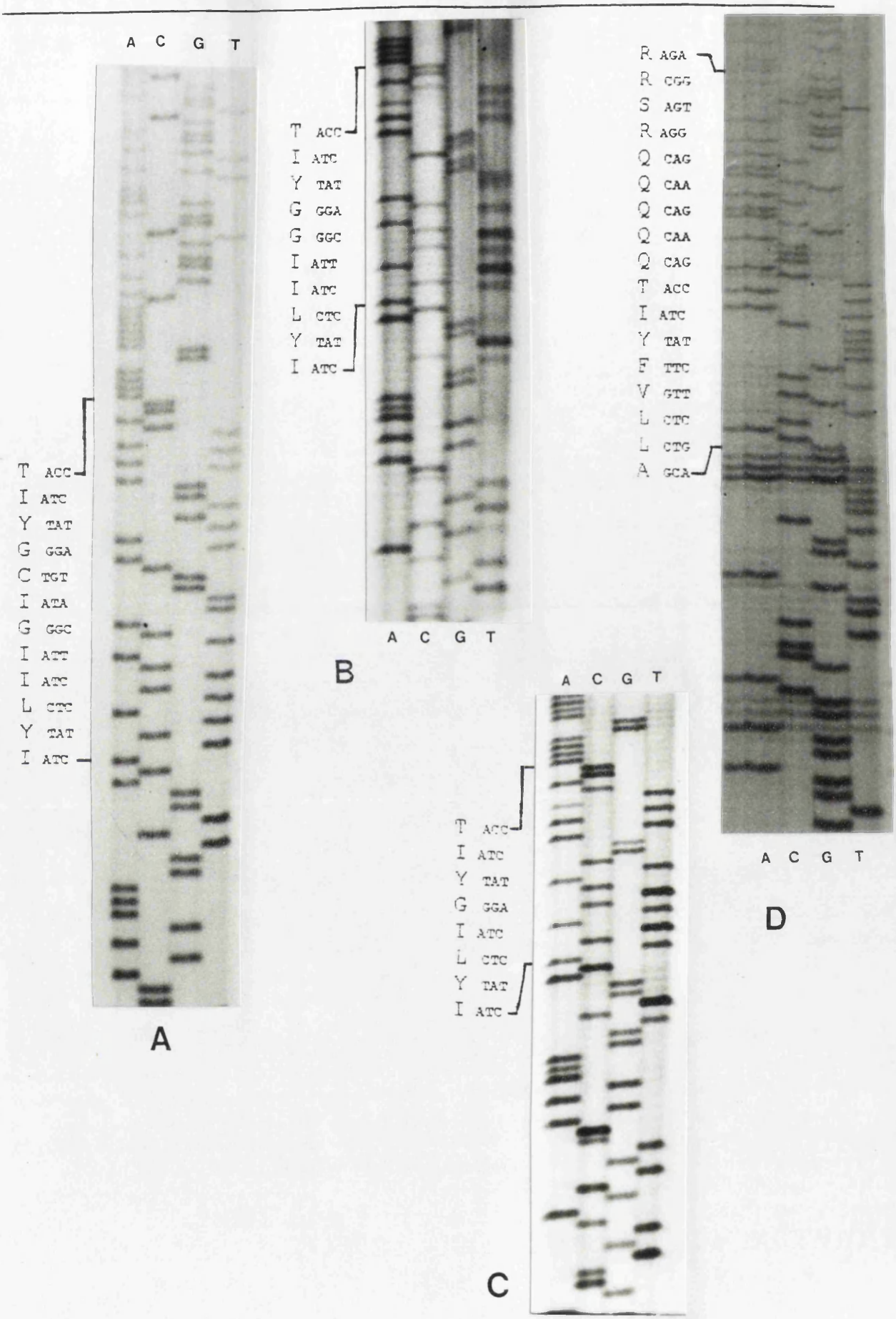


Figure 3.10 - Plasmid sequence of CD2 mutants in PT7Blue generated PCR mutagenesis, using CD2B7 as a sequencing primer. **A** - CD2 12TM mutant. **B** - CD2 10TM mutant. **C** - CD2 8TM mutant. **D** - CD2+VE mutant

410bp fragments were obtained, respectively (Fig. 3.11b). Each of these fragments was cloned into wild type CD2 (previously cloned into Hind III site of pAT153), digested with PvuII /NcoI (Fig. 3.11a). This constructions were denominated pATCD2WT, pATCD214TM, pATCD212TM, pATCD210TM, pATCD28TM and pATCD2+VE, respectively (Fig. 3.11b).

The CD2 fragment from pATCD2WT, pATCD14TM and pATCD212TM was cloned into pcDNAINeo digested with HindIII and treated with calf intestine alkaline phosphatase (CIP). These constructions were called pcDNAINeoCD2WT, pcDNAINeoCD214TM and pcDNAINeoCD212TM (Fig 3.12). Because of difficulties like high background of religated vector and orientation of the insert, the cloning strategy was changed to eliminating the 3' end HindIII site by doing a partial digestion with HindIII enzyme. The digestion was done using buffer selected to yield a partial digestion and suitable amount of enzyme in 20 ul reaction. Different aliquots were left for 60, 40, 30, 20 and 15 minutes at 37°C. Immediately after, dNTPs in a final concentration of 2mM/ul and 1.0 ul of Klenow were added to each aliquot and the mix was left for 30 minutes at room temperature. The samples were run on a gel and the 5.1 kb fragment, that corresponded to the linearized one, was band purified by QIAEX method and religated (Fig. 3.13a). The ligation mix was transformed into DS941 cells and transformants were screened on ampicilin plates. The efficiency of the reaction was checked by digesting the DNA of some candidates with HindIII, if the plasmid linearized, one of the HindIII sites was eliminated. This plasmids were digested with HindIII and BamHI, in order to confirm the orientation of the disrupted HindIII site (Fig 3.13b). The CD2 fragment was lifted by HindIII / BamHI from pATCD2 constructs and ligated into pcDNAINeo HindIII / BamHI. The ligated DNA was transformed into DS941 cells and

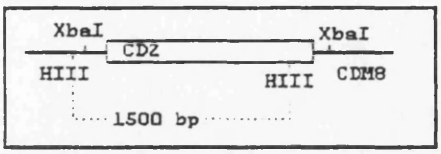
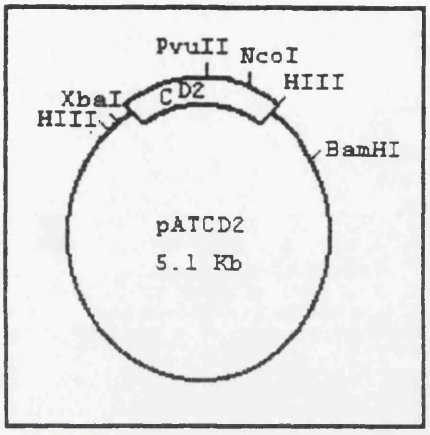


Figure 3.11a - Diagram of 1.5 Kb CD2 WT fragment excised from CDM8 by HIII digestion.



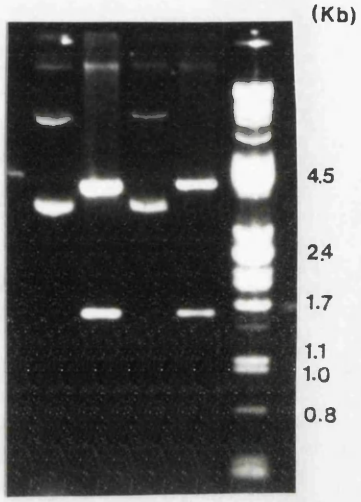
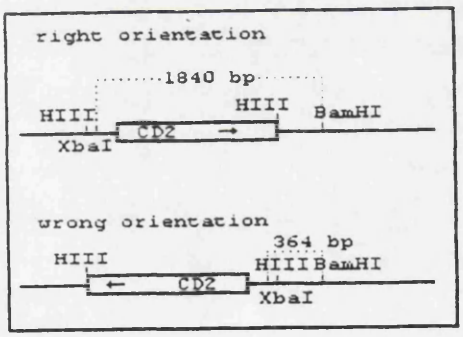


Figure 3.11a - Diagnostic restrition of pAT153CD2 with HindIII to verify cloning of CD2 fragment into pAT153. The 1.5 Kb fragment corresponds to CD2 wild type and the 3.6 fragment to pAT153.



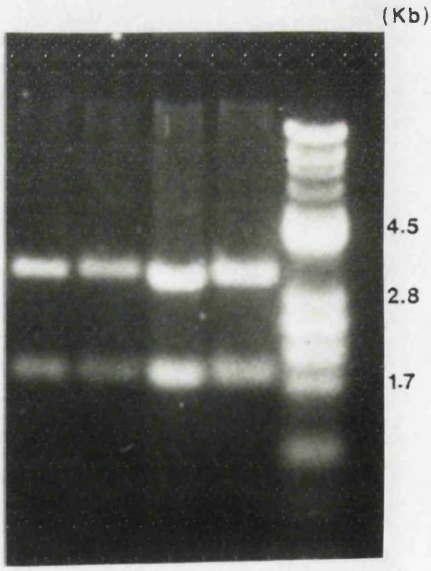
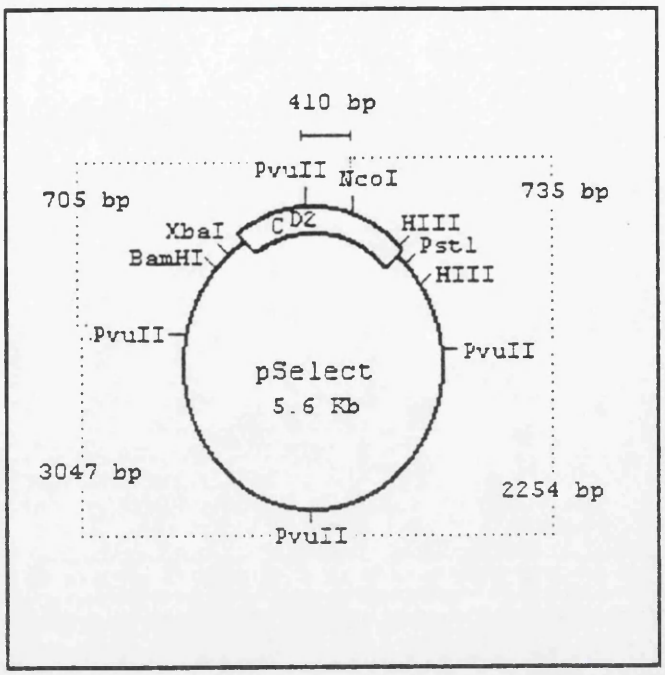


Figure 3.11a - Diagnostic restriction with XbaI/BamHI to check orientation of the insert into pAT153. The 1846 bp fragment corresponds to XbaI/BamHI fragment at the right orientation and the 3.3Kb one corresponds to pAT153.



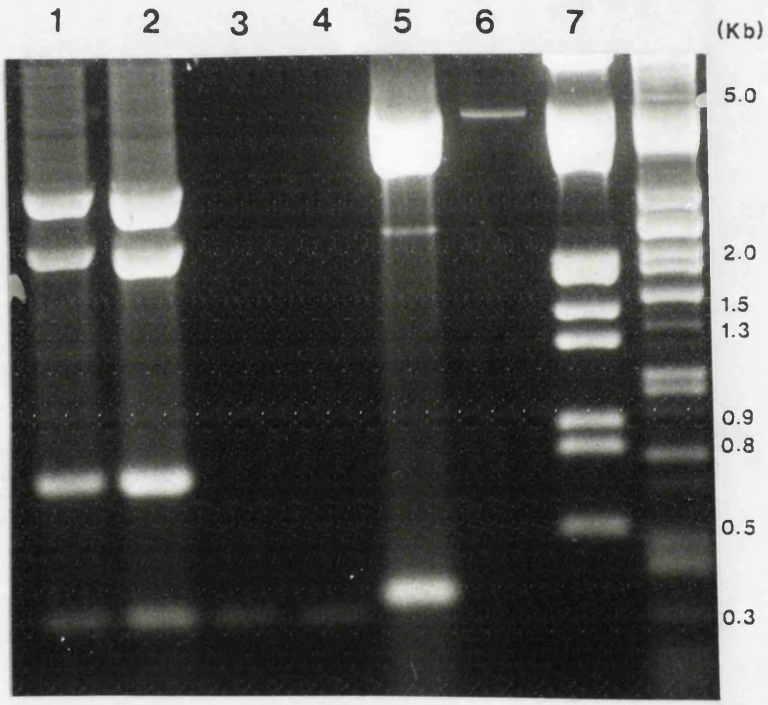
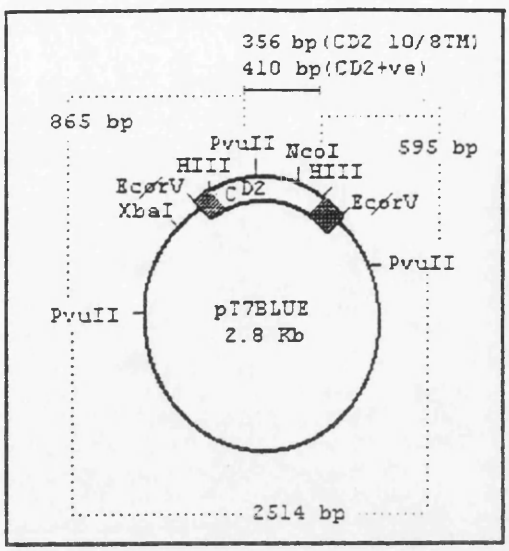
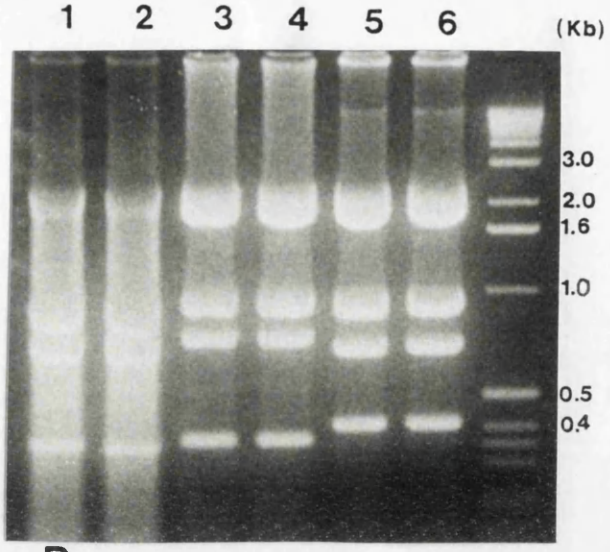


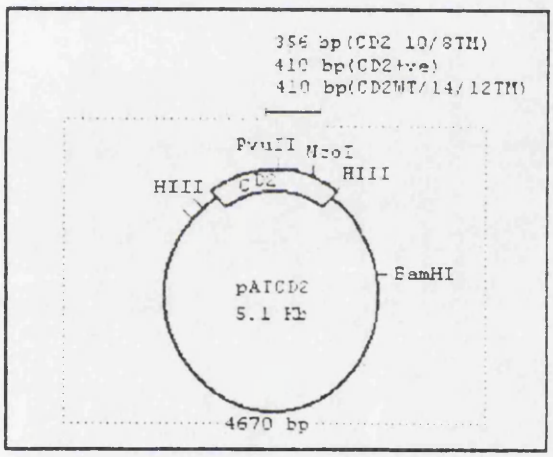
Figure 3.11b - Top - Diagram of pSelect/CD2 14TM and CD2 12TM digested with PvuII/NcoI, showing the size of the expected fragments. Bottom - Digestion of pSelect/CD2 14TM, pSelect/CD2 12TM and pATCD2 WT plasmids with PvuII/NcoI. Lane 1 and 2 pSelectCD2 14TM and 12TM. Lane 3 and 4 - 410 bp fragment of CD2 14TM and CD2 12TM, respectively, band purified by glass wool method. Lane 5 - pATCD2 WT digested with PvuII/NcoI. Lane 6 - The 4670 bp fragment band purified by glass wool method. CD2 14TM and CD2 12TM fragments were ligated to CD2 WT 4670bp fragment.



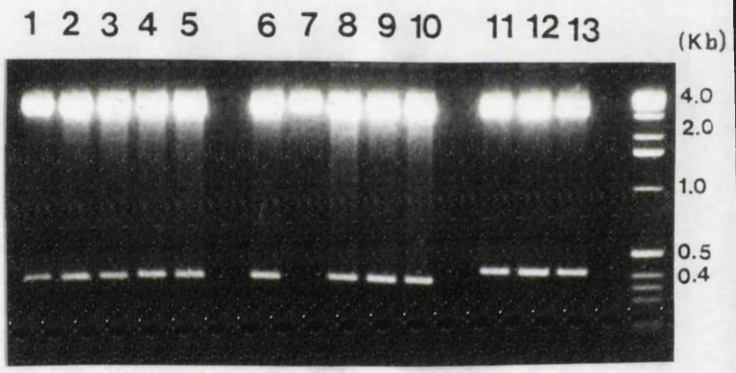
A



B



C



D

Figure 3.11c - **A** - Diagram of pT7Blue/CD2 10TM, CD2 8TM and CD2+VE with the expected sizes of fragments generated by PvuII/NcoI digestion. **B** - PvuII/NcoI digestions of plasmid DNA from pT7Blue/CD2 mutants. Lane 1 and 2 pT7Blue CD2 8TM. Lane 3 and 4 pT7Blue CD2 10TM. Lane 5 and 6 pT7Blue CD2+VE. The 356 bp and 410bp fragments were band purified by glass wool method and ligated to pATCD2 WT 4670 fragment. **C** - Diagram of CD2 mutants cloned into pATCD2. **D** - PvuII/NcoI diagnostic restriction of pATCD2 mutants. Lanes 1 to 5 pATCD2 8TM, Lanes 6 to 10 pATCD2 10TM and lanes 11 to 13 pATCD2+ve.

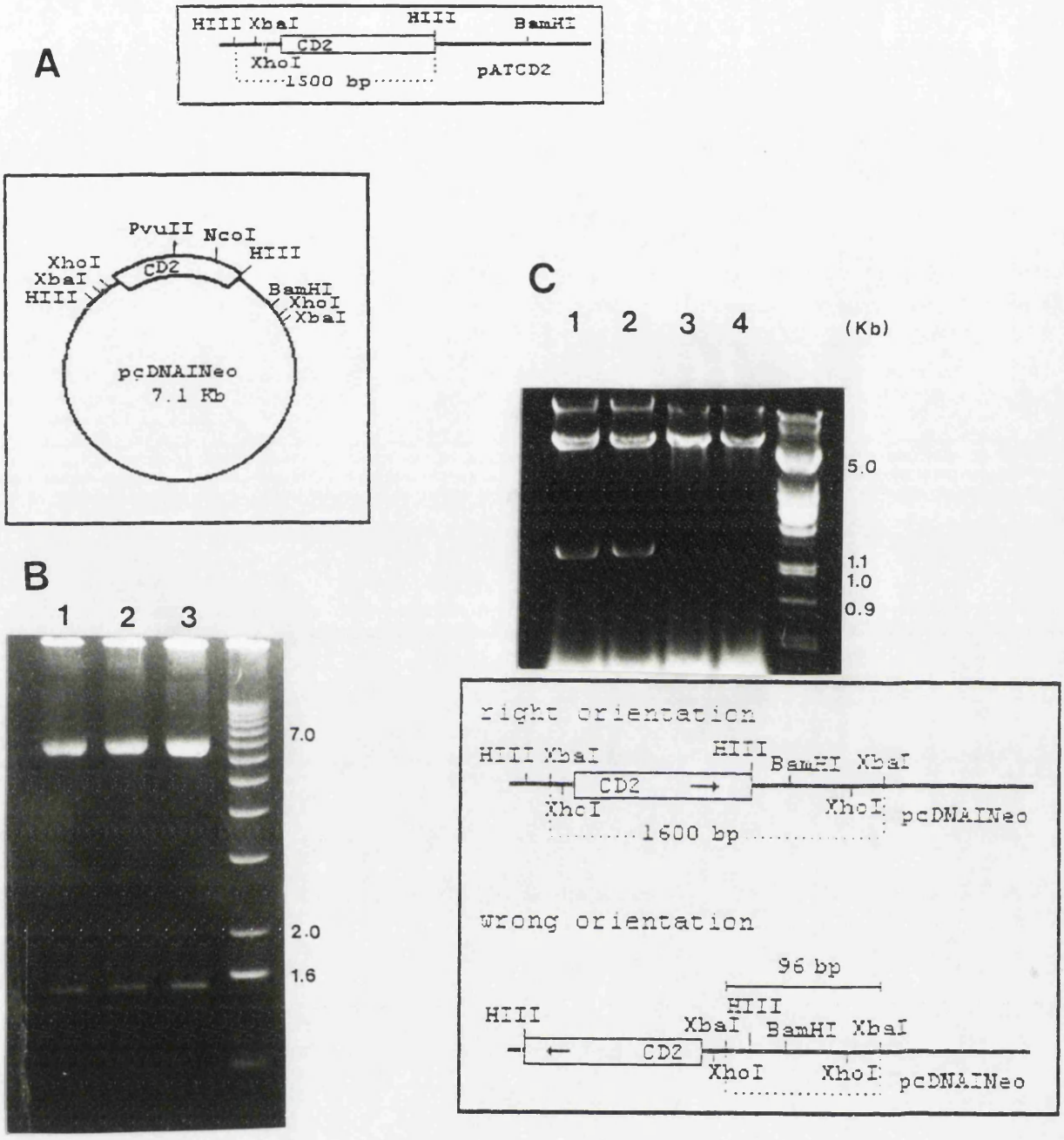
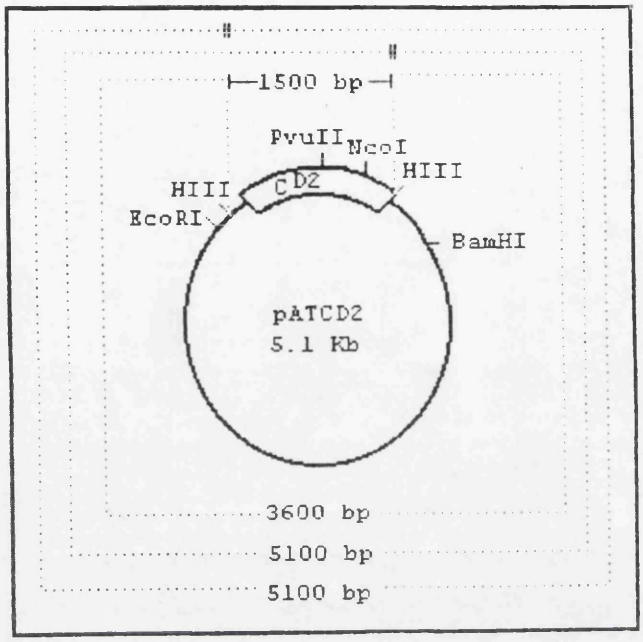


Figure 3.12 - Cloning of CD2 WT, CD2 14TM and CD2 12TM into pcDNAINeo. **A** - Diagram representing the 1.5Kb fragment excised from pATCD2 WT, 14TM and 12TM respectively, by HindII digestion. **B** - Plasmid DNA from a mini prep digested with HindIII, the 1.5 Kb fragment corresponds to CD2 fragment and the 7.1 one to pcDNAINeo. Lane 1 pcDNAINeo CD2 WT, lane 2 pcDNAINeo CD2 14TM, lane 3 pcDNAINeo 12TM. **C** - XbaI digestion of CD2 14TM and CD2 12TM to check orientation into pcDNAINeo. Lanes 1 and 2 corresponds to right orientation because of the 1.5 Kb fragment, in lanes 3 and 4 the plasmid linearized because of the wrong orientation.



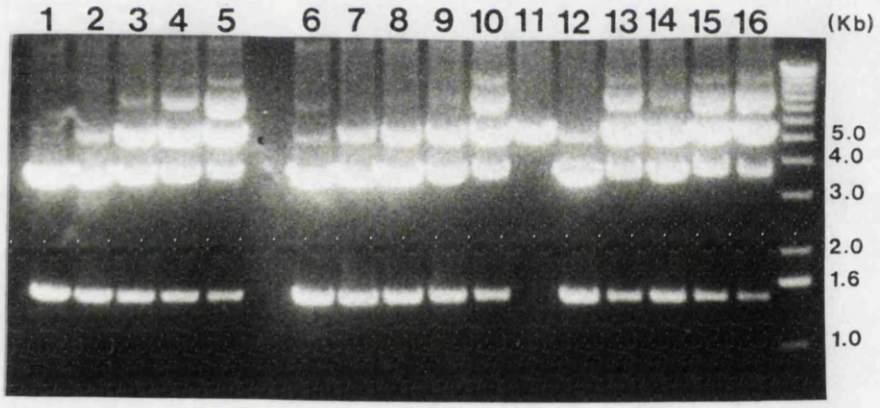


Figure 3.13a - Partial digestion of pATCD2 10TM, 8TM and +VE with HindIII. **Top** - Diagram of the plasmid with HindIII digestions showing the expected sizes for partial (5.1Kb) and complete digestion (3.6Kb/1.5Kb). **Bottom** - Hind III partial digestion of the plasmid DNA. Lane 1 to 5 pATCD2 10TM, lane 6 to 10 pATCD2 8TM, lane 12 to 16 pATCD2+VE and lane 11 the plasmid digested with BamHI as a control to the 5.1 Kb linearized fragment.

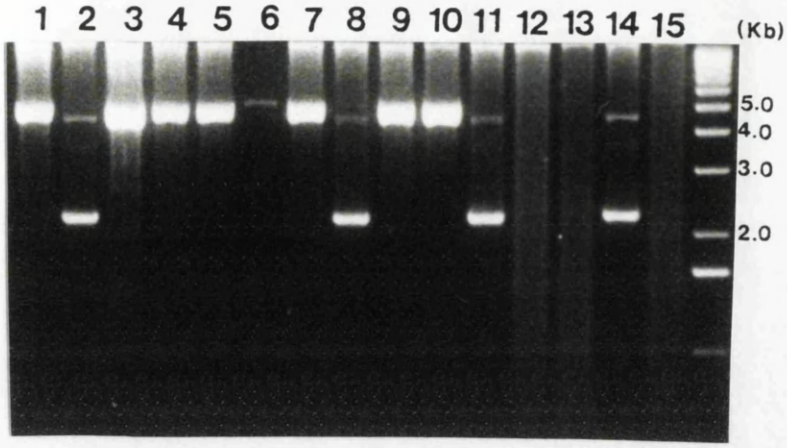


Figure 3.13b - The religated plasmic was digested with HindIII to check the disruption of the 3' HindIII sit The linearized plasmids corresponds the ones with only one HindIII site (lanes 2, 8, 11 and 14). Lanes 1 to CD2 10TM, lanes 6 to 10 CD2 8TM and lanes 11 to 15 CD2+VE.

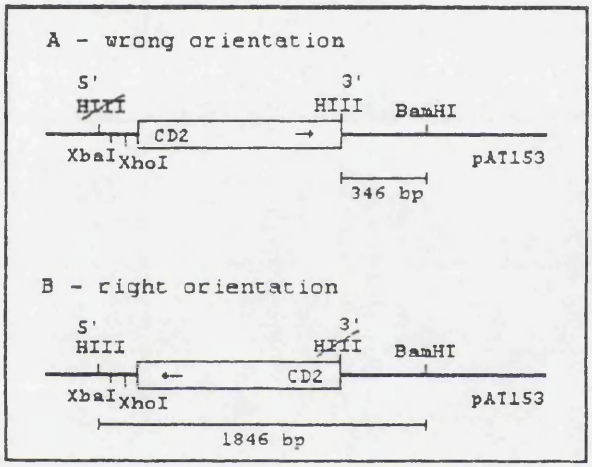


Diagram representing the sizes of fragments resulting from HindIII/BamHI digestion of the plasmid for right and wrong orientation of the disrupted HindIII site.

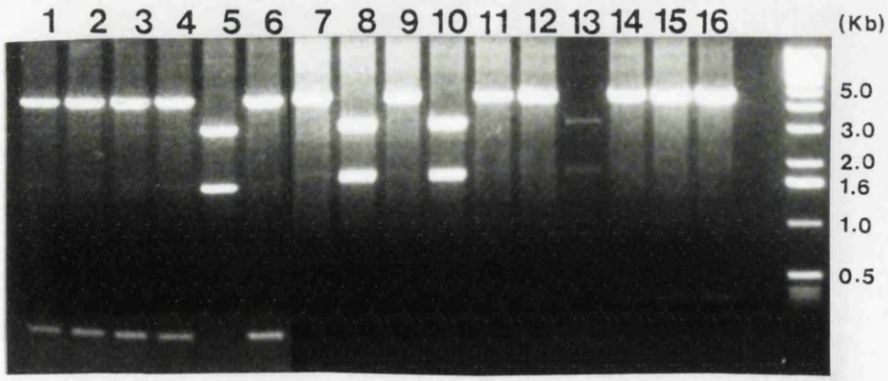


Figure 3.13b - Plasmid DNA digested with HindIII/BamHI. Lanes 1 to 5 CD2 10TM, lanes 6 to 10 CD2 8 TM and lanes 9 to 16 CD2+VE. In lanes 5, 8 10 and 14 two fragments of 3.9Kb and 1.8Kb were observed, these corresponds to the right orientation of the disrupted HindIII site at the 3' end.

transformants were screened on kanamycin plates. Plasmid DNA was made from the colonies, and the presence of insert was checked by cutting it with HindIII/BamHI (Fig. 3.14). These constructions were called pcDNAINeo CD28TM, pcDNAINeoCD210TM and pcDNAINeoCD2+VE. Once the CD2 mutants were cloned into pcDNAINeo, they were sequenced again in order to check any possible mistake during cloning. The fusion protein CD2B7CDM8 was cloned into pcDNAINeo digested with HindIII/Not-1 (Fig 3.15).

3.3 Transfection of CD2 mutants into mammalian cells

Once the CD2 WT and mutants were cloned into the expressing vector pcDNAINeo, the plasmids were transfected into mammalian cells with the purpose of observing cell expression of the mutant proteins. The established system in the laboratory for transient transfection of COS-7 was DEAE-dextran. As DOTAP is a more straight forward method and the transfection efficiency (see Material and Methods) obtained was 3%-5%, that was satisfactory for the purposes of the project, transient transfection into COS-7 cells was changed to the DOTAP system. Transfection of CD2 WT, CD2 14TM and CD2 12TM into CHO for selection of stable cell lines were performed using the $CaPO_4$ system. Because the frequency of transfected clones(see Material and Methods) was very low (0.004%), the system was changed to DOTAP and the frequency increased to 0.6%. Consequently, CD2 10TM, CD2 8TM, CD2+VE and CD2B7 were transfected into CHO cells for selection of stable cell lines by using DOTAP.

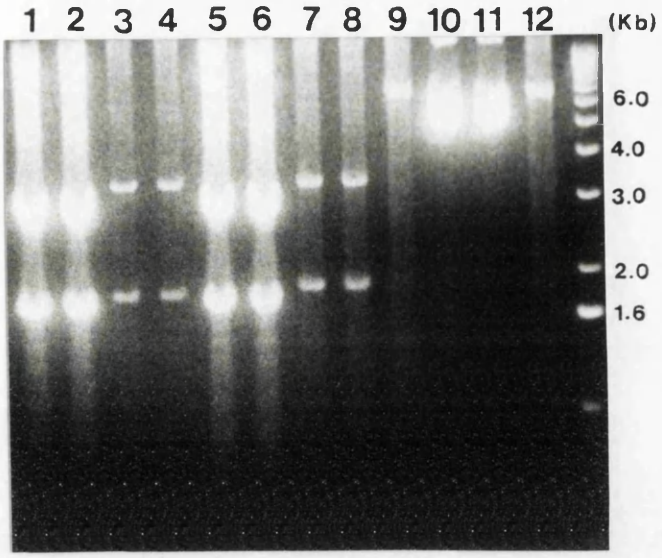
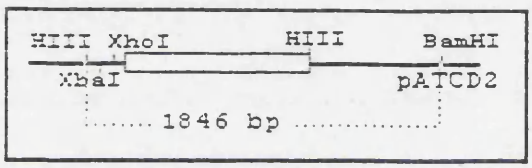


Figure 3 .14a - Digestion of pATCD2 10TM 8TM and +VE and pcDNAINeo with Hind III BamHI, the 1.8Kb and 7.1Kb fragments were band purified by QIAEX and ligated.
Lanes 1 to 4 - pATCD2 10TM
Lanes 5 and 6 - pATCD2 8TM
Lanes 7 and 8 - pATCD2+VE
Lanes 9 to 12 - pcDNAINeo



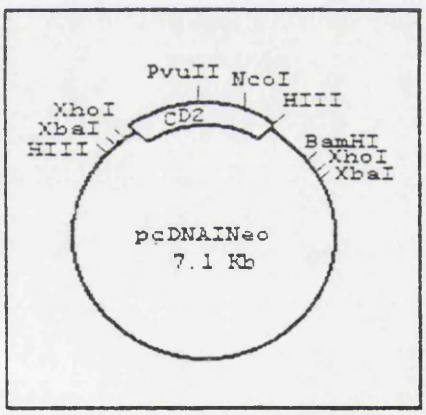


Figure 3.14b - Diagram representing the excised fragment from pATCD2 contructs and the cloning of these fragments into the expressing vector pcDNAINeo.

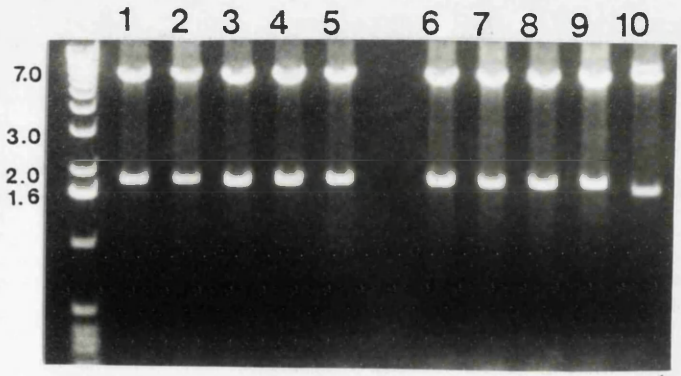


Figure 3.14c - Diagnostic restriction of the plasmid with HindIII/BamHI to check if the vector (7.1 Kb) has the insert (1.8Kb).

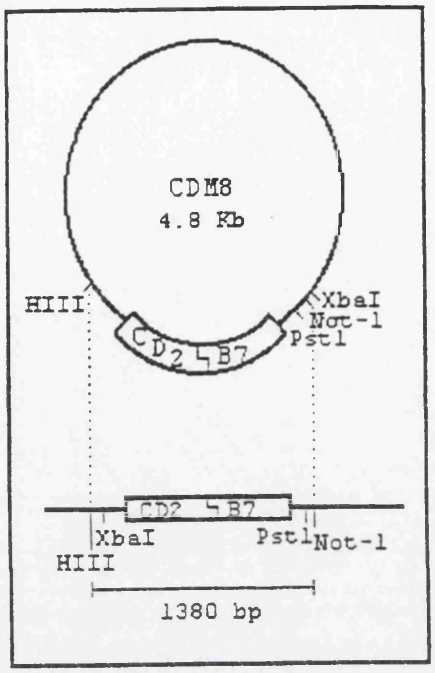


Figure 3. 15a - Schematic representation of the 1380bp CD2B7 fragment excised from CDM8 by HindIII/Not-1 digestion.

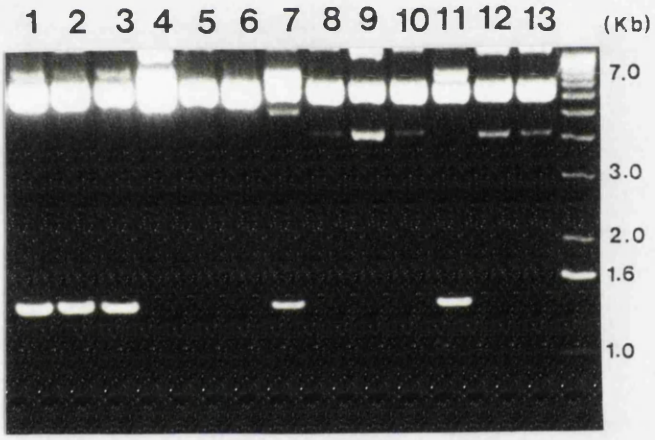


Figure 3.15b - Diagnostic digestion of plasmid DNA by HindIII/Not-1 to check presence of insert. Lanes 1,2 3,7 and 11 show the 1380 bp band that corresponds to CD2B7, the 7.1 band corresponds to pcDNAINeo.

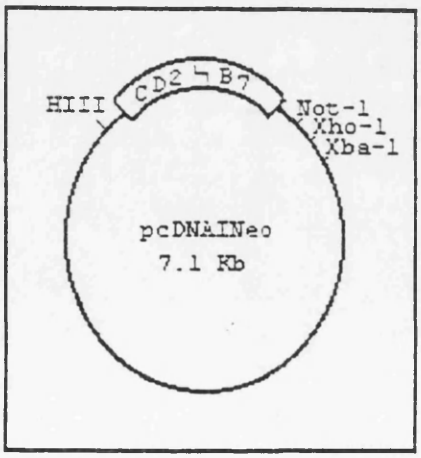


Figure 3.15c - Schematic representation of CD2B7 cloning into pcDNAINeo.

3.4 Cellular expression and distribution of CD2 mutants

Panning experiments were carried out to determine if CD2 mutants could be expressed on the cell surface. COS-7 cells were transiently transfected by DOTAP with DNA encoding the wild type and mutant forms of CD2. After 72 hours of transfection the cells were harvested, labelled with mouse anti-CD2 MAb (SeraLab) and distributed into panning plates coated with goat anti-mouse IgG antibody. COS-7 cells untransfected labelled with W6/32 were included as a positive control and COS-7 untransfected and unlabelled, as a negative control. CD2 WT, CD2 +VE, CD2 14TM and CD2 12TM transfected cells attached to the panning plates, indicating cell surface expression of CD2. On the other hand, CD2 10TM and CD2 8TM showed negative result for this experiment. These results were confirmed by panning CHO stable cell lines of CD2 mutants with RFT11 and GT2 antibodies (Table 3.1).

Table 3.1 - Panning results for the 3 antibodies tested on transient transfected COS-7 cells and CHO stable cell lines of CD2 wild type (WT) and mutants (14TM, 12TM, 10TM, 8TM, CD2+VE and CD2B7)

	Anti-CD2 Seralab (COS-7)	Anti-CD2 RFT11 (CHO)	Anti-CD2 GT2 (CHO)
COS-7/CHO	-VE	-VE	-VE
CD2 WT	+VE	+VE	+VE
CD2 14TM	+VE	+VE	+VE
CD2 12TM	+VE	+VE	+VE
CD2 10TM	-VE	-VE	-VE
CD2 8TM	-VE	-VE	-VE
CD2 +VE	+VE	+VE	+VE
CD2B7	+VE	+VE	+VE

Once some of the CD2 mutants exhibited cell surface expression and others not, indirect immunofluorescence microscopy experiments were performed to find out their distribution in the cell. Transient transfected COS-7 cells were passed to slides flasks 48 hours after transfection, they then adhered to it and were fixed after further 24 hours. First, cells were stained with mouse anti-CD2 MAb from Seralab (1:10 dilution) and anti-mouse FITC second antibody (1:100 dilution). As a positive control untransfected cells were stained with mouse W6/32 MAb (1:50 dilution) and anti-mouse FITC . The mutants CD2 10TM and CD2 8TM did not show cell surface labelling, as CD2 WT and other mutants and it was not possible to observe internal staining (Fig 3.16). So, immunostaining with a anti-CD2 antibody (RFT11) that is able to bind both the native and denatured conformation of CD2 external domain, were carried out. CD2 WT and other mutants were included as a control for surface staining. An internal staining pattern were observed for both CD2 10TM and CD2 8TM, and surface staining for CD2WT, CD214TM, CD212TM, CD210TM, CD28TM and CD2 +VE (Fig 3.17). This result indicates that the truncated forms of CD2 are not folding correctly, once the epitope for the first antibody (Seralab CD2) was not recognized.

Western blotting experiments were performed in order to estimate sites of post-translational modification and elucidate about the intracellular localization. Protein were extracted from CHO stable cell lines of each mutant with FSB. The lysate were run on a 12% SDS-PAGE gels and electroblotted. The blot was hybridized with RFT11 at room temperature and developed with Protoblot alkaline phosphatase AP system (Promega). The CD2 WT, CD2 14TM, CD2 12TM and CD2+ve cells produced multiple bands ranging from 45kDa to 55kDa (Fig 3.18), that correlates with the sizes described in

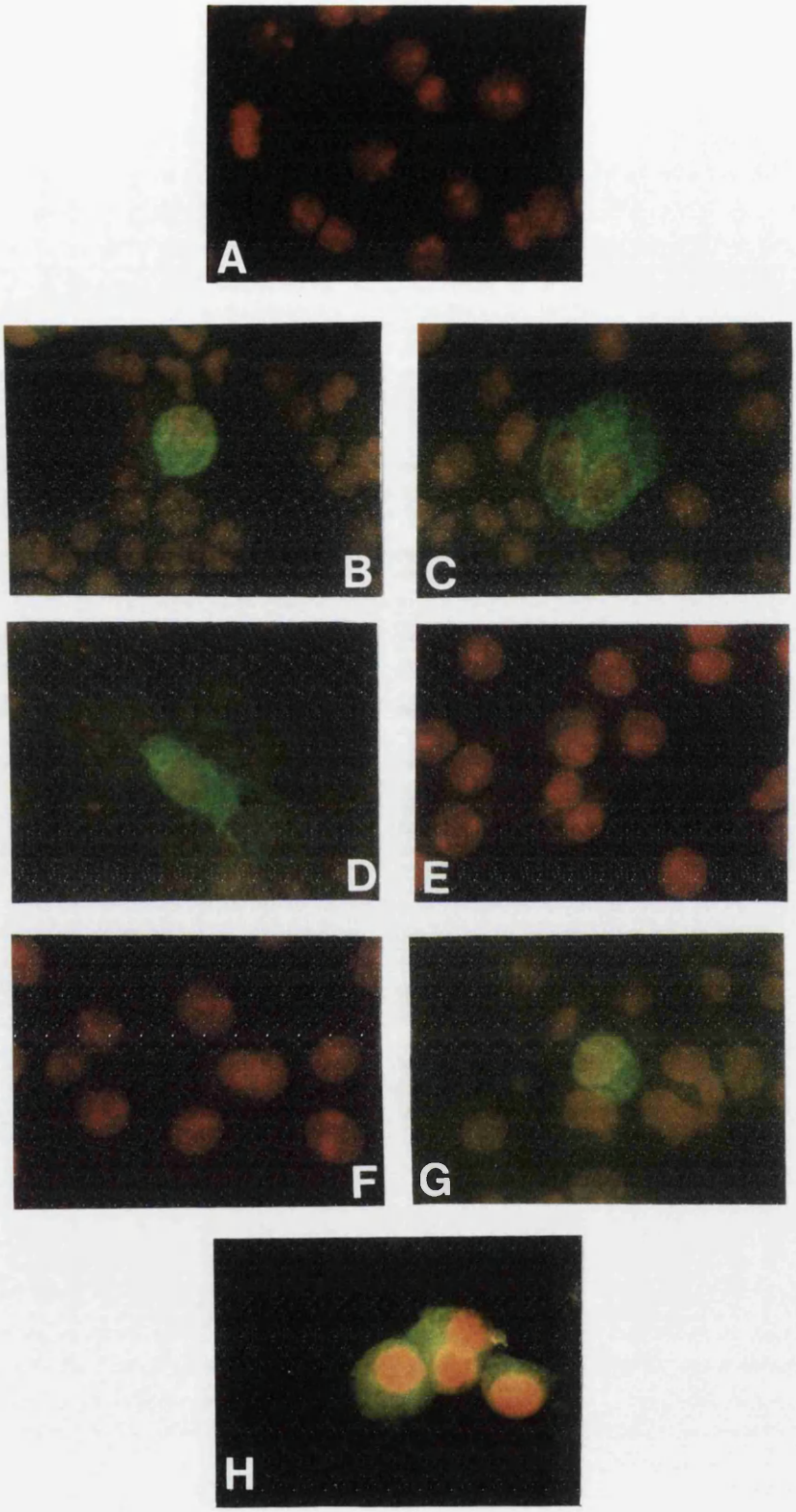


Figure 3.16 - Staining of COS-7 cells transiently transfected with CD2 wild type and mutants, with anti-CD2 from SeraLab. A - No DNA, B - CD2WT, C - CD2 14TM, D - CD2 12TM, E - CD2 10TM, F - CD2 8TM, G - CD2 +VE and H - COS-7 cell stained with W632.

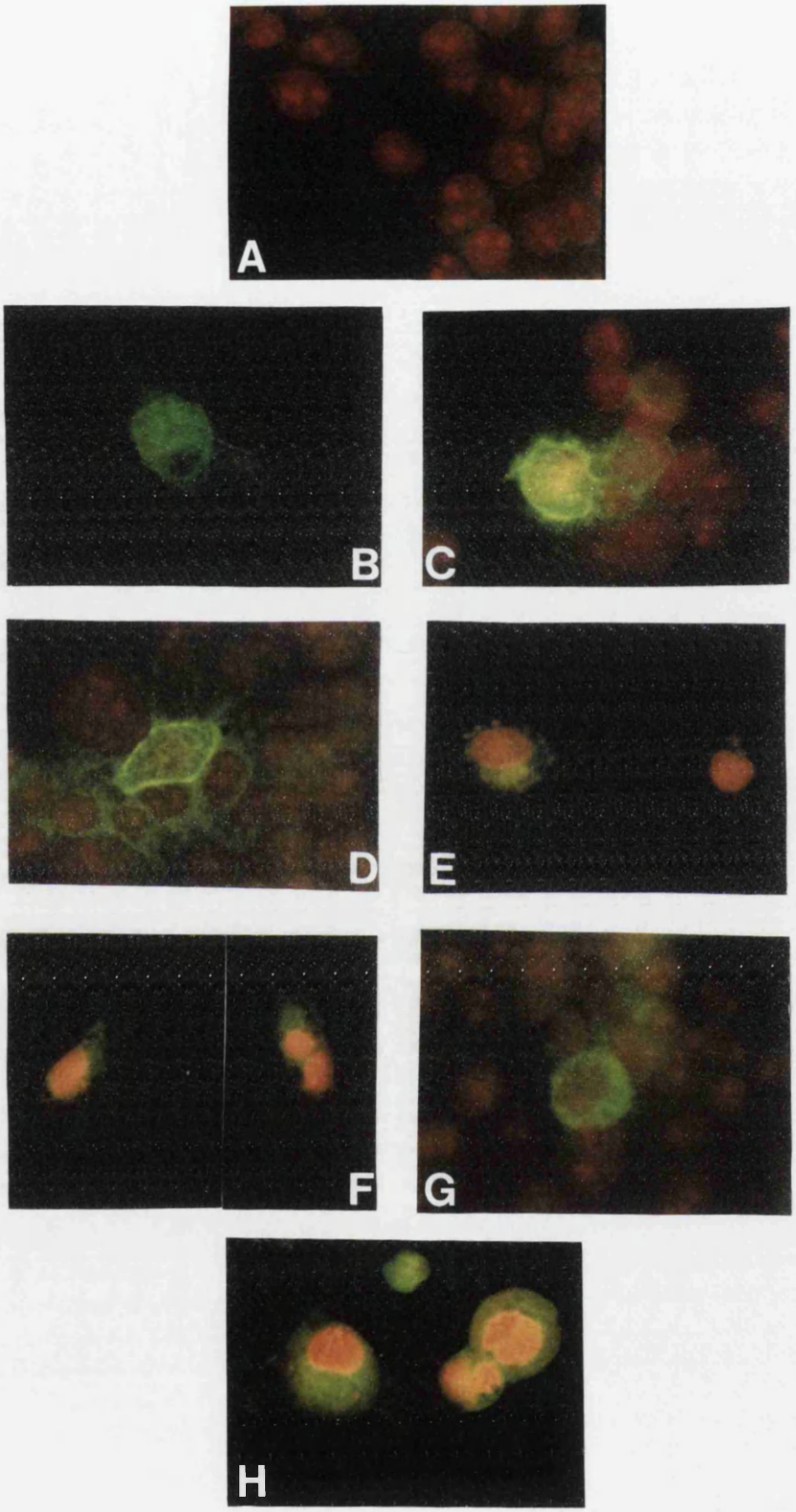


Figure 3.17 - Staining of COS-7 cells transiently transfected with CD2 wild type and mutants, with RFT11 anti-CD2. A - No DNA, B - CD2WT, C - CD2 14TM, D - CD2 12TM, E - CD2 10TM, F - CD2 8TM, G - CD2 +VE and H - COS-7 cell stained with W632.

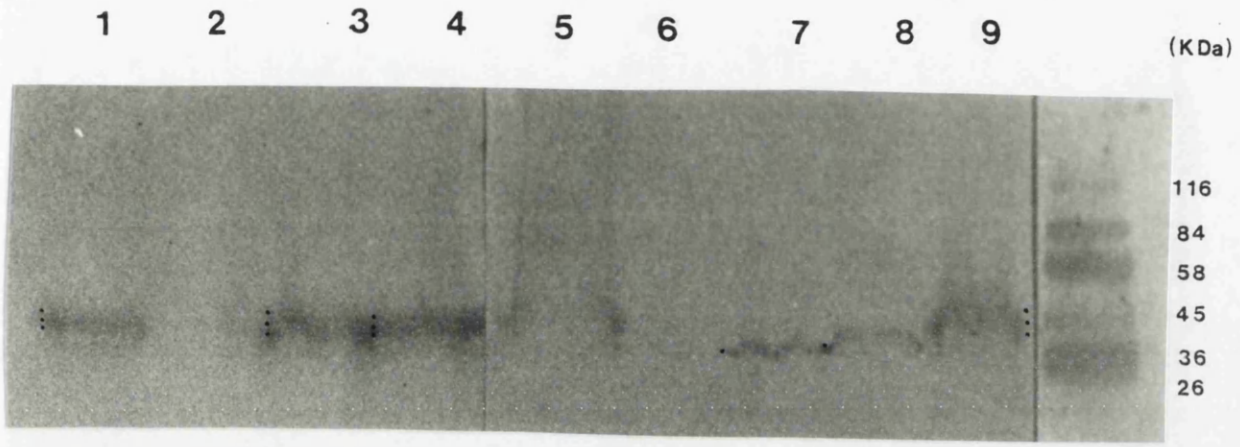


Figure 3.18 - Western blotting of CD2 wt and mutants. The protein was extracted from CHO cells and blot with RFT11 anti-CD2.

Lane 1 - CD2 WT
Lane 2 - CHO cells (negative control)
Lane 3 - CD2 14TM
Lane 4 - CD2 12TM
Lane 5 - CD2 +VE
Lane 6 - CHO cells (negative control)
Lane 7 - CD2 8TM
Lane 8 _ CD2 10TM
Lane 9 - CD2 +VE

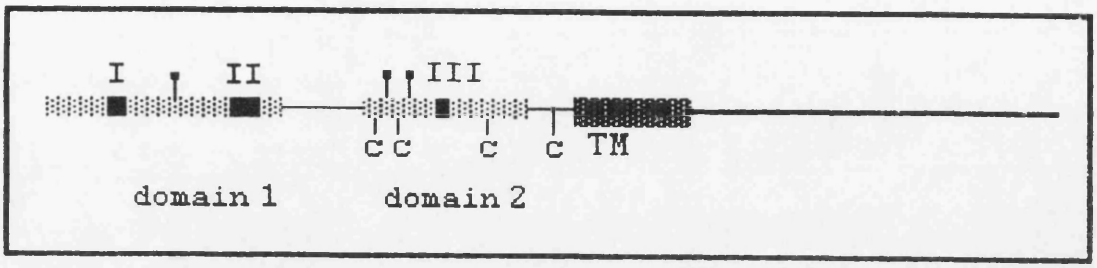
	Molecular weight of the unglycosylated form
CD2 WT	36.96 KDa
CD2 14TM	35.56 KDa
CD2 12TM	35.29 KDa
CD2 10TM	35.03 KDa
CD2 8TM	34.03 KDa
CD2 +VE	36.96 KDa

the literature (50, 53 and 55kDa, Sayre et al., 1987). These various bands observed should correspond to differential glycosylation products, since the protein possesses three putative N-terminal Asn-linked glycosylation sites. CD2 10TM and CD2 8TM produced a single band around 35-38kDa (Fig 3.18).

3.5 Further studies of epitope presentation in CD2 external domain

Once CD2 WT, CD2 14TM, CD2 12TM, CD2 +VE and CD2B7 (Hundal,1991) have shown cell surface expression by panning and immunofluorescence microscopy, the external domain conformation of these mutants was analysed. This was done by immunostaining with antibodies to different epitopes of CD2, situated in domain 1 and domain 2, according to Peterson and Seed (1987) (Fig 3.19).

COS-7 cells 48 hours after transfection were passed to slides flasks, after 24 hours they were fixed and stained with anti-CD2 GT2, specific to domain 2 and anti-mouse FITC second antibody (Fig 3.20). The wild type, +VE, 14TM, 12TM and CD2B7 mutants were positive for surface staining with anti-CD2 for domain 2. The antibody used in previous staining anti-CD2 from Seralab (clone F923A11)is specific to epitope in region II of domain 1 . As observed before CD2 WT, 14TM, 12TM, CD2 +VE (Fig 3.16) and CD2B7 (Hundal,1991) showed surface staining with this antibody. These results indicate that there is no change in the conformation of the external domains of CD2.



- extracellular domain
- transmembrane domain
- cytoplasmic domain
- linker region between extracellular domains
- glycosylation sites (N^{62}, N^{114} and N^{123})
- c - disulphide bonds (Cys^{112}/Cys^{176} and Cys^{119}/Cys^{159})

Region I - residues 48
Region II - residues -90
Region III - residues 135-136

Figure 3.19 - Schematic representation of CD2 molecule with the epitope regions of the extracellular domain.

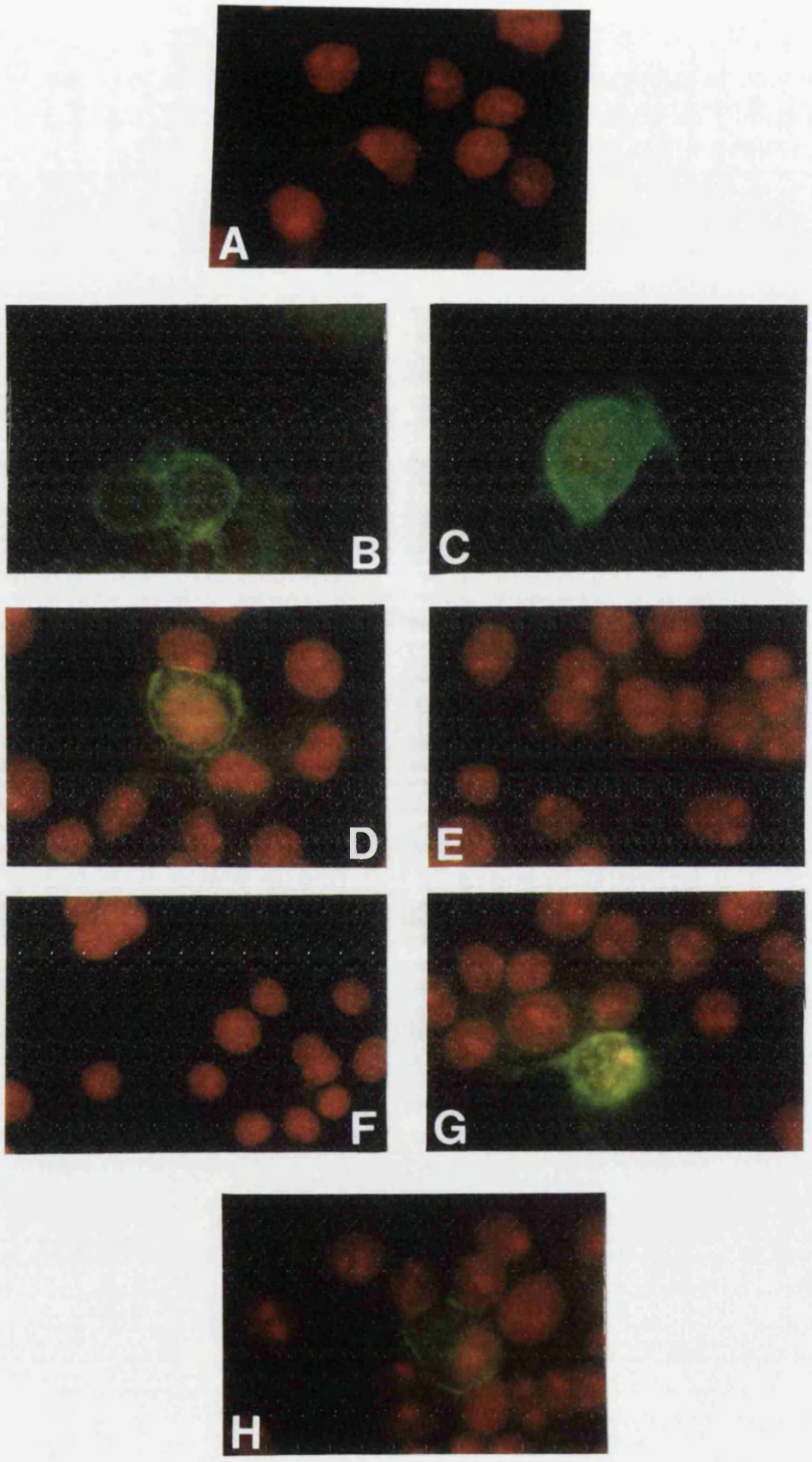


Figure 3.20 - Staining of COS-7 cells transiently transfected with CD2 wild type and mutants, with anti-CD2 GT2. A - No DNA, B - CD2WT, C - CD2 14TM, D - CD2 12TM, E - CD2 10TM, F - CD2 8TM, G - CD2 +VE and H - CD2B7.

3.6 Function of CD2 external domain

As mentioned before the first domain of CD2 carries the E rosetting receptor. In order to determine if the mutations had affected the adhesion function of the molecule, rosetting experiments with SRBC were carried out. Stable CHO cell lines of CD2 mutants were harvested and treated with SRBC. The mutants that had showed surface expression (CD2 WT, CD2 +VE, CD2B7, CD2 14TM and CD2 12TM) formed rosettes with SRBC, on the other hand CD2 10TM and CD2 8TM were negative for rosetting (Fig 3.21). These results showed that the first domain exhibits the right conformation to bind red cells, when the TM is reduced to 12 amino acids.

3.7 Lateral mobility of CD2 mutants on CHO cells

Preparation of CD2 antibody for photobleaching

Normally in photobleaching experiments the cells are labelled with the F(ab) fragment of the required antibody, to avoid cross reactions, what will interfere with the measurements of lateral mobility. The F(ab) fragment from CD2 was prepared (Fig 3.22) and labelled with FITC as described, but the staining of the cells with this antibody was very poor and the signal was not enough to the photobleaching analysis. So it was decided to use the whole CD2 IgG molecule labelled with FITC. Because the FITC labelling can be inhibited by the presence of BSA in the storage buffer of the antibody, CD2 MAb (Boehringer Mannheim) was purified by ammonium sulphate precipitation prior to FITC labelling (Fig 3.23).

Staining of the cells and photobleaching experiment

The following experiment was designed to have an estimation of the general lateral mobility of membrane glycoproteins proteins on surface of CHO cells. Cell surface

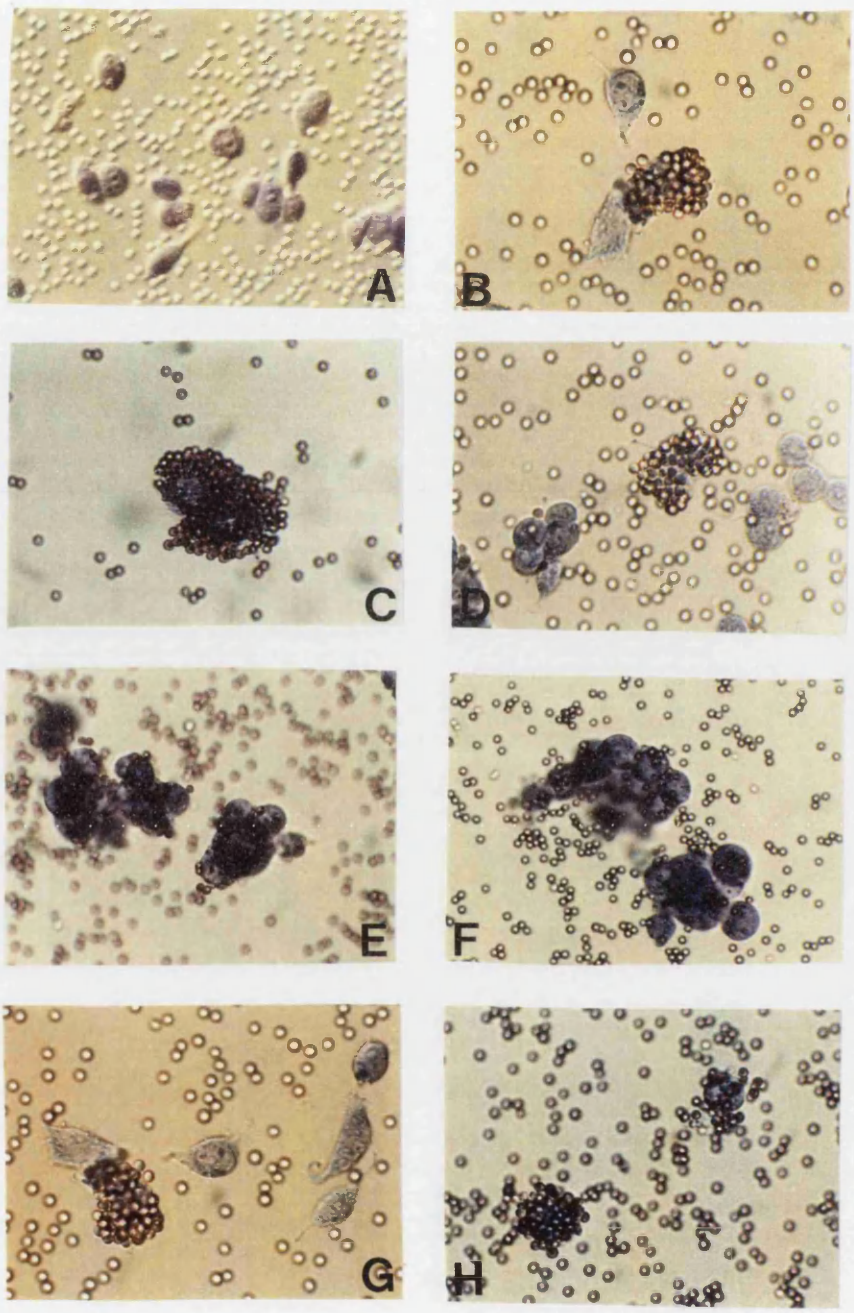


Figure 3.21 - Rosetting of CHO cells expressing CD2 wild type and mutants with sheep red blood cells. A - CHO cells, B - CD2 WT, C - CD2 14TM, D - CD2 12TM, E - CD2 10TM, F - CD2 8TM, G - CD2 +VE and H - CD2B7.

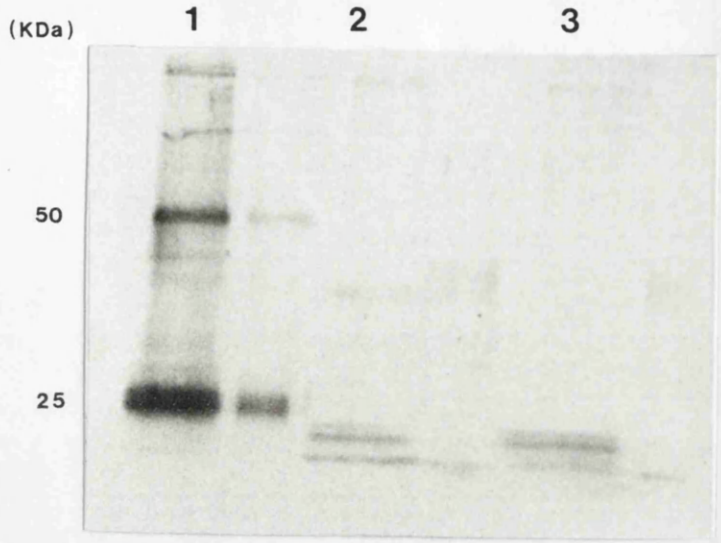


Figure 3.22 - Preparation of $F(ab)_2$ fragment from W632 and anti-CD2. SDS PAGE blot with anti-mouse AP conjugated. Under reducing conditions IgG yields two bands, one about 50 KDa and one at 25KDa; $F(ab)_2$ yield a doublet of bands at about 25KDa and Fc fragments yield one band at about 25KDa that runs slower than $F(ab)_2$ doublet.

Lane 1 W6/32 IgG

Lane 2 CD2 $F(ab)_2$ fragment

Lane 3 CD2 Fc fragment

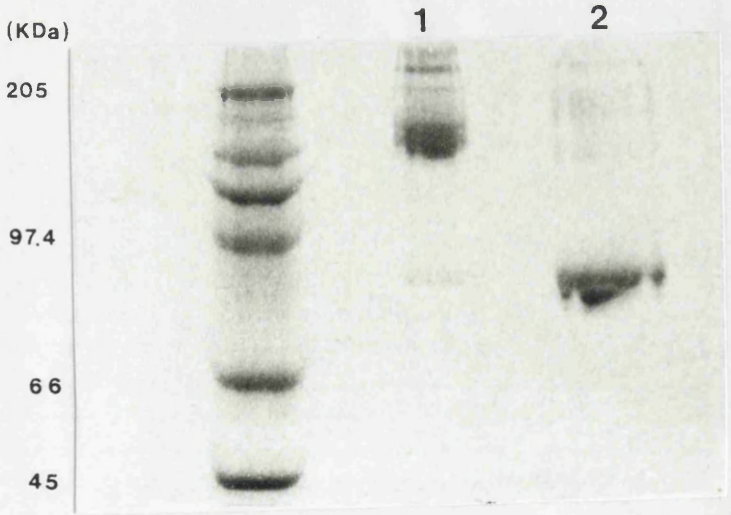


Figure 3.23 - Purification of anti-CD2 by ammonium sulphate. SDS PAGE , commassie blue staining , run under nonreducing conditions.

Lane 1 anti-CD2

Lane 2 BSA

carbohydrates were detected with succynyl concanavalin A (1:10 dilution), simultaneously the cells were stained with Hoescht probe (1:100) and propidium iodide (0.01%) to observe damage of the cells during the staining and washing process, (Fig 3.24). The Hoescht probe is a nucleus stainer and when there is a damage in the cellular membrane the inside structures will be also stained. If the cell membrane is damaged propidium iodide entries and stained the nucleus. Since photobleaching analysis has to be carried out on intact cells, propidium iodide was always used together with the FITC staining; red labelling of the nucleus is observed under the laser and those cells were discarded . The cells expressing the WT and CD2 mutants (14TM, 12TM and +ve) were stained with anti-CD2/FITC (1:10 dilution) (Fig 3.25) and the slides mounted on media containing propidium iodide. The measurements were carried out within 2 hours after staining, an average of 4 pulses/cell was stored in the computer and the curve was analysed by a computer program as described before.(Fig 3.26) The quantitative fluorescence was measured for each cell chosen to receive the bleach pulse. The data of D.L. coefficient, % of recovery and quantitative fluorescence for each mutant is summarized at Tables 3.2, 3.3, 3.4, 3.5 and 3.6. The mean, standard deviation (sd) and standard error of the mean (sem) from D.L. coefficient, % of recovery and quantitative fluorescence for each mutant and CHO labelled with concanavalin A was calculated (Table 3.7). To verify if the differences between the calculated means from CD2WT and mutants were significant or not, paired data for the wild type and mutants was analysed by Wilcoxon's signed rank and student's *t* test and $p < 0.05$ was considered significant(Table 3.9).

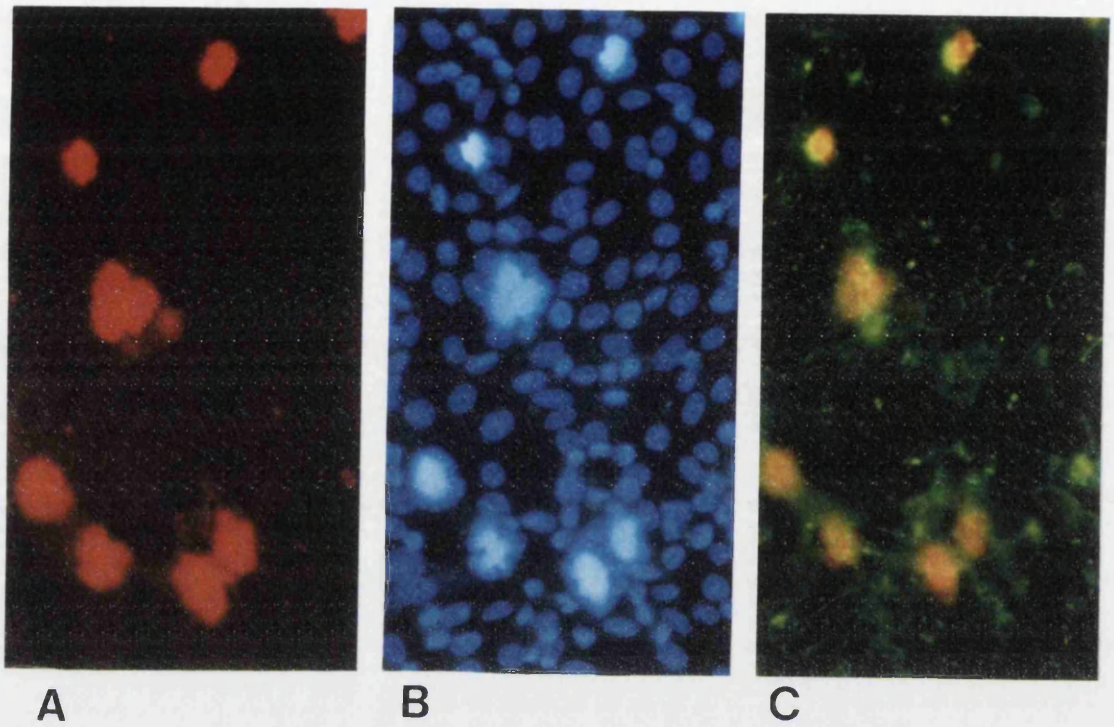


Figure 3.24 - Staining of CHO cells with:
A - Propidium Iodide
B - Hoescht probe
C - Succynyl Concanavalin A

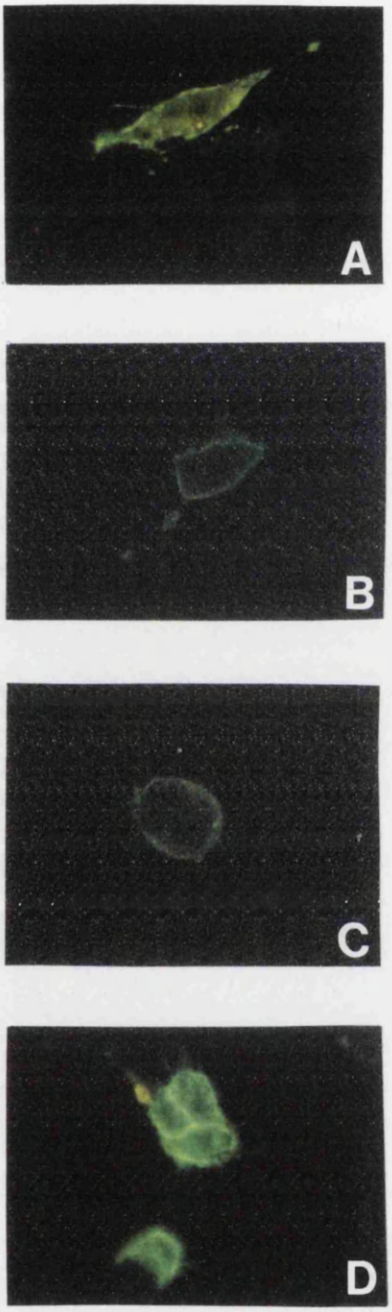


Figure 3.25 - Staining of CHO cell lines expressing CD2 wild type and mutants with anti-CD2 FITC from Boheringer Mannheim. A. - CD2 WT, B - CD2 14TM, C - CD2 12TM and D - CD2 +VE.

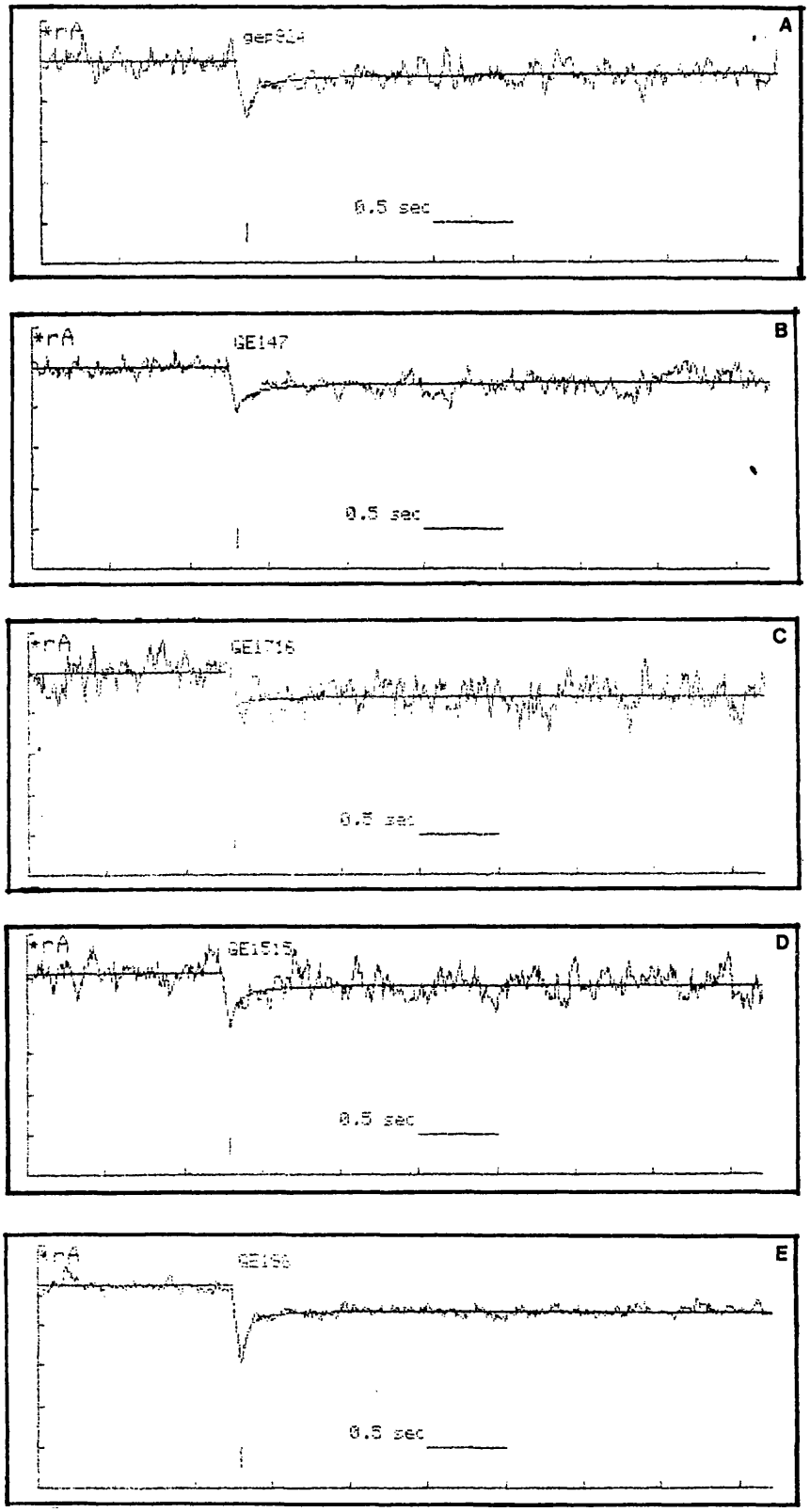


Figure 3.26 - FRAP curves for CHO cell stained with Succinyl ConA and CHO cell lines of CD2 wild type and mutants stained with anti-CD2. A - CHO, B - CD2 WT, C - CD2 14TM, D - CD2 12TM and E - CD2 +VE.

Table 3.3 - Values of D.L., % of recovery and quantitative fluorescence for CD2 WT stained with anti-CD2 (Boheringer Mannheim)

	DL ($x10^{-9}cm/s^{-1}$)	% of recovery	Quantitative fluorescence
GE622	2.18	79.5	134.9434
GE653	1.59	82.4	174.5214
GE721	0.48	79.0	214.2305
GE134	1.29	51.4	123.5566
GE1313	2.18	35.1	141.5957
GE145	1.16	81.7	32.4453
GE147	0.93	64.2	94.7851
GE1410	0.82	52.2	109.5996
GE1412	0.60	70.2	60.8125
GE165	3.35	36.1	58.3886
GE1622	6.74	54.0	116.0895
GE1627	0.29	35.0	124.1171

Table 3.2 - Values for D.L. and % recovery for CHO stained with Succynyl Concanavalin A.

	D.L. ($x10^{-10}cm^2/s^{-1}$)	% recovery
GE115	6.91	91.9
GE118	7.06	95.2
GE119	3.45	92.1
GE1110	6.82	79.9
GE1122	1.36	58.2
GE122	3.42	86.7
GE922	7.23	85.2
GE924	1.68	81.0
GE925	7.01	90.8
GE927	1.34	87.9
GE928	1.41	89.0
GE931	7.29	92.4

Table 3.4 - Values of D.L., % of recovery and quantitative fluorescence for CD2 14TM stained with anti- CD2 (Boheringer Mannheim).

	DL ($x10^{-10} cm^2 s^{-1}$)	% of recovery	Quantitative fluorescence
GE168	0.18	36.8	107.0605
GE1610	7.13	74.3	48.2597
GE1613	0.94	30.2	50.8808
GE1614	3.27	10.2	50.1660
GE1617	0.25	21.3	71.6523
GE1716	1.29	37.2	127.9238
GE1718	0.04	21.7	49.3027
GE171	3.54	79.6	30.7460
GE173	2.20	72.1	35.7539
GE175	0.40	78.0	128.3574

Table 3.5 - Values of D.L., % of recovery and quantitative fluorescence for CD2 12TM stained with anti-CD2 (Boheringer Mannheim).

	DL ($x10^{-10} cm^2 s^{-1}$)	% of recovery	Quantitative fluorescence
GE812	1.23	94.5	202.0488
GE813	9.33	99.1	63.1015
GE819	7.54	98.9	91.6757
GE822	3.74	99.3	143.5253
GE1510	7.26	91.1	50.7167
GE1513	1.33	94.8	46.5566
GE1514	0.64	88.5	58.0078
GE1515	3.47	85.3	47.0117
GE1517	0.79	40.9	39.2500
GE177	3.53	95.1	198.5390
GE1710	0.73	70.3	54.3144

Table 3.6 - Values of D.L., % of recovery and quantitative fluorescence for CD2 +VE stained with anti-CD2 (Boheringer Mannheim)

	DL($x10^{-10}cm^2s^{-1}$)	% of recovery	Quantitative fluorescence
GE1637	6.45	50.4	81.1015
GE1638	1.61	21.6	110.3398
GE1640	0.11	38.3	142.3554
GE1714	2.11	69.6	127.9238
GE191	0.11	30.6	130.8378
GE192	0.04	18.4	141.2246
GE194	0.20	75.6	113.6308
GE195	6.84	72.9	149.6914
GE198	7.64	79.5	109.4179
GE1914	1.07	62.9	114.1210
GE1915	68.69	74.5	126.1562

Table 3.7 - Means values and standard deviation (s) of D.L. coefficient, % of recovery and quantitative fluorescence calculated for CHO - Con A, CD WT, CD2 14TM, CD2 12TM and CD2 +VE.

Cell line - staining	D.L. coefficient ($10^{-10}cm^2/s^{-1}$) mean (sd/sem)	% recovery (mobile fraction) mean (sd/sem)	Quantitative fluorescence mean (sd/sem)
CHO - Con A (n = 12)	4.48 (2.58/0.84)	85.8 (10.3/3.1)	nt
CD2WT CHO - CD2 (n = 12)	1.80 (1.78/0.51)	60.0 (18.6/5.4)	115.4(50.5/14.5)
CD214TM CHO - CD2 (n = 10)	1.93 (2.22/0.70)	46.1 (26.9/8.5)	70.0 (37.3/11.8)
CD212TM CHO - CD2 (n = 11)	3.60 (3.12/0.94)	87.0 (17.4/5.3)	90.4 (61.7/18.6)
CD2+VE CHO - CD2 (n = 11)	3.00 (3.20/0.97)	54.0 (23.1/6.9)	122.4(19.4/5.8)

Table 3.8 - Values of lateral difusion(D.L) and percentage of recovery (%R) for membrane proteins referred in the literature.

Protein	D.L.(x$10^{-10}cm^2/s$) (sd/sem)	% of recovery (R)	Reference
Class -I MHC antigens	0.12(0.16/0.32)	31	Edidin and Zuniga, 1984
EGF receptor	1.2(3.56/0.7)	61 (106.9/21)	Livneh et al., 1986
Class II MHC molecules	1.0(2.05/0.56) to 3.0(2.42/0.67)	36 (25.9/8) to 73(50.5/14)	Wade et al., 1989
Human insulin receptor	6.0(5.39/0.5)	51(81.36/12)	Goncalves et al., 1993

Table 3.9 - Statistical analysis by Wilcoxon's rank signed test and student *t*-test of D.L. coefficient, % of recovery and quantitative fluorescence for paired data of CD2 WT, CD2 12TM, CD2 14TM and CD2 +VE.

	D.L. coefficient		% of recovery		Quantitative fluorescence	
	Wilcoxon's test	t'student test	Wilcoxon's test	t'student test	Wilcoxon's test	t'student test
CHO ConA/ WT	8.000' $p < 0.025$	2.989** $p < 0.01$	1.000'' $p < 0.005$	4.037*** $p < 0.001$	nt	nt
CHO ConA/ 14TM	8.000' $p < 0.05$	2.494* $p < 0.02$	0.000'' $p < 0.005$	4.537*** $p < 0.001$	nt	nt
CHO ConA/ 12TM	21.000 $p > 0.05$	0.863 $p > 0.2$	14.500 $p > 0.05$	0.211 $p > 0.5$	nt	nt
CHO ConA/ +VE	22.000 $p > 0.05$	1.316 $p > 0.1$	5.000' $p < 0.05$	4.160*** $p < 0.001$	nt	nt
WT/ 14TM	24.000 $p > 0.05$	0.146 $p > 0.5$	11.000' $p < 0.05$	1.430 $p > 0.1$	13.000 $p > 0.05$	2.355* $p < 0.05$
WT/ 12TM	18.000 $p > 0.05$	1.715 $p > 0.1$	7.000' $p < 0.05$	3.577*** $p < 0.001$	21.000 $p > 0.05$	1.067 $p > 0.1$
WT/ +VE	28.000 $p > 0.05$	1.122 $p > 0.2$	27.000 $p > 0.05$	0.693 $p > 0.4$	29.000 $p > 0.05$	0.432 $p > 0.5$
14TM/12TM	8.000' $p < 0.05$	1.400 $p > 0.1$	4.000'' $p < 0.01$	4.173*** $p < 0.001$	13.000 $p > 0.05$	0.906 $p > 0.2$
14TM/ +VE	25.000 $p > 0.05$	0.884 $p > 0.2$	22.000 $p > 0.05$	0.722 $p > 0.4$	5.000' $p < 0.05$	4.098*** $p < 0.001$
12TM/ +VE	26.000 $p > 0.05$	0.442 $p > 0.5$	7.000' $p < 0.05$	3.784** $p < 0.01$	20.000 $p > 0.05$	1.641 $p > 0.1$

* and ' - significant at 5%

** and '' - significant at 1%

*** and ''' - significant at 0.1%

Quantitative fluorescence analysis

The quantitative fluorescence data is a measure of the quantity of CD2 on the cell surface and the only difference observed was between CD2 14TM and CD2+ve. The low level of fluorescence with 14TM expressed in CHO cell line was confirmed by fluorescence microscopy. This is likely to reflect the copy number of the plasmid in CHO cels rather tahn an effect on the protein level. When COS-7 cells were transfected with CD2 14TM plasmid DNA and immunostained, no detectable difference could be observed by fluorescence microscopy between CD2 14TM and the other surface staining mutants.

Lateral mobility and percentage of recovery analysis

From the D.L. coefficient data, no significant difference was observed between the lateral mobility of the wild type and mutant proteins. When the mobile fraction was analysed, differences between CD2 WT/ CD2 12TM, CD2 14TM/ CD2 12TM and CD2+VE/CD2 12TM were considered significant. When the means for these coefficients were analysed (Table 3.10), it was noticed that CD2 12TM presents the higher percentage of recovery.

Distribution of photobleaching data

The means and standard deviations values of the present FRAP data show a scattered distribution. This is also observed in data from literature for membrane proteins (Table 3.8) and in lateral diffusion studies of lipid probes in the surface membrane of *Schistosoma mansoni* (John Kusel, personal communication).It is widely regarded that the scattered distribution of FRAP results is due to the photobleaching method which requires the analysis of repeat samples over

several days. Different days of analysis, different samples and different levels of protein expression from cell to cell are just some of the factors which may contribute to the wide variation in the data.

Table 3.10 - Comparisons of means for % of recovery and quantitative fluorescence, that were statistically significant.

Cell line	D.L. coefficient ($10^{-10}cm^2/s^{-1}$) mean (s)	% recovery (mobile fraction) mean (s)	Quantitative fluorescence mean (s)
CHO WT/14TM	ns	ns	ns
CHO WT/12TM	ns	60.0(18.6)/ 87.0(17.4)	ns
CHO WT/+VE	ns	ns	ns
CHO 14TM/12TM	ns	46.1(26.9)/ 87.0(17.4)	ns
CHO 14TM/+VE	ns	ns	70.0/122.0
CHO 12TM/+VE	ns	87.0(17.4)/ 54.0 (23.1)	ns

CHAPTER 4

DISCUSSION

4.1 Background and present study

Previous experiments have been done to determine the minimal length TM which can anchor in the cell membrane. Davis and colleagues (1985), using a prokaryotic system, made a series of overlapping deletions in the TM region of bacteriophage f1 gene III protein from 23 amino acids (wild type) to 22, 20, 16, 11, 6 and 4 amino acids. The mutants were expressed on *E. coli* and because of the presence of the outer cell wall the anchoring properties could only be investigated by alkali fractionation. The mutant proteins with TM region 22 to 16 amino acids long were still able to span the cell membrane. Mutants 11 to 6 exhibited a residual membrane association and the 4 amino acids long mutant lost the anchoring properties completely. Due to the indirect methodology used in these experiments to assay the membrane bound fraction of proteins, it was difficult to differentiate cell surface membrane signals.

Adams and Rose (1985b) created mutant forms of VSV G proteins with shortened TM domains to determine the structural requirements for a functional membrane spanning domain. The TM domain was deleted from 20 amino acids (wild type) to 18, 16, 14, 12 and 8. The mutant proteins were expressed in COS cells. Cellular localization was verified by immunostaining. Transport through the secretory pathway was determined by acquisition of endoglycosidase H (endo H) resistance. Simple oligosaccharide units are added to the polypeptide in the ER,

at this stage such units are susceptible to endo H digestion. After transport to the Golgi apparatus, these oligosaccharides are converted to a complex type which is resistant to endo H cleavage (Tarentino and Malley, 1974). The VSV G proteins with TM of 18, 16 and 14 amino acids were transported to the cell surface and were resistant to endo H. The mutant protein with 12 amino acids was not detected at the cell surface and was only about 30% resistant to endo H, indicating that a fraction of the protein reaches as far as the Golgi region. The 8 amino acid form was not detected at the cell surface and was not endo H resistant, indicating a failure in the transport to Golgi. These results demonstrated that in the case of VSV G protein, at least 14 amino acids become necessary to permit expression on the cell surface.

Doyle and colleagues (1986) introduced translational termination codons at four sites of the HA transmembrane region (composed of 27 amino acids), producing truncated forms that lack the entire cytoplasmic domain and with TM domains of 17, 14 and 9 amino acids long. The mutants were expressed on CV-1 cells and by immunostaining, hemagglutination and acquisition of endo H resistance the cellular localization and transport rate were determined. When the protein had a normal TM (27 amino acids) but no cytoplasmic domain it was detected at the cell surface and exhibited erythrocyte binding as the wild type. A low percentage of the mutant protein with TM 17 amino acids long was anchored at the cell surface at a concentration sufficient for erythrocyte binding. Some internal staining was also observed. The acquisition of resistance to endo H by this mutant was detectable only after 4 hours of chase, while in the wild type it was detected after 30 minutes. So the transport of the majority of these mutant protein between the endoplasmic reticulum and the medial Golgi is blocked or severely delayed. The 14 and 9 amino acids proteins exhibited intracellular immunofluorescent staining that was confirmed to be a ER region by endo H digestion. They

were not able to bind erythrocytes. From these results it was possible to conclude that 17 hydrophobic amino acids are sufficient to anchor HA in the membrane. This assóciation was not considered stable, maybe because this mutant also lacked the cytoplasmic domain. HA is a trimeric protein, and trimerization is a prerequisite for intracellular transport. Alterations in the cytoplasmic and transmembrane domains possibly interfere with this assembly process causing a delayed or failure in the transport to the cell surface.

In the present study the human CD2 molecule was studied. This is a eukaryotic membrane protein, that assembles in a monomeric form in the cell membrane and its structure has been determined (Sayre and Reinherz, 1988). Crystallography studies on the rat CD2 extracellular domain contributed to better understanding of this structure (Jones et al., 1992). A series of deletions were made on the TM domain shortening it from 26 amino acids (wild type) to 14, 12, 10 and 8 amino acids long. The mutant proteins were expressed on mammalian cells (COS-7 and CHO). The CD2 14TM and 12TM mutants showed cell surface expression, as demonstrated by panning and immunostaining. Quantitative fluorescence measurements indicated that these two mutants are being expressed at the cell surface at the same level that the wild type. Rosetting with SBRC confirmed the integrity of the adhesion domain of CD2 14TM and CD2 12TM, corroborating the idea that these mutants were able to span cellular membrane in a functional way. The mutant proteins were also detected on the cell surface by immunostaining with antibodies specific to domain 1 and domain 2. In the case of CD2, it seems that the TM region has to be at least 12 amino acids long to be anchored to the cell membrane in a functional state.

4.2 Comparisons between CD2 and VSV G mutants

CD2 TM mutants (This study)

CD2 WT
E^{172}PVSC^{176}PEKGLD **IYLIIGICGGGSLLMVFVALLVFYIT** KRKKQRSRR^{217}

CD2 14TM
E^{172}PVSCPEKGLD **IYLIIGICGGFYIT** KRKKQRSRR

CD2 12TM
E^{172}PVSCPEKGLD **IYLIIGICGYIT** KRKKQRSRR

CD2 10 TM
E^{172}PVSCPEKGLD **IYLIIGGYIT** KRKKQRSRR

CD2 8TM
E^{172}PVSCPEKGLD **IYLIGYIT** KRKKQRSRR

CD2B7
E^{172}PVREFPVVVPSGEEQRYTCHVQHEGLPKPLTL **GWEPSSQGAVVAAVMC** RRKSS
CD2 HLAB7 TM

VSV G mutants (Adams and Rose, 1985b)

VSV G WT
E^{455}GWFSSWK**SSIASFFFII**^{472}**GLIIGLFLVL**RVGIHLC^{489}IKLKHTKKR^{498}
TM

VSV G 18TM
E^{455}GWFSSWK **SSIASFFFIIGLGLFLVL** RVGIHLCIKLKHTKKR

VSV G 16 TM
E^{455}GWFSSWK **SSIASFFFIIGLFLVL** RVGIHLCIKLKHTKKR

VSV G 14TM
E^{455}GWFSSWK **SSIASFFFIIFLVL** RVGIHLCIKLKHTKKR

VSV G 12TM
E^{455}GWFSSWK **SSIASFFFILVL** RVGIHLCIKLKHTKKR

VSV G 8TM
E^{455}GWFSSWK **SSIAFLVL** RVGIHLCIKLKHTKKR

The numbers indicate amino acids positions, the transmembrane domain is highlighted in bold. In CD2B7 construction, the CD2 sequence is underlined with a double line and HLAB7 with single line.

Comparing the amino acids sequence of CD2 and VSV G protein mutants, it is noticeable that the hydrophobic domains from both are flanked by positively charged amino acids. Adams and Rose (1985b) suggested that an additional short hydrophobic stretch from the cytoplasmic domain could be buried into the lipid bilayer, to complete the enough length require to form an α-helix. This was verified when the mutants proteins were labelled with [^{3}H] palmitic acid. Palmitic acid is esterified to the VSV G protein at Cys^{489} at the cytoplasmic domain and it is believed to occur just as the protein enters the Golgi. The 12TM mutant showed a reduced level of labelling, which indicates that either the cysteine is not available or the mutant protein failed to reach the compartment where the palmitic acid is added. In the case of CD2, it is unlikely that additional residues could be incorporated into the membrane spanning region from the cytoplasmic domain, because they are so highly charged.

Amino acids from the external domain could be buried inside the lipid bilayer. The TM region of VSV G protein is preceded by a Lys residue at the extracellular domain, immediately before the TM region. CD2 molecule has a stalk of 11 amino acids that separates the external domain from the TM region (Glu^{172}-Pro-Val-Ser-Cys^{176}-Pro^{177}-Glu-Lys-Gly-Leu-Asp). The Tm domain is preceded by an Asp residue. The Cys^{176} together with C^{112} forms one of the disulphide bonds characteristic of CD2 external domain, that brings the bulk of the molecule close to the C-terminal. The other dis ulphide bond between C^{119} and C^{159} corresponds to the standard immunoglobulin di sulphide bond, both contribute to the tight conformation of CD2 external domain 2 (Jones et al.,1992). Such conformation did not suffer any change, as verified by immunostaining with antibody against domain 2. It seems unlikely that any of the residues from the stalk would be buried inside the lipid bilayer because they are mainly charged residues or weakly hydrophobic.At this step, the CD2B7 fusion mutant was

included. It is composed of the external domain of CD2 up to Glu^{170}, followed by The HLAB7 sequence which provided a long stalk before the B7 TM, which is 17 amino acids in length. The cytoplasmic domain was the wild type B7 domain. Cys^{174} residue from the CD2 stalk was deleted in this new construction, but another Cys 14 amino acids downstream from this site is present at the new 25 amino acid stalk. This may have contributed by forming a di sulphide bond with C^{110} to maintain the conformation of CD2 external domain. However, it was not deleted to test whether it was essential for di sulphide bo nd function. Therefore, it seems that the foreign TM region and the 45 amino acids stalk does not interfere with CD2 insertion in the membrane, or even with a different conformation the adhesion domain and epitopes are still available.

4.3 Lateral diffusion studies of CD2 mutants with short TM

The lateral mobility for membrane proteins may differ by three orders of magnitude, 10^{-8} to 10^{-11} cm^2/s (Chan et al., 1991). Membrane proteins are assumed to have a slow lateral mobility because of the interactions of the positively charged regions of the cytoplasmic domain with the acidic lipid head groups of the lipid bilayer. Interactions with other membrane proteins that are themselves anchored in the bilayer or interactions of of the molecule with cytoskeletal elements could also contribute to decrease the lateral mobility. Comparing previous observations of lateral diffusion in membrane proteins , the values vary from $0.12x10^{-10}$ cm^2/s to $6.4x10^{-10}$ cm^2/s, and the percentage of recovery, from 31% to 73% (Table 4.1). The lateral diffusion of CD2 wild type in CHO cells is $1.8x10^{-10}$ cm^2/s with 60.07% of mobile fraction, similar to the observed for membrane proteins.

Table 4.1 - Values of lateral diffusion (D.L.) and percentage of recovery (%R) for membrane proteins referred in the literature.

Protein	D.L. ($x10^{-10}cm^2/s$) (SEM)	% of recovery (R)	Reference
Class -I MHC antigens	0.12 (0.32)	31	Edidin and Zuniga, 1984
EGF receptor	1.2 (0.7)	61 (21)	Livneh et al., 1986
Class II MHC molecules	1.0 (0.56) to 3.0 (0.67)	36 (8) to 73(14)	Wade et al., 1989
Human insulin receptor	6.0 (0.5)	51 (12)	Goncalves et al., 1993

There is no significant difference between the CD2 wild type and CD2 14TM, CD2 12TM mutants lateral mobility coefficients. This suggests that large deletions in this domain do not affect the lateral diffusion of the mutant proteins in the lipid bilayer. Glycine and proline residues are considered to be classical helix breakers. Goncalves and colleagues (1993) observed that when the residues Gly^{933} - Pro^{934} within the transmembrane domain of human insulin receptor are mutated to Ala, the lateral mobility increased 2 to 3 fold. These results suggests that in this case the lateral mobility is retarded by a kinked TM domain in the wild type receptor. Comparing CD2 12TM with CD2 WT, CD2+ve and 14TM, the number of Gly residues has reduced by half, without significant effect on the lateral mobility. This implies that the interaction of the CD2 12TM transmembrane domain is not as stable as the wild type and other mutants, maybe due to an extension of the α-helix to accommodate the mutant form into the lipid bilayer. Although this seems to not interfere with the lateral mobility, it could play a role in the interaction of the mutant protein with the other membrane proteins or elements of the lipid bilayer. However, when the means for the mobile fraction are compared, there is no linear increase of such values from CD2 wild type to CD2 12TM. These results could also reflect an artificial situation, where the recovery coefficient is altered because the protein has a TM region in a limit length to be detected in the cell surface.

4.4 Intracellular localization of CD2 mutants with short TM

CD2 10TM and 8TM mutants fail to anchor the cell membrane and the immunostaining indicated an intracellular localization. It is not possible to differentiate by immunofluorescence microscopy the actual site of intracellular distribution of CD2 10TM and 8TM mutants. Poruchynsky and colleagues (1985) have shown by electron microscopy that in COS-7 cells the Golgi stacks in the juxtanuclear region are spatially interwined with an extensive endoplasmic reticulum region and the Golgi apparatus can surround concentrated elements of the ER. Human CD2 has three potential sites of N-linked glycosylation N^{62}, N^{114} and N^{123} (Bierer et al., 1989) which give to the mature protein molecular weight of approximately 45-55 KDa. On SDS-PAGE gels CD2 12TM and 14TM proteins showed patterns of bands similar to CD2 wild type,which is consistent with these mutant proteins being glycosylated and transported to cell surface through the Golgi apparatus. The mutants CD2 10TM and 8TM exhibited single bands with higher molecular weight than the expected for unglycosylated forms. It is not clear whether these mutants reached the medial Golgi apparatus, where glycosylation is completed. Addition of oligosaccharide units to the polypeptide takes place in the ER and trimming of these units begins in the ER and is completed after the glycoprotein reaches the medial Golgi apparatus, whereupon the glycoproteins become endoglycosidase H sensitive. Without data about sensitivity to this enzyme we cannot be sure how far the 10TM and 8TM passed into the Golgi. We only know they do not reach the surface in significant quantity.

It was observed that CD2 10TM and 8TM could be not stained with antibodies against epitopes localized on the extracellular domains 1 (Seralab) and 2 (GT2), the intracellular staining pattern could be visualized only when the cells were stained with the antibody against the denatured

protein (RFT11). This indicates that such domains are in a different conformation and therefore, the epitopes are not available anymore. Mutants with short TM regions, like the VSV G protein 8 amino acids mutant (Adams and Rose, 1985b) and the HA 9 amino acids mutant (Doyle, 1986) were able to be transported until the Golgi apparatus (as observed by endo H digestion), although in a slow rate. Such observations suggest that even proteins with short TM domains are able to anchored cellular membranes, but their transport to cell surface is defective maybe due to failure in the folding process, causing their transport to a degradation pathway.

4.5 The role of positively charged residues

The positive inside rule suggests that membrane proteins orientate themselves with the most positively charged end in the cytoplasm. This charged residues cluster is related with orientation of the protein and not with translocation. Studies where the topology of integral membrane proteins were altered when this positive stretch was deleted, support this idea (von Heijne, 1989; Parks and Lamb, 1991; Nilsson and von Heijne, 1990). Cutler and Garoff (1986) created mutants of Semliki Forest Virus (SFV) p62 polypeptide by altering the two basic amino acid in the cytoplasmic domain adjacent to the TM region. The wild type charge cluster of Arg-Ser-Lys was changed to Gly-Ser-Met (hydrophobic) or to Gly-Ser-Glu (weakly hydrophobic). The mutants were expressed on COS-7 cells and showed membrane association in a functionally unaltered fashion. Cutler and colleagues (1986) demonstrated by carbonate extraction of the mutants from the membrane, that the stability of the mutants in the cell membrane was reduced. This raises the question of the role of the positive charges. The current dogma is probably inapplicable to membrane proteins with one spanning domain, when the leader sequence should orientate the rest of the protein across the membrane.

We mutated the cytoplasmic positive cluster Lys-Arg-Lys-Lys, immediately adjacent to CD2 TM , to something less positive like Gln-Gln-Gln-Gln. The mutant reached the cell membrane in a functional state as observed by panning, immunostaining and western blotting. Rosetting experiments demonstrated that the extracellular domain did not exhibit any conformational change or signs of inverted topology. This implies that substituting these residues for something less positive does not alter the orientation of CD2 molecule in the lipid bilayer. This result could be also due to the other sequence of positively charged amino acid (Arg-Ser-Arg-Arg) situated immediately downstream this mutated sequence. This sequence was left behind because it was assumed that the positively charged amino acids (Lys-Arg-Lys-Lys) interact with the acidic heads of the lipid components of the membrane, contributing to stabilization of the molecule within the membrane. The substitution of these amino acids would disrupt this interaction, consequently even the Arg-Ser-Arg-Arg sequence left behind would not be in contact with the membrane anymore. It was also possible that removal of the further positively charged amino acids would disrupt the organization of the cytoplasmic domain, changing the behaviour of the molecule. We have disrupted the positive cluster without phenotypic effect. Therefore, to know if this downstream positively charged sequence is stabilizing the protein insertion into the membrane, further mutagenesis in this sequence should be done.

The CD2+VE mutant have not shown any significant difference as regards to lateral mobility and percentage of mobile fraction in comparison with the wild type CD2 protein. This lead us to deduce that there is a stable association of this mutant with the membrane even when the number of positively charged amino acids flanking the the TM domain at the cytoplasmic side was reduced. One of the reasons membrane proteins show a slow

lateral diffusion is because interactions of the cytoplasmic domain with the lipid bilayer can retard the lateral mobility of the molecule (Jacobson et al., 1987). Large deletions in this domain do not affect the lateral mobility of epidermal growth factor (EGF) receptor (Livneh et al., 1986) and class I MHC antigen (Edidin and Zuniga, 1984), although in none of these cases the positive cluster situated at the cytoplasmic side of the TM domain was deleted. Wade and colleagues (1989) observed that deletion of all amino acids of the cytoplasmic domain of both α and β chains of the murine class II MHC antigen, resulted in a 10 fold increase of lateral mobility coefficient. This indicates that this domain is important in constraining the lateral mobility of these molecules. Wier and Edidin (1988) noticed that the loss of glycosylation of the glycoprotein L^d of the class I MHC increased the lateral mobility coefficient. This protein has three sites of glycosylation, and such results indicates that the external domains of cell surface glycoproteins also interact with other nearby molecules. Chang and cols (1989) in an attempt to demonstrated the role of the cytoplasmic domain of CD2 in signal transduction, created some mutants with deletions in this domain. A particular mutant with a cytoplasmic domain composed only by two residues Lys-Arg has shown an unstable surface expression and functional analysis was not possible. This should be due to misfolding because of the short cytoplasmic domain, although VSV G proteins mutants with only three residues in the cytoplasmic domain (Arg-Val-Gln and Lys-Val-Lys) were still able to anchor the cell surface (Puddington et al., 1986).

4.6 Hydrophobic and positively charged sequences within mature secreted proteins

The way a stop transfer signal halts the translocation of the polypetide across the membrane is related to its association with protein-conducting pore (channel) situated in the membrane. There are some hypothesis about the interaction of the protein with components of this pore (see Introduction, Lingappa, 1991; Simon and Blobel, 1991). The hydrophobic domain stop the translocation of the polypeptide chain, disrupting such structure. However, in the case of secreted proteins, the hydrophobic and positively charged stretches are passing across the pore without disruption. This implies that either the length of the hydrophobic stretch or the position of the positively charged residues should interfere with the translocation.

Computer searches was done in order to look for hydrophobic sequences and positively charged residues in secreted proteins. Hypothetical sequences of highly hydrophobic 18, 16, 14, 12, 10 and 8 amino acids were created and matched sequences in secreted proteins were searched in a data base. Hydrophobic sequences that vary from 7 to 8 amino acids long (excluding signal sequences) were found (Table 4.2). Hypothetical sequence of at least 4 positively charged amino acids (similar to the observed in CD2) was created and matched to sequences in the data base. Sequences varying from 4 to 6 positively charged amino acids were found (Table 4.3).

Table 4.2 - Hydrophobic stretches (in bold) in secreted proteins, excluding signal sequences.

Secretory glycoprotein gX (Δ=498) (Rea et al., 1985)	V^{146}LQPGLYDASG**LYIVVLVF**GDDAYL^{170}
Serum albumin precursor - pig (Δ=605) (Weinstock and Baldwin, 1988)	E^{162}IARRHPYFYAPE**LLYYAIIY**KDVF^{186}
Serum albumin precursor - human (Δ=558) (Eiffert et al., 1984)	G^{171}RIRLDIQGTGQ**LLFSVVI**DELRLS^{195}
Secretory component - human (Δ=609) (Lawn et al., 1981)	K^{341}NYAEAKD**VFLGMFLY**EYARRHPD^{364}
Secretory component - human (Δ=693) (Krajci et al., 1989)	E^{86}NAKQKRKGLYKQIG**LYPVLVI**DSS^{110}
Beta lactoglobulin I - donkey (Δ=162) (Godovac et al., 1988)	A^{94}LDSDYKN**YLLFLCM**KNATPGQSLV^{118}

Table 4.3 - Sequences of positively charged residues (in bold) in secreted proteins.

Human RD protein (Δ=382) (Speiser and White, 1989)	E^{11}EALQKKFNKL**KKKKK**ALLALKKQSS^{37}
Human ovarium inhibins (Δ=426) (Mason et al., 1986)	E^{249}SGASLVLLG**KKKKK**EEEGEKKKGG^{274}
Human ovarium inhibins (Δ=426) (Mason et al., 1986)	R^{298}QSEDHPH**RRRRR**GLECDGKVNICC^{322}
Bovine interferon - gamma (Δ=166) (Cerreti et al., 1986)	N^{141}DLSPKSNL**RKRKR**SQNLFRGRRAS^{165}
Platelet - derived growth factor (Δ=211) (Betsholtz et al., 1986)	K^{72}HVPEKRPLPI**RRKR**SIEEAVPAVCK^{97}
Platelet - derived growth factor (Δ=211) (Betsholtz et al., 1986)	L^{184}NPDYREEDTGRPRESG**KKRKRKR**L^{209}

Δ represents the amino acid length of the protein and numbers represent the amino acids position.

Comparing sequences of table 4.2 with CD2 8TM transmembrane region (EPVSCPEKGLD**IYLIGYIT**KRKKQRSRRND, the bold represents the TM region) not much difference is observed, apart from the positively charged cluster immediately after the hydrophobic stretch. At the secretory glycoprotein gX, the hydrophobic sequence could be extended to 12 amino acids, flanked by Asp residues at both sides, and the sequence is similar to CD2 12TM. In the case of the three mutants CD2 12TM, 10TM and 8TM, the positively charged sequence could be contributing to their membrane anchoring, since translocation of positively charged amino acids through the hydrophobic domain uses considerable free energy. Some of the secreted proteins exhibit the hydrophobic domain flanked by positive charge like serum albumin precursor (pig), human secretory component and beta lactoglobulin (donkey), it seems that one or two positive charges adjacent to a short hydrophobic domain does not have the same effect.

Table 4.3 highlights a further feature of the protein translocation apparatus, namely strings of positively charged sequences that can translocate the cell membrane, through the protein conducting pore. However, in the examples above the positive charges were not observed adjacent to hydrophobic domains. It may be that in the case of CD2 12TM, CD2 10TM and 8TM, the positively charged sequence that follows the TM prevents the polypeptide from translocating through the pore. Consequently the pore structure would disassembled and the polypeptide chain will interact with the lipid bilayer with a transmembrane topology of a mature protein. Its subsequent fate (export or degradation) will then be influenced by conformational considerations in the flanking domains. This may help explain why hydrophobic runs of amino acids can be secreted (Table 4.2) unless they are present in membrane proteins. To determine if the positive cluster adjacent to the TM is influencing in the insertion of CD2 12TM or in the

intracellular localization of CD2 10TM and 8TM, deletion or substitution of such positive sequence should be done.

CHAPTER 5

CONCLUDING REMARKS

The topology of integral membrane proteins is determined in the early stages of the polypeptide translocation across the protein-conducting channel in the ER membrane. The localization and structure of the signal peptide and transmembrane domain contribute to this orientation, and positively charged amino acids situated at the cytoplasmic side of the TM is more likely to be responsible by the stabilization of this interaction.

CD2 transmembrane domain has to be composed of at least 12 hydrophobic amino acids to span the lipid bilayer in a stable association. Although it was not determined if 12 amino acids are enough to stop translocation and disrupt the pore structure. Maybe the positively charged amino acids situated immediately downstream of this domain could be contributing to this insertion. Mutants forms with shorter TM domains have their transport to the cell surface blocked, and the truncate forms should be degraded. It was assumed that the positive cluster situated immediately after the TM region of mutants CD2 10TM and 8TM, was also responsible for their intracellular localization. It is unlikely that the α-helix structure of the TM of these mutants is halting the translocation of the molecule across the cell membrane. Additional mutagenesis studies, where this positive cluster in the CD2 12TM, 10TM and 8TM cytoplasmic domain is either removed or substituted by something less positive would bring more information about the transport of these mutants through the

secretory pathway and the length of the hydrophobic sequence necessary to stop translocation across the protein-conducting pore. Further studies will be also necessary to establish the intracellular localization of the CD2 10TM and CD2 8TM. Electron microscopy of structure of the endoplasmic reticulum from cells expressing these two mutants should reveal more about their intracellular localization .

The lateral diffusion experiments suggested that larger deletions in the TM domain do not interfere with the lateral mobility of a membrane protein. CD2 12TM exhibited a higher percentage of molecules free for diffusion. This suggests that this molecule is not associated to the lipid bilayer in such a stable way as the CD2 wild type, 14TM and CD2+ve. Maybe due to an alteration of this structure to accommodate the molecule into the membrane in a functional state. Both ends of the transmembrane domain are marked by positively charged amino acids (a higher number is found at the cytoplasmic side) and it seems unlike that any of these would be buried inside the lipid bilayer as part of the transmembrane domain.

The common basic box situated in the cytoplasmic domain adjacent to the TM was disrupted. It was expected either changes in the orientation of this mutant within the membrane, or a less stable interaction of the molecule with the lipid bilayer. It is assumed that these positively charged amino acids will interact with the acidic heads of the lipid components of the membrane. The substitution of these amino acids should interfere with this interaction. The stable association of CD2+ve mutant with the cell membrane, revealed by lateral diffusion studies, lead us to two conclusions. The 26 amino acids TM domain is enough to maintain a stable association with the lipid bilayer and the positive charged amino acids are not required for this. The positive sequence

left behind may still have been able to interact with the lipid bilayer, stabilizing this association. Further deletions of the positively charged sequence left behind should also be carried out to verify if any change in the topology would occur.

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APPENDIX

I - Abbreviations

Ab	- Antibody
Amp	- Ampicilin
APS	- Ammonium persulphate
ATP	- Adenosine triphosphate
Bis-acrylamyde	- N,N' methylene-bisacrylamyde
BiP	- H chain binding protein
BSA	- Bovine serum albumin
CD2	- Cluster of differentiation molecule 2
cDNA	- copy DNA
CIP	- calf intestinal phosphatase
dH_2O	- distilled water
DMSO	- Dimethyl sulfoxide
DNA	- Deoxyribonucleic acid
dNTPs	- Deoxynucleotides triphosphates
DTT	- Dithiothreitol
EDTA	- Ethylenediaminetetra acetic acid
ER	- Endoplasmic reticulum
FRAP	- Fluorescence recovery after photobleaching
FITC	- Fluorescein isothiocynate
GT	- Glucose transporter
HA	- Hemagglutinin
HEPES	- N-2- Hydroxyethylpiperazine-N-2-ethane Sulphonic acid
HLA	- Human leukocyte antigen
Ig	- Immunoglobulin
IgG	- Immunoglobulin G
Il-2	- Interleukine 2
IPTG	- Isopropylthio-B-D-Galactoside
Km	- Kanamycin

LFA	- Lymphocyte function associated
MAb	- monoclonal antibody
MHC	- Major histocompatibility complex
mRNA	- messenger RNA
Page	- Polyacrylamyde gel electrophoresis
PBS	- Phosphate buffered saline
PCR	- Polymerase chain reaction
RER	- Rough endoplasmic reticulum
RNA	- Ribonucleic acid
RNase	- Ribonuclease
rRNA	- Ribosomal RNA
RT	- Room temperature
SDS	- Sodium dodecyl sulphate
SRBC	- Sheep blood red cells
SRP	- Signal recognition particle
SV40	- Simian virus 40
TBS	- Tris buffered saline
TCR	- T cell receptor
TEMED	- N,N,N',N' - tetramethylenediamine
Tet	- Tetracycline
TM	- Transmembrane
TRAM	- Translocating chain-associating membrane protein
TRAP	- Translocating assembly protein
tRNA	- Transfer RNA
T/E	- Trypsin / EDTA
VSV G	- Vesicular stomatitis virus G protein
Xgal	- 5-bromo-4-chloro-3-indolyl-β-galactosidase

II Units

A	- amps
mA	- miliamps
bp	- base pair
Kb	- Kilobase pair

°C	- degrees centegrade
Da	- Daltons
KDa	- Kilodaltons
g	- grammes
Kg	- Kilogrammes
mg	- milligrammes
µg	- microgrammes
ng	- nanogrammes
Hz	- Hertz
l	- litres
ml	- millilitres
µl	- microlitres
m	- metres
cm	- centimetres
µm	- micrometres
min	- minutes
M	- Molar
mM	- millimolar
µM	- micromolar
pmoles	- picomoles
sec	- seconds
ms	- miliseconds
µs	- microseconds
rpm	- revolutions per minute
krpm	- kilorevolutions per minute

III - Chemicals

General chemicals and solvents	- BDH
Media	- Difco, Oxoid
Biochemicals	- BDH, BRL, Sigma
Agarose	- BRL
Antibiotics	- Gibco-BRL, Sigma
Restriction enzimes	- BRL, Promega
DNA modifying enzymes	- BRL, Promega, USB

Antibodies	- SeraLab, Serotec, Boehringer Manheim

The CD2GT2 antibody was kindly given by Prof. Alain Bernard.

Immunological reagen	- Sigma, Promega
Tissue culture media	- Gibco
Fetal Calf Serum	- Gibco

IV Equipment

Centrifuges	- Eppendorf 5413, 54145 and 5415, MSE microcentrifuge, Beckman microfuge E, MSE minor S bench top centrifuge
Spectrophotometers	- Beckman Du 50, LKB ultraspec 4050
Sequencing apparatus	- IBI and BRL LKB 2197 power supply
Agarose gel kits	- IBI
Protein gel kit	- Biorad
Electroblotter	- Biorad
Microscopes	- fluorescent - Leitz phase contrast - Zeiss inverted - Leitz
Power supplies	- Consort - Flowgen
Balances	- Sartorius
Membranes	- Nitrocellulose - Hybaid
Pipetmans	- Finnpipettes
X-ray film	- Kodak hyper film