Characterisation of Protease Activated Receptors

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Preface

The work presented in this dissertation is the result of my own work except where otherwise indicated. No part of it has been submitted to any other board for any other qualification.

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Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCA	bicinochoninic acid
BCIP	bromochloroindolyl phosphate
bp	base pair
BSA	bovine serum albumin
[Ca2+]i	intracellular calcium concentration
cAMP	adenosine 3':5'-cyclic monophosphate
CCL39	Chinese hamster lung fibroblast cell line
cDNA	complementary deoxyribonucleic acid
CDR(s)	complementarity-determining region(s)
Ci	Curie
CNBr	cyanogen bromide
DAB	diaminobenzidine
DEPC	diethylpyrocarbonate
DIP	diisopropylfluorophosphate
dCTP	deoxy-cytosine triphosphate
DMEM	Dulbecco's modified minimum essential medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxy-nucleotides
D1.3	anti-hen egg lysozyme antibody
D1.3Loop	recombinant mutant scFv anti-hen egg lysozyme antibody incorporating a loop from thrombin
(dT)3	poly-deoxythymidine
(dG)6	poly-deoxyguanidine
EDTA	ethylenediaminetetra-acetic acid
EGTA	[ethylenebis(oxyehylenenitro)] tetra-acetic acid
ELISA	enzyme-linked immunosorbant assay
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FR(s)	framework region(s)
GAPDH	glyceraldehyde phosphate dehydrogenase
GST	glutathione S-transferase
HAT	hypoxanthine, aminopterin, thymidine
HBSS	Hank's balanced salt solution

HEL	hen egg lysozyme
HEPES	N-(2-hydroxyethyl)-1-piperazine-N'-2-ethanesulphonic acid
hirugen	N-acetyl hirudin 53-64; Ac-NGDFEEIPEEY^{SO3}L
hirulog1	hirulog-1; D-FPRPGGGGDGDFEEIPEEYL
HPRT	hypoxanthine phosphoribosyl transferase
HRP	horse-radish peroxidase
hPAR-2	human proteinase activated receptor-2
hr	hour
HUVECs	human umbilical vein endotheial cells
Ig	Immunoglobulin
IFA	Incomplete Freunds adjuvant
IMAC	immobilised metal affinity chromatography
InsP3	inositol 1,4,5-trisphosphate
IPTG	isopropylthiogalactoside
Kanr	Kanamycin resistance
Kb	kilo base pairs
kDa	kilodalton
KLH	Keyhole limpet haemocyanin
L	light
LPS	lipopolysaccaharide
Mabs	monoclonal antibodies
MAP	multiple antigenic peptide
MAPKs	mitogen activated protein kinases
M-MLV	moloney murine leukemia virus
MOPS	3-[N-morpholino] propanesulphonic acid
mPAR-2	mouse proteinase activated receptor-2
mRNA	messenger RNA
NBT	nitro-blue tetrazolium
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAP	peroxidase-anti-peroxidase
PAR-2	proteinase activated receptor-2
PBS	phosphate buffered saline
PBSBT	phosphate buffered saline plus 1%(w/v) BSA and 0.1%(v/v) Tween-20
PBST	phosphate buffered saline plus 0.1%(v/v) Tween-20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGI2	prostacyclin

PI3K	phosphoinositide 3-kinase
PI-3,4,5-trisP	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PNGase F	peptide N-glycosidase F
preproET1	pre-pro-endothelin-1
PIP2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PMSF	phenylmethane-sulphonyl-fluoride
PRP	platelet rich plasma
RGD	Arg-Gly-Asp
rIDP	poly-inosine
RF	replicative form
RNA	ribonucleic acid
RnaseA	ribonuclease A
RNasin	ribonuclease inhibitor
RT	reverse transcriptase
Ser195Ala	mutated thrombin (Ser195 substituted by Ala)
scFv	recombinant single chain antibody variable region fragment
SDS	sodium dodecyl sulphate
Sf9	*spodoptera frugiperda* 9 cells
SOE	splicing by overlap extension
ss	single stranded
SSC	standard sodium citrate/sodium chloride
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TRAP	thrombin receptor agonist peptide
TR 38-60	thrombin receptor peptide comprising residues 38-60
TR 42-50	thrombin receptor peptide comprising residues 42-50
TR 53-64	thrombin receptor peptide comprising residues 53-64
TBSBT	Tris buffered saline plus 1% (w/v) BSA and 0.1% (v/v) Tween-20
TBST	Tris buffered saline plus 0.1%(v/v) Tween-20
TLCK	tosyl-lysine-chloromethylketone
TMB	tetramethylbenzidine
Tos-Lys-CH2Cl	tosyl-lysine-chloromethylketone
Tos-Arg-CH2Cl	tosyl-arginine-chloromethylketone
TPCK	tosyl-phenylalanine-chloromethylketone
t-PA	tissue plasminogen activator

TRED	thrombin receptor extracellular domain
T7 POL	T7 polymerase
Tween-20	polyoxyethylene(20) sorbitan monolaurate
V	variable
VH	variable heavy
VL	variable light

Summary

The thrombin receptor is a seven transmembrane G-protein-coupled receptor that is activated by thrombin cleavage. In order to characterise the thrombin receptor three mouse monoclonal antibodies were isolated by using a recombinant fusion protein incorporating the extracellular domain of the human thrombin receptor as an antigen.

An affinity matrix prepared from one monoclonal antibody was used in the successful purification of the native receptor from human platelet membranes. Two of the monoclonal antibodies were capable of blocking thrombin-induced platelet aggregation, and this may lead to their use as possible platelet thrombin receptor antagonists. To characterise further the monoclonals, their epitopes were mapped. In addition the cDNA encoding the antibodies variable regions were cloned and sequenced. By using this procedure, the amino acid sequence of the antibody complementarity determining regions (CDRs) involved in binding to the target epitope were determined.

The recently discovered human protease activated-2 receptor (hPAR-2) was studied using rabbit polyclonal antibodies generated with a multiple antigenic peptide comprising a region around the protease cleavage site. Immunocytochemistry and flow cytometry using affinity purified antibodies detected expression of hPAR-2 on human umbilical vein endothelial cells, keratinocytes and granulocytes. The expression of both PAR-2 and thrombin receptor were analysed using Northern analysis in a wide variety of murine haematopoietic cell lines. This analysis revealed the widespread distribution of the thrombin receptor. In contrast expression of PAR-2 was not observed in haematopoietic cells, suggesting that it is a marker of terminal differentiation in granulocytes. To identify potential activators of PAR-2, a variety of serine proteases were tested using a chloromethylketone inhibitor based on the peptide sequence of the cleavage site. These experiments revealed three potential trypsin-like enzymes that may activate PAR-2 *in vivo* namely, pancreatic trypsin, mast-cell tryptase and acrosin.

Thrombin is a serine protease that plays a central role in blood coagulation. Its specificity is determined by interactions with both the active site and a region distant from the active site called the anion-binding exosite. A surface loop from the thrombin anion-binding exosite, involved in binding to the hirudin-like domain of the thrombin receptor was grafted onto a recombinant single chain antibody using protein engineering. Molecular modelling indicated that incorporation of this loop into a framework region of the well-characterised anti-lysozyme antibody D1.3 would result in a chimeric molecule that would both bind its target antigen hen egg lysozyme, and also bind the platelet thrombin receptor through the engineered loop. The mutant antibody was unfortunately expressed as an incorrectly folded protein.

Chapter 1

Introduction

1.1 Serine Proteases

1.1.1 Roles of serine proteases

Serine proteases were some of the first enzymes to be extensively studied (Neurath, 1984). Interest in this group of enzymes has been maintained, in part, by an increasing recognition of their involvement in a host of physiological processes, ranging from generalised protein digestion to more specific regulated functions such as blood coagulation, fibrinolysis, the complement cascade the release of hormones and pharmacological active peptides from precursor proteins, fertilisation, and the control of cell differentiation and development (Neurath, 1989; Stroud, 1974). The basic function of a serine protease is the cleavage of peptide bonds and this enzymatic mechanism has been well characterised (Kraut, 1977) (Figure 1.1). The mammalian serine proteases (such as chymotrypsin, trypsin and elastase) differ in their cleavage specificity, although each has the same catalytic residues present in its active site (Perona and Craik, 1995).

The interaction of proteases with their substrates and, by analogy, with their inhibitors can be described using the system of nomenclature introduced by Schechter and Berger (1967). The substrate residues are numbered as follows Pn -···P3 - P2 - P1 - P1'- P2' - P3'-···Pn' where cleavage occurs at the P1 - P1' bond. Subsites in the catalytic site of the protease that interact with the substrate are designated S and are numbered to complement those that interact with the substrate; S3, S2, S1, S1', S2', S3'. The S1 subsite is often termed the "primary specificity pocket" of the protease. Enzymes described as "trypsin-like" cleave preferentially after arginine or lysine and these residues bind in the S1 subsite pocket. Whereas, "chymotrypsin-like" enzymes cleave after large hydrophobic residues such as phenylalanine, tyrosine and tryptophan, so that chymotrypsin-like proteases have a large non-polar S1 pocket. "Elastase-like" enzymes cleave after small

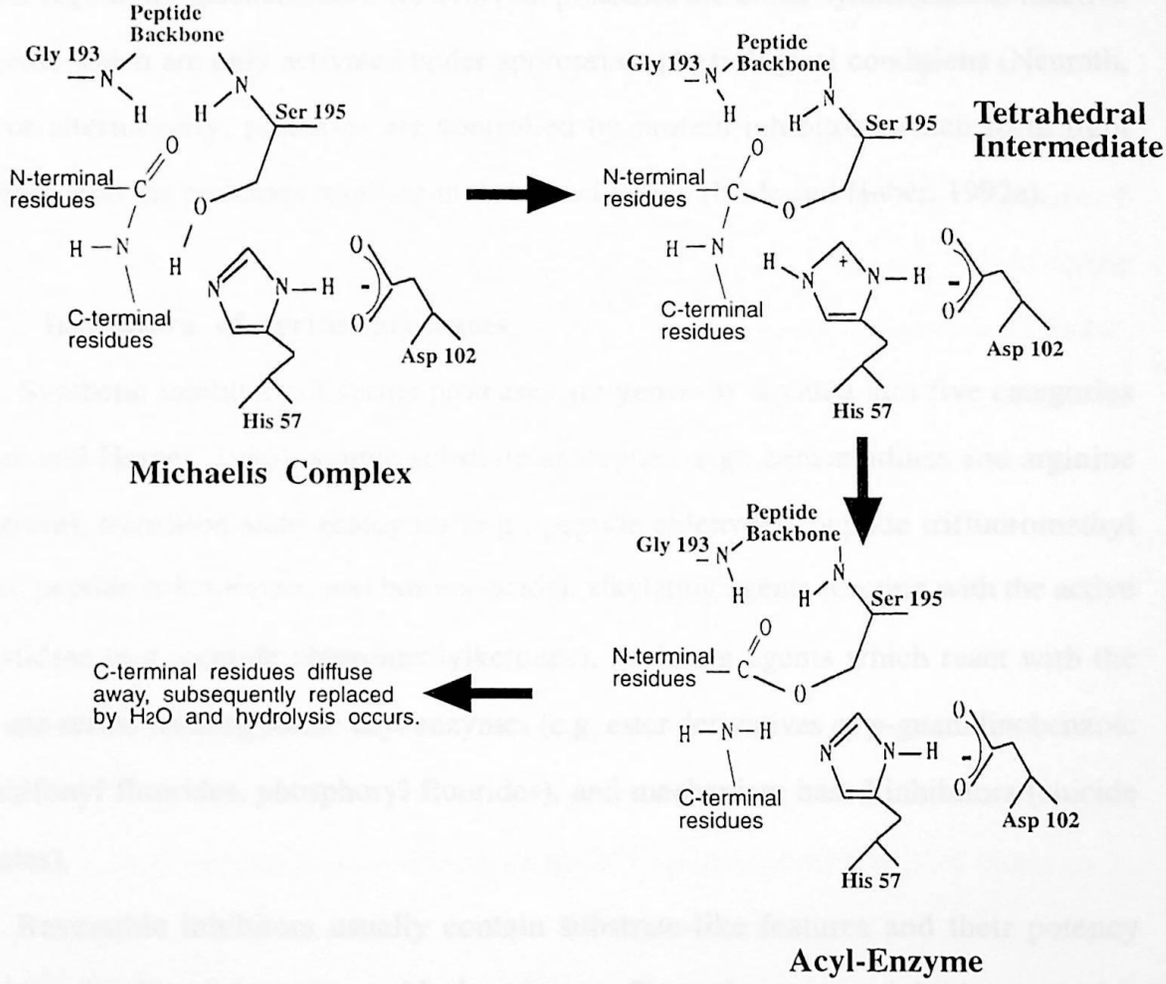


Figure 1.1 *Serine protease catalytic mechanism.*
Serine proteases possess a catalytic triad comprising His 57, Asp 102 and Ser 195 (chymotrypsin numbering system). During catalysis, the substrate first binds to form a non-covalent complex known as the Michaelis complex. It is then attacked by the Oγ of the catalytic serine to form a tetrahedral intermediate and a proton is transferred from Ser 195 to His 57. The resulting positively charged His 57 is stabilised due to an ion pair with Asp 102. The negative charge on the carbonyl oxygen in the tetrahedral complex is stabilised by hydrogen bonding interactions to backbone amide groups in the so called "oxyanion hole". The tetrahedral intermediate subsequently forms an acyl complex with the enzyme and the scissile bond of the peptide is broken. C-terminal residues of the substrate are released after protonation by His 57. Deacylation proceeds essentially by a reversal of the same mechanism; His 57 activates a water molecule. The hydroxyl of the water molecule attacks the carbonyl carbon of the acyl intermediate to form a tetrahedral intermediate. The proton of the water molecule is first transferred to His 57 and then to Ser 195 as the tetrahedral intermediate breaks down to yield free peptides.

residues such as alanine (Stroud, 1974).

The activity of proteases must be controlled, since unregulated proteolytic activity would be capable of destroying the protein components of cells and tissues. Hence two principal regulatory mechanisms have evolved: proteases are either synthesised as inactive zymogens, which are only activated under appropriate physiological conditions (Neurath, 1989) or alternatively, proteases are controlled by protein inhibitors which form tight complexes with the proteases resulting in their inactivation (Bode and Huber, 1992a).

1.1.2 Inhibitors of serine proteases

Synthetic inhibitors of serine proteases are generally divided into five categories (Powers and Harper, 1986): simple substrate analogues (e.g., benzamidines and arginine derivatives), transition state analogues (e.g., peptide aldehydes, peptide trifluoromethyl ketones, peptide α-ketoesters, and boronic acids), alkylating agents reacting with the active site histidine (e.g, peptide chloromethylketones), acylating agents which react with the active site serine forming stable acyl enzymes (e.g, ester derivatives of *p*-guanidinobenzoic acid, sulfonyl fluorides, phosphoryl fluorides), and mechanism based inhibitors (suicide substrates).

Reversible inhibitors usually contain substrate-like features and their potency depends on binding interactions with the enzyme. Since the enzyme-inhibitor complex formed with reversible inhibitors can dissociate, restoration of enzymatic activity may occur as the inhibitor is destroyed or cleared from the system. The strength of the binding is reflected in K_i, the equilibrium dissociation constant for the enzyme-inhibitor complex EI. Irreversible inhibitors usually inactivate serine proteases by first forming a reversible EI complex followed by covalent bond formation. The potency of an irreversible inhibitor therefore depends on both the strength of reversible binding to the enzyme (K_i) and the rate of the inactivation step (k_{on}).

Peptide chloromethylketone inhibitors are a well-understood class of substrate-derived irreversible inhibitors of serine proteases which alkylate the active site His 57 and form a tetrahedral adduct with Ser 195. Their value in the identification and characterisation

of proteases in their physiological environments has been clearly demonstrated by the use of Tos-Lys-CH_2Cl and Tos-Arg-CH_2Cl. These reagents distinguish trypsin-like activities by conforming to the primary specificity requirements of the protease (Kettner and Shaw, 1981). These irreversible inhibitors both utilize the binding specifity of their target enzyme and the catalytic apparatus for chemical inactivation. Further work has concentrated on molecules with greater degrees of specificity towards specific proteases. This has largely been accomplished by using small peptides rather than amino acids as the base structure of the chloromethylketone. Such efforts have been particularly important in studies of serine proteases and the design of selective protease inhibitor compounds (Kettner and Shaw, 1981). A second major advance has been the use of the covalent linking properties of chloromethylketones to carry reporter groups such as fluorescent probes and biotin groups into the enzyme active site (Williams and Mann, 1993).

1.1.3 Thrombin function and structure

Thrombin is a multifunctional trypsin-like serine protease (Fenton et al., 1977; Guillin et al., 1995). Formed during the final stage of the blood clotting cascade, thrombin converts the soluble plasma protein fibrinogen to the insoluble fibrin matrix of blood clots. It also activates the blood coagulation factor XIII which cross-links the fibrin network to stabilise the blood clot. By cleaving the factors V and VIII, thrombin amplifies its own production; activated factors Va and VIIIa are essential cofactors in the coagulation cascade. Thrombin also regulates its own production when bound to thrombomodulin (a membrane-bound cofactor) on the vascular endothelium. Thrombin converts protein C (another serine protease) to its activated form. Together with the co-factor protein S, activated protein C catalyses the degradation of factors Va and VIIIa and the hydrolysis of these cofactors in turn shuts down the clotting cascade see (Figure 1.2). Thrombin's catalytic activity is further controlled by the plasma serine protease inhibitors antithrombin III and heparin cofactor II (Travis and Salvesen, 1983).

The thrombin molecule (Figure 1.3) has a canyon-like active site cleft, mainly shaped by two prominent insertion loops around Trp 60D and Trp 148 (Stubbs and Bode,

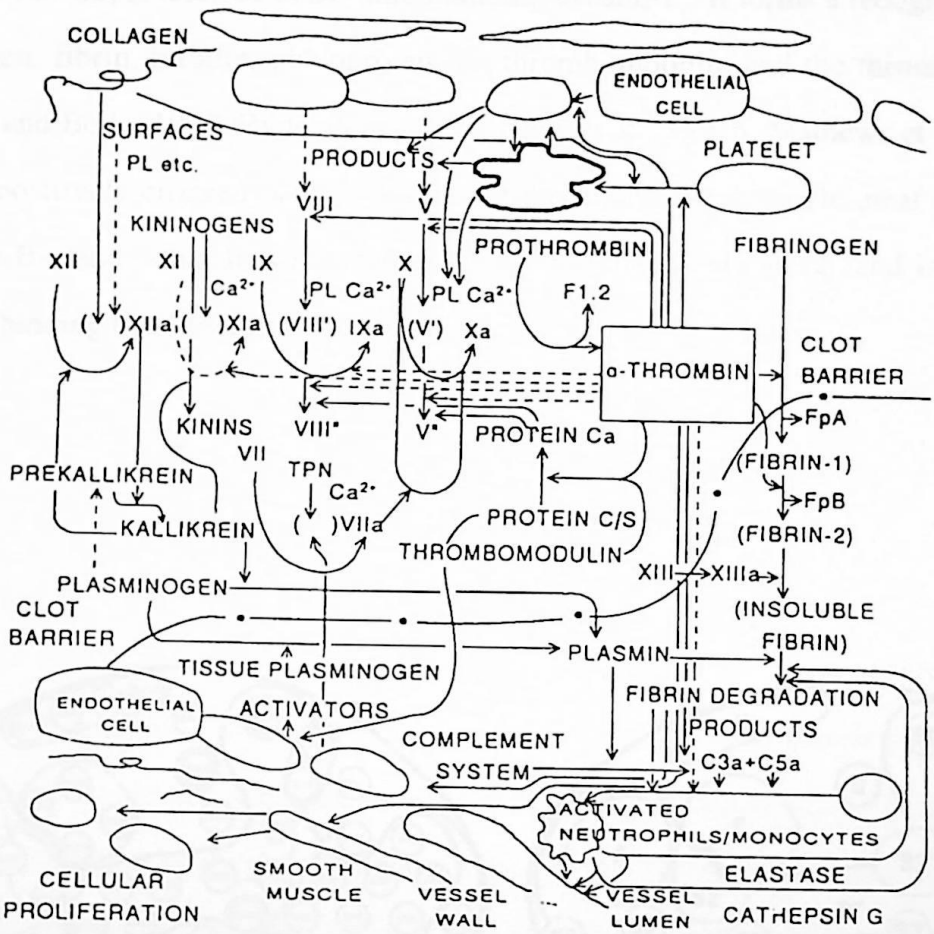


Figure 1.2 *A depiction of the functions of α-thrombin in haemostasis.*
Roman numerals are used for coagulation factors and are followed by "a" for the activated serine proteinases, whereas primes and double primes signify activated and inactivated forms of factors V and VIII, respectively. Other abbreviations include; F1.2 for the prothrombin activation fragment, FpA for fibrinopeptide A, FpB for fibrinopeptide B, PL for phospholipid and TPN for thromboplastin (tissue factor). The clot barrier is indicated by a dash-dot line. The upper left-hand-facing corner shows pre-thrombotic, while the lower right-hand-facing corner portrays post-thrombotic events. From Fenton (1995).

1995). The most important features of the active site are the catalytic triad (His 57, Asp 102, Ser 195) and the primary specificity pocket, with Asp 189 at the bottom to facilitate binding of basic residues. The 60D insertion loop occludes the active site to form a particularly hydrophobic S2 site. The extended surface groove rich in basic amino acids, segment 70 - 80, is referred to as "anion binding exosite-1". It forms a recognition site for fibrinogen, fibrin, hirudin, glycoprotein Ib, thrombomodulin and the thrombin receptor (Stubbs and Bode, 1995; Rydel et al., 1991; Bode et al., 1992b; Mathews et al., 1994). A second positively charged patch is located at the top of the molecule, near the carboxy-terminal B chain helix. It is referred to as "anion binding exosite-2" and is involved in heparin binding (Stubbs and Bode, 1995).

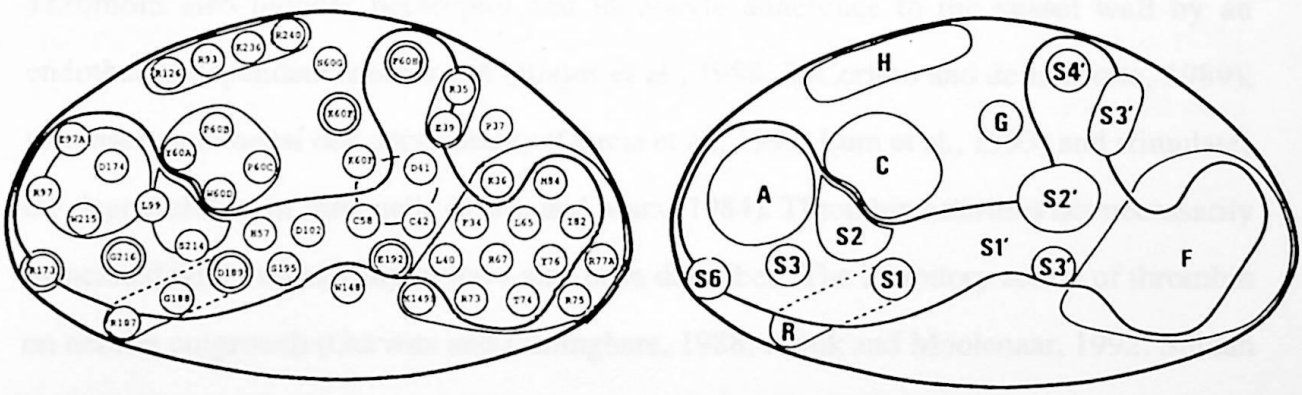


Figure 1.3 *Schematic representation of human α-thrombin.*
Surface residues appear in their approximate locations in the standard orientation (left). The right-hand side shows the division of the surface into functional regions. The active site triad (H57, D102 and S195) is shown in the centre of the left diagram. Key: S1-S6, specificity sites amino terminal to cleavage; A, aryl-binding site (the aryl-binding site and the S2 site together form the "apolar binding site"); F, fibrinogen-recognition exosite; H, heparin-binding site; G, glycosylation site; S1'-S3', putative specificity sites carboxy-terminal to cleavage; C, chemotactic region; R, RGD sequence. Redrawn from Stubbs and Bode (1995).

1.1.4 Cell-activating functions of thrombin

In addition to cleaving fibrinogen to fibrin, thrombin exhibits important "cell-activating" functions (Figure 1.4). First and foremost, thrombin is a potent stimulator of platelet aggregation (Berndt et al., 1995), an activity critical in the formation of a blood clot. A variety of other thrombin effects on cells have been defined, largely *in vitro*, suggesting an important role for thrombin in controlling the inflammatory and proliferative responses associated with wound healing. Thrombin is chemotactic for monocytes and neutrophils (Bar Shavit et al., 1983a; Bizios et al., 1986), mitogenic for lymphocytes and for mesenchymal cells including vascular smooth muscle cells (Chen et al., 1976: Chen and Buchanan, 1975) and has a number of effects upon the vascular endothelium. These include stimulating the production of prostacyclin (Weksler et al., 1978; Jaffe et al., 1987), platelet-activating factor (Prescott et al., 1984), tissue plasminogen activator (Levin et al., 1986), plasminogen activator inhibitor (Gelehrter and Sznycer-Laszyk, 1986) and the potent smooth muscle cell mitogen platelet-derived growth factor (Harlan et al., 1986). Thrombin also induces neutrophil and monocyte adherence to the vessel wall by an endothelial-dependent mechanism (Bizios et al., 1988; DiCorleto and de la Motte, 1989), increases endothelial cell permeability (Garcia et al., 1993; Lum et al., 1993) and stimulates the degranulation of mast cells (Razin and Marx, 1984). Thrombin activities not necessarily associated with vascular injury have also been described. The inhibitory action of thrombin on neurite outgrowth (Gurwitz and Cuningham, 1988; Jalink and Moolenaar, 1992; Suidan et al., 1992) and the apparent expression of prothrombin mRNA in the brain (Dihanich et al., 1991; Soifer et al., 1994) highlight potential roles for thrombin in neuronal development.

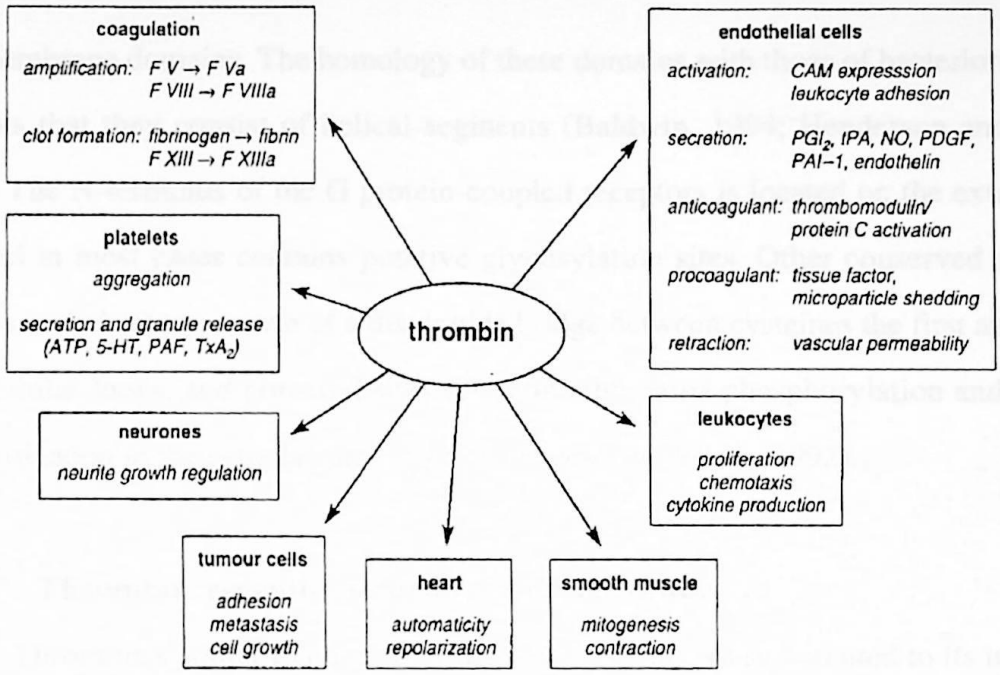


Figure 1.4 *Scheme depicting the multiple enzymatic and cellular actions of thrombin.* Abbreviations used: F V, clotting factor V; F Va, activated clotting factor V; 5-HT, 5-hydroxytryptamine; PAF, platelet activating factor; TxA2, thromboxane A2; PGI2, prostaglandin I2; tPA, tissue plasminogen activator; NO, nitric oxide; PDGF, platelet derived growth factor; PAI-1, plasminogen activator inhibitor-1. From Taparelli et al. (1993).

1.2 Thrombin Receptor

1.2.1 The seven transmembrane domain family of receptors

A large number of peptide hormones and neurotransmitters elicit intracellular changes by interaction with cell membrane receptors that are coupled to intracellular effector proteins, so called guanine-nucleotide-binding regulatory proteins (G proteins), (reviewed in Savarese and Fraser, 1992; Strader et al., 1994). Comparison of the deduced amino acid sequences of these G protein-coupled receptors reveals a similar secondary structure with a single polypeptide chain containing seven hydrophobic domains. Since the seven hydrophobic domains in each receptor are of similar size (20 - 28 amino acids) and of sufficient length to span the membrane lipid bilayer, they have been postulated to form

transmembrane domains. The homology of these domains with those of bacteriorhodopsin suggests that they consist of helical segments (Baldwin, 1994; Henderson and Unwin, 1975). The N-terminus of the G protein-coupled receptors is located on the extracellular side and in most cases contains putative glycosylation sites. Other conserved structural features include the presence of a disulphide bridge between cysteines the first and second extracellular loops, and potential sites for serine/threonine phosphorylation and cysteine -palmitoylation in the cytoplasmic regions (Savarese and Fraser, 1992).

1.2.2 Thrombin receptor structure and activation

Thrombin's ability to elicit a cellular response has been attributed to its interaction with the recently cloned thrombin receptor. Thrombin receptors from human platelets (Vu et al., 1991a; Vu et al., 1991b), rat aortic smooth muscle cells (Zhong et al., 1992), hamster lung fibroblasts (Rasmussen et al., 1991) and *Xenopus laevis* (Gerszten et al., 1994) have been cloned and sequenced. The results show that the thrombin receptor is a member of the seven transmembrane G-protein coupled receptor family, with a similarity to receptors for neuropeptides and glycoprotein hormones (Vu et al., 1991a). The human thrombin receptor consists of seven helical hydrophobic transmembrane regions giving rise to three intra- and extra-cellular loops, a C-terminal intracellular tail and a long N-terminal extracellular domain (Figure 1.5). The cDNA sequence revealed an open reading frame coding for 425 amino acids. A disulphide link between Cys 175 in the first extracellular loop and Cys 254 in the second extracellular loop has been proposed by analogy with rhodopsin and the β2-adrenergic receptor (Vu et al., 1991a). The large number of serine and threonine residues in the receptor's carboxyl tail are potential phosphorylation sites for a β-adrenergic receptor kinase-like enzyme (Ishii et al., 1994). Compared to other seven-transmembrane receptors, a relatively long extracellular amino-terminal extension precedes the first transmembrane domain and contains five potential glycosylation sites (Vouret-Craviari et al., 1995a). Thrombin recognises the amino-terminal exodomain of its receptor via two different types of interactions: the LDPR amino acid sequence docks in the active site of thrombin and the

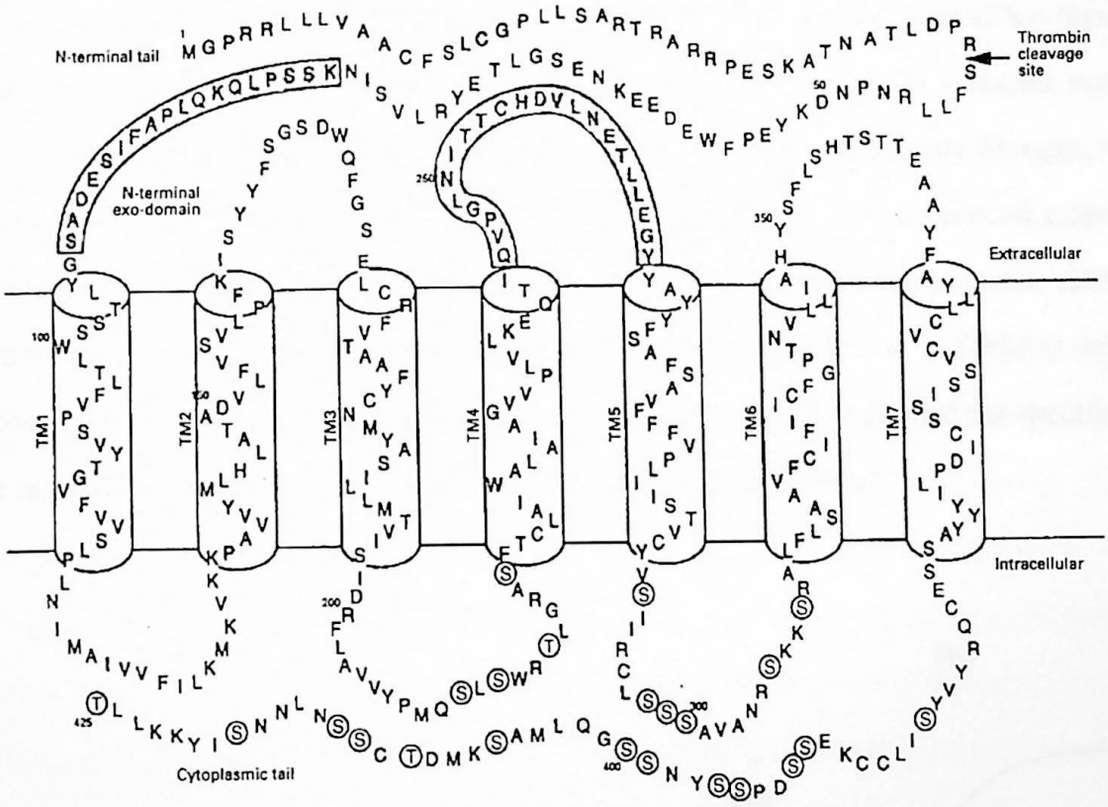


Figure 1.5 *Structure of the human thrombin receptor.*
The amino acid sequence is arranged through the membrane, as suggested by Vu et al. (1991a) and Gerszten et al. (1994). The thrombin cleavage site (←), N- terminal extracellular domain, extracellular loops, cytoplasmic loops and the seven transmembrane helical domains (TM1 - TM7) are shown. Potential intracellular phosphorylation sites (encircled letters) and amino acid residue numbers are marked. The extracellular domains involved in the interaction with the tethered ligand are indicated by filled in boxes. Redrawn from Grand et al. (1996).

DKYEPF sequence binds to the thrombin anion-binding exosite-1. Thrombin then cleaves receptor extracellular domain between residues Arg 41 and Ser 42 to unmask a new amino terminus beginning with the sequence SFLLRN. This new amino terminus serves as a tethered ligand, binding to other receptor domains to effect receptor activation (Figure 1.6).

Thrombin's proteolytic activity is crucial for activation of the thrombin receptor. A proteolytically inactive mutant of thrombin (Ser195Ala) failed to activate platelets or invoke a calcium mobilisation in oocytes expressing the thrombin receptor. Ser195Ala thrombin was also a potent antagonist of thrombin, but not agonist peptide-induced receptor activation on platelets (Hung et al., 1992a). The hirudin-derived peptide hirugen, which binds to thrombin's anion-binding exosite-1, also blocked thrombin-induced responses, pointing to a role for thrombin's anion binding exosite-1 in thrombin receptor activation (Vu et al., 1991a). Replacing the receptor's thrombin cleavage site (LDPR/S) with the recognition site for the serine protease enterokinase (DDDDK/S) switched the specificity of the receptor to an enterokinase-activated receptor (Vu et al., 1991b).

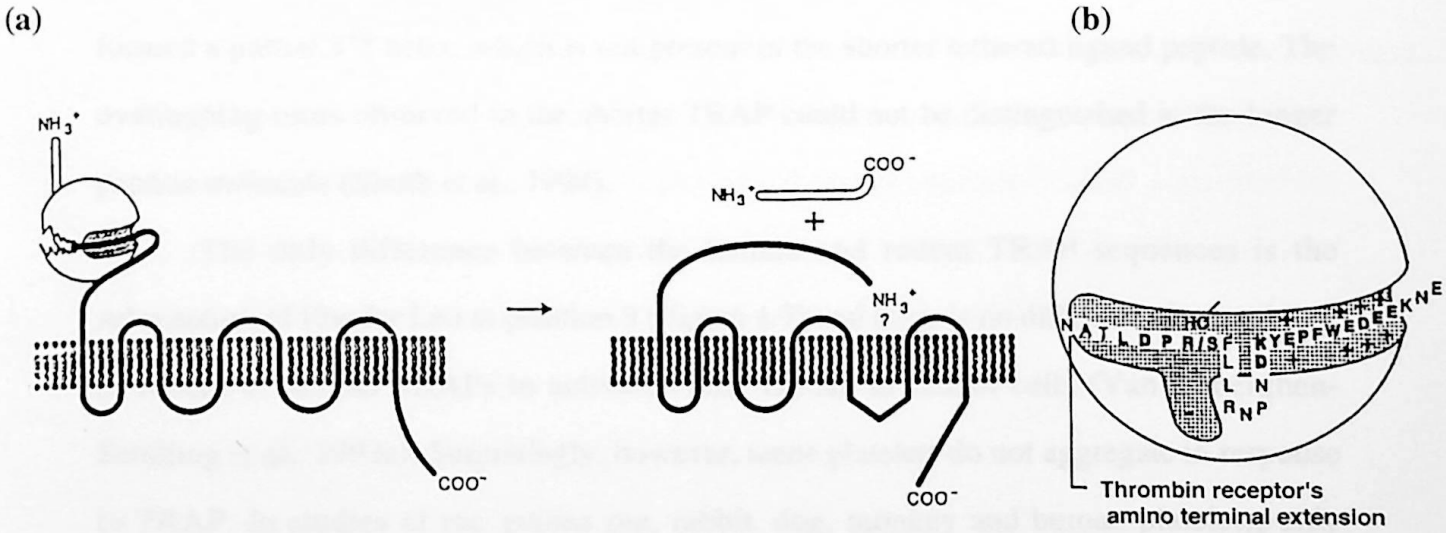


Figure 1.6
(a) *Model for the activation of the thrombin receptor.* After thrombin cleavage of the receptor, a 16 amino acid residue peptide is released and the newly created N-terminus then functions as a tethered ligand to effect receptor activation.
(b) *Interaction of the thrombin receptor's extracellular domain with thrombin.* Thrombin has an extended substrate binding surface. The receptor's hirudin-like domain (KYEPF) interacts with thrombin's anion-binding exosite, while its cleavage site (LDPR/S) interacts with thrombin's S1-S4 subsites. From Vu et al., (1991a).

1.2.3 Thrombin receptor activating peptides (TRAPs)

Peptides identical to the tethered ligand sequence can duplicate the actions of thrombin on a variety of cells. The amino acid residues within the tethered ligand that are necessary for receptor activation and the minimal length required have been determined (Chao et al., 1992; Vassallo et al., 1992; Scarborough et al., 1992).

The α-amino group of the serine at position 1 of the tethered ligand (Coller et al., 1992), as well as the side chain of phenylalanine in the second position appear to be essential for binding. Leucine and arginine at positions 4 and 5 have shown to be necessary for full activity (Natarajan et al., 1995) and the minimal sequence required to mimic the effects of thrombin resides in the first 6 residues (Chao et al., 1992; Vassallo et al., 1992).

The NMR structure of a 14 mer TRAP (S^{42}FLLRNPNDKYEPF^{55}) has been determined and found to have two overlapping turns (amino acid residues Arg 46 to Asn 49 and Asn 47 to Asp 50 in the TRAP sequence); the five N-terminal residues considered to be important for biological activity were principally unstructured. A longer peptide covering the thrombin cleavage site was found to be more highly structured, namely A^{33}TNATLDPRSFLLRNPNDKYEPF^{55}. The seven residues from Pro 40 to Arg 46 formed a partial 3_{10} helix, which is not present in the shorter tethered ligand peptide. The overlapping turns observed in the shorter TRAP could not be distinguished in the longer peptide molecule (Smith et al., 1994).

The only difference between the human and rodent TRAP sequences is the substitution of Phe for Leu at position 3 (Figure 1.7) and there is no difference in the ability of rodent or human TRAPs to activate either human or rodent cells (Van Obberghen-Schilling et al., 1993a). Surprisingly, however, some platelets do not aggregate in response to TRAP. In studies of rat, guinea pig, rabbit, dog, monkey and human platelets, only guinea pig, monkey and human platelets were responsive to human TRAP (Derian et al., 1995). Platelets in plasma from rabbit, dog, pig and hamster undergo a shape change but fail to aggregate in response to human TRAP, despite being fully responsive to human thrombin (Connolly et al., 1994). Paradoxically, other rodent cells respond to TRAP (Suidan et al., 1992). This heterogeneity of responses, highlights potential problems in

using animal models to evaluate the design of potential human thrombin receptor antagonists. The *Xenopus* TRAP has the sequence TFRIFD (Figure 1.7); this peptide is able to activate the human receptor, but is 30 fold less potent compared to SFLLRN (Gerszten et al., 1994).

The ability of small peptides to mimic thrombin's catalytic action has provided a focus for the design of better agonists and possible antagonists of this receptor. The synthetic peptide YFLLRNP induces a state of partial activation in human platelets, but antagonises aggregation induced by low concentrations of α-thrombin or TRAP (Rasmussen et al., 1993). The ability of TRAP to tolerate amino acid substitutions in non-critical residues has been exploited in the generation of photo-activatable human thrombin receptor agonists, useful in the study of the receptor (Bischoff et al., 1994). The selective removal of the N-terminal serine by either membrane bound or serum aminopeptidases, inactivates TRAP (Coller et al., 1992). This observation led to the development of active analogues of TRAP, with the N-terminal serine replaced with isoserine (Coller et al., 1993) making them resistant to cleavage and enhancing their plasma half life. A prototype thrombin receptor antagonist, the decapeptide analogue 3-mercapto-propionyl-Phe-cyclohexylalanine-cyclohexylalanine-Arg-Lys-Pro-Asn-Asp-Lys-amide, has been demonstrated to block thrombin receptor-mediated vascular relaxation in pig coronary artery rings (Tesfamariam, 1994). The use of a "tethered ligand library" bearing random pentapeptides in place of the native sequence identified the sequence GFIYF which was capable of activating the receptor (Chen et al., 1995a). Recombinant phage display technology (Smith, 1985) has also been exploited to isolate both peptide agonists and antagonists to the platelet thrombin receptor (Doorbar and Winter, 1994).

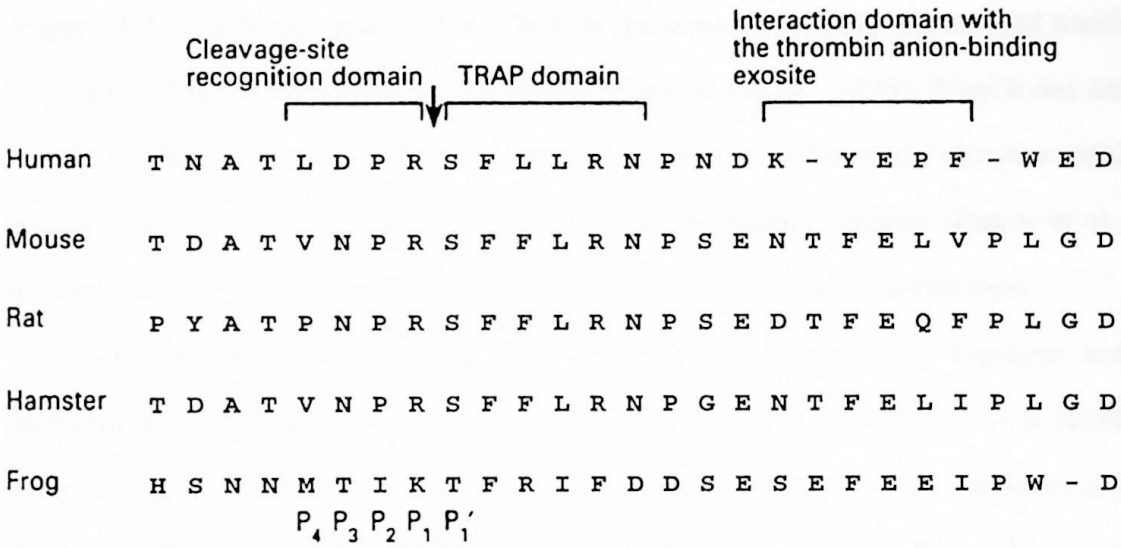


Figure 1.7 *Comparison of the N-terminal extracellular domains of the thrombin receptor from different species.*

1.2.4 Mapping of the thrombin receptor tethered ligand binding site

Seven transmembrane domain G-protein-coupled receptors respond to a structurally diverse set of ligands (Savarese and Fraser, 1992). Catecholamines and certain other small ligands elicit responses by binding to their receptor's transmembrane regions (reviewed in Dohlman et al., 1992; Savarese and Fraser, 1992). The amino acid residues involved in binding to some peptide ligands have been identified using site directed mutagenesis of their corresponding receptors (Fong et al., 1992a; Fong et al., 1992b; Huang et al., 1994; Lee et al., 1994a; Lee et al., 1994b).

There is evidence that thrombin receptor specificity is determined by its extracellular surface (Gerszten et al., 1994; Nanevicz et al., 1995). In experiments with chimeras of human and *Xenopus laevis* thrombin receptors, which are activated selectively by different agonist peptides (SFLLRN and TFRIFD, respectively), the receptor domains responsible for agonist specificity have been identified. A replacement of *Xenopus* with human receptor sequence suggested that two regions accounted for this change in specificity to either agonist peptide; residues 244 - 268 in the second extracellular loop and residues 76 -

93 located in the amino-terminal exodomain near the start of transmembrane domain 1 (Figure 1.5) (Gerszten et al., 1994). Within these two domains, amino acid residues Phe 87 and Glu 260 made major contributions (Nanevicz et al., 1995). Polyclonal antibodies directed against the amino-terminal region of the human thrombin receptor (amino acid residues 76 - 93) blocked receptor activation by the agonist peptide (Bahou et al., 1993a; Bahou et al., 1994), consistent with a role for this region in agonist function.

To define further the agonist-receptor interactions, an "arginine scan" was performed on the human receptor's extracellular surface and amino acid residues that complemented a gain of function with a mutant agonist peptide (SFLLEN) were observed (Nanevicz et al., 1995). "Alanine scanning" of the same regions of the receptor, revealed the importance of aromatic or hydrophobic residues in the receptor binding site (Nanevicz et al., 1995). While constructing the chimeric receptors, it was noted that substitution of eight amino acids from the *Xenopus* receptor's second extracellular loop for the cognate sequence in the human thrombin receptor was sufficient to confer robust constitutive activity in this mutant human thrombin receptor (Nanevicz et al., 1996).

1.2.5 Thrombin receptor intracellular responses

Thrombin receptors are present on a wide variety of cells, and exhibit quite different responses upon activation. Since different receptor subtypes have not been identified, these observed responses reflect differences in the G-proteins and effectors down-stream from the receptor. In most cells, thrombin stimulates phospholipases C, A2 and D, activates protein kinase C, mitogen activated protein kinases (MAPKs) and tyrosine kinases, and raises the intracellular calcium concentration ($[Ca^{2+}]_i$) (reviewed in Van Obberghen-Schilling and Pouyssegur, 1993b; Brass et al., 1993; Grand et al., 1996).

Many seven transmembrane domain receptors, such as the thrombin receptor, through coupling of heterotrimeric G-proteins stimulate phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to generate the second messenger molecules inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol. Thrombin stimulation of PLC activity in platelets (Crouch and Lapetina, 1988), human erythroid leukemia cells

(HEL) (Brass et al., 1991), Chinese hamster lung fibroblasts (CCL39) (Paris and Pouyssegur, 1986) and vascular smooth muscle cells (Huang and Ives, 1989) is sensitive to pre-treatment with pertussis toxin, suggesting a role for G_i subunits in stimulating PLC activity in these cells. The study of thrombin receptor mediated $[Ca^{2+}]_i$ in CCL39 cells combined with microinjection of specific anti-G-protein subunit antibodies, showed that the thrombin receptor in these cells is coupled to two different types of G-proteins, G_q and G_o (Baffy et al., 1994). In platelets, functional coupling of the thrombin receptor to members of the G_q family has been demonstrated (Benka et al., 1995). TRAP can both activate protein kinase C and inhibit adenylate cyclase in a pertussis toxin-sensitive manner in CCL39 cells (Vouret-Craviari et al., 1992). Thrombin activation also inhibits adenyl cyclase activity in platelets (Aktories and Jakobs, 1984), but in a human erythroid leukemia cell line, thrombin causes an increase in cAMP levels (Brass and Woolkalis, 1990).

Expression of antisense mRNA to the protein kinase-C β isoenzyme (PKC-β) in human erythroid leukemia cells suggested that PKC-β was a specific negative regulator of thrombin receptor-mediated Ca^{2+} entry (Xu and Ware, 1995). Overexpression of PKC-β in Rat6 fibroblasts led to the conclusion that PKC-β may act as a switch to up-regulate phospholipase-D and down regulate phospholipase-C activity (Pacter et al., 1992). TRAP has been shown to potentiate the activation of phospholipase-C in platelets (Huang et al., 1991).

Activation of the thrombin receptor can also stimulate the production of phosphoinositide 3-kinase (PI3K), which specifically phosphorylates phosphatidylinositol 4,5-bisphosphate (PI-4,5 bisP). Upon platelet thrombin stimulation, increased PI3K activity can be detected resulting in the formation of phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-trisP) (Carter et al., 1994; Toker et al., 1995). This general PI3K activity has been further shown to result from the activity of PI3K-γ and P85/PI3K (Zhang et al., 1996). PI3K has also been shown to play a role in promoting pleckstrin (p47) phosphorylation through activation of a calcium independent PKC isoform (Zhang et al., 1995; Toker et al., 1995). Following stimulation by either thrombin or TRAP, specific inhibitors of PI3K activity inhibited platelet aggregation, but not secretion. This

demonstrates that PI3K activity is necessary for irreversible platelet aggregation (Kovacsovics et al., 1995).

The thrombin receptor can activate non-receptor tyrosine kinases. Thus, thrombin receptor activation induces the tyrosine-specific phosphorylation of a large number of intracellular proteins, as assessed by anti-phosphotyrosine immunoblotting (Ferrell and Martin, 1988; Golden and Brugge, 1989). Both thrombin and TRAP treatment leads to rapid phosphorylation of proteins with apparent molecular masses of 42, 44, 75, 120 and 190 kDa in Swiss 3T3 fibroblasts (Molloy et al., 1996) and phosphorylation of cytoskeletal proteins in the platelet (Pumiglia and Feinstein, 1993). The tyrosine kinase inhibitors genistein and tyrphostin A23 were able to inhibit platelet shape change induced by the agonist peptide YFLLRNP (Negrescu et al., 1995). The induction of the pre-pro-endothelin-1 (preproET1) gene in human umbilical endothelial cells (HUVECs) by thrombin has been showed to involve tyrosine kinase activity, as the use of tyrosine kinase inhibitors herbimycin A and genistein blocked preproET1 mRNA production and peptide secretion (Marsen et al., 1995). Thrombin stimulates tyrosine phosphorylation of several proteins of molecular mass 40, 42, 70, 120, and 130 kDa in Jurkat T-cells (Mari et al., 1994) and 65 - 70 and 110 - 120 kDa in mouse BC3H1 muscle cells (Offermanns et al., 1993). Thrombin can mediate the mitogenic response in human glomerular mesangial cells through the activation of tyrosine kinases (Grandaliano et al., 1994). The Src tyrosine kinase has proved to be active in growth responsive CCL39 cells (Chen et al., 1994).

Thrombin is a mitogenic factor, invoking the activation of the Ras proto-oncogene protein. With the direct injection of dominant Ras interfering mutant proteins and inhibitory antibodies to Ras, DNA synthesis in response to thrombin was inhibited (LaMorte et al., 1993). TRAP activates p21ras in fibroblasts, which is not inhibited by genistein and was pertussis toxin sensitive, highlighting a role for a heterotrimeric G protein of the G_i subfamily (Van Corven et al., 1993). The Ras related protein Rho, a small GTP-binding protein, has been implicated in neurite retraction in both N1E-115 and NG108-15 neuronal cells, and inactivation of Rho by ADP-ribosylation by Clostridium botulinum C3 exoenzyme results in neurite outgrowth (Jalink et al., 1994).

MAPKs, which possess serine, threonine and tyrosine kinase activity, are important in mediating responses to both tyrosine kinase receptors and G-protein coupled receptors (Van Obberghen-Schilling et al., 1993b). Thrombin and TRAP stimulate p44mapk (ERK) in G_0-phase arrested CCL39 cells (Vouret-Craviari et al., 1993). In addition to p44mapk, p70 S6 kinase activity is increased in CCL39 cells stimulated by thrombin (Kahan et al., 1992). Activation of both p44(mapk) and p42(mapk) was observed in thrombin treatment of quiescent rat aorta smooth muscle cells (Molloy et al., 1996). A p38 mapk has also been found in platelets after thrombin receptor activation (Kramer et al., 1995).

Thrombin receptor activation upregulates the expression of many genes such as c-fos and platelet-derived-growth-factor in human smooth-muscle cells (Kanthou et al., 1995), the urokinase-type plasminogen activator receptor in bovine vascular smooth muscle cells (Reuning et al., 1994), pre-pro-endothelin-1 in HUVECs (Marson et al., 1995) and CD69 and the nuclear transcription factor NF-κB in the Jurkat T-cell line (Mari et al., 1994).

Since gross morphological changes can accompany thrombin stimulation in terminally differentiated cells (for example, thrombin-stimulated cell motility in U937 human monocyte/macrophage-like cells (Joseph and MacDermot, 1992), human monocytes (Crago et al., 1995) and thrombin-stimulated neurite retraction (Gurwitz and Cunningham, 1988; Jalink and Moolenaar, 1992; Suidan et al., 1992), thrombin receptor activation must also regulate cytoskeleton assembly and disassembly. The ability of thrombin receptor activation to initiate changes in the cytoskeletal reorganisation processes has been demonstrated in platelets (Hartwig et al., 1995).

1.2.6 Desensitisation of the thrombin receptor

Most cell surface receptors, including those that interact with G-proteins, are subject to internalisation, recycling, degradation, and eventual replacement. Recent evidence suggests that thrombin receptors are subject to the same events. Like other G-protein-coupled receptors, activated thrombin receptors are quickly desensitised, leaving the cell in a state in which a subsequent encounter with thrombin invokes little or no response. Desensitisation appears to involve several events, including phosphorylation of the receptor by one or more protein kinases (Ishii et al., 1994). In a broad sense, cleavage of the receptor N-terminus by thrombin or other proteases is also a type of desensitisation, since the cleavage reaction is irreversible and cleaved receptors cannot therefore be activated a second time by thrombin. Cleavage by other proteases may also cause desensitisation without activation. Cleavage of the receptor on the C-terminal side of the thrombin activation site will remove this site without activating the receptor. The neutrophil protease cathepsin-G has been identified as a potential desensitising protease (Molino et al., 1995). Whether they are recycled or degraded, cleaved thrombin receptors have to be replaced before the responsiveness of the cell to thrombin can be restored. This is in contrast to other G-protein-coupled receptors, which typically can be activated more than once. In theory, the rate of thrombin receptor replacement is limited by the rate of receptor synthesis unless the cells contain a pool of receptors that can move to the cell surface. Such a pool of receptors has been identified using antibodies directed to the thrombin receptor (Brass et al., 1994).

1.3 Proteinase Activated Receptor-2 (PAR-2)

1.3.1 Mouse PAR-2 (mPAR-2)

Recently, a reduced stringency hybridisation search of a mouse genomic library with a bovine substance K oligonucleotide probe serendipitously led to the identification of a G-protein-coupled receptor related to, but distinct from, the thrombin receptor (Nystedt et al., 1994). The open reading frame encoded a 395 amino acid protein with seven putative transmembrane domains and other features common to G-protein coupled receptors. This putative receptor contained in its sequence a motif for trypsin cleavage (Figure 1.8) that would generate a potential tethered ligand (SLIGRLETQP-) homologous to TRAP. Indeed, when expressed in *Xenopus* oocytes, this receptor was activated not only by low concentrations of trypsin (0.3-30 nM), but also by the receptor derived peptide SLIGRL with an EC_{50}= 5 μM (Nystedt et al., 1994). This new receptor, which was relatively resistant to the action of thrombin compared to trypsin, was designated as "protease-activated receptor number 2" as the putative enzyme that cleaves and activates the receptor *in vivo* has yet to be identified.

With the cloning of the full length PAR-2 cDNA from mouse stomach, it became apparent that the 5' region of the mRNA is derived from a separate exon located about 10 kilobases (Kb) away from the exon encoding the bulk of PAR-2 (Nystedt et al., 1995a). Comparing the mPAR cDNA sequence with the genomic sequence, codons 30 to 399 are identical, but upstream from codon 30 the two sequences are completely different (Nystedt et al., 1995a), diverging at the splice site identified using an exon trap experiment (Nystedt et al, 1995a). The peptide sequence encoded by the 5' end of the cDNA appears to represent a signal peptide. The postulated signal peptidase cleavage site is between Thr-25 and Glu-26 and if this is correct there are 5 amino acid residues in the mature receptor which are not present in the mouse genomic clone originally identified by Nystedt et al. (1994).

1.3.2 Human PAR-2 (hPAR-2)

Similar to the mouse PAR-2, the human PAR-2 (hPAR-2) is divided into two exons separated by about 14 Kb of intronic DNA (Nystedt et al., 1995b). The deduced protein sequence is 83% identical to the mouse receptor sequence. Residues within the human and mouse tethered ligand differ from the thrombin agonist peptide (Figure 1.8). Using fluorescent *in situ* hybridisation, hPAR-2 has been mapped to chromosomal region 5q13 (Nystedt et al., 1995b), where the human thrombin receptor gene has also been previously found to be located (Bahou et al., 1993b; Demetrick et al., 1996).

Although the physiological role of PAR-2 remains to be elucidated, it is found on human keratinocytes along with the thrombin receptor (Santulli et al., 1995). The presence of both of these protease receptors on keratinocytes could play a role in the post-clotting events of wound repair and inflammation. PAR-2 is involved as well as the thrombin receptor in endothelium-dependent rat aorta relaxation, which is blocked by a nitric-oxide-synthase inhibitor, and also in gastric longitudinal muscle contractions (Al-Ani et al., 1995). Thus, PAR-2 activation may exert a direct physiological influence on the intact vascular and gastrointestinal system. *In situ* hybridisation revealed high expression in intestinal epithelial cells throughout the gut (Bohm et al., 1996) and correspondingly PAR-2 may serve as a trypsin sensor in the small intestine (Bohm et al., 1996). It has also been localised along with the thrombin receptor on human umbilical endothelial cells (HUVECs) (Mirza et al., 1996) so could have a significant role along with the thrombin receptor in the vascular system. The detection of PAR-2 mRNA in peripheral blood leucocytes (Nystedt et al., 1995b) and selective T-cell lines (Mari et al., 1996) indicates that it may play an important role along with the thrombin receptor in haematopoiesis and the cells involved with the immune response.

Using Northern blot mRNA analysis there is conflicting data suggesting the presence of PAR-2 in human tissues. Nystedt et al. (1995b) found PAR-2 to be highly expressed in the human pancreas, kidney, colon, small intestine and liver and moderately expressed in prostate, heart and lung, with no expression in the testis, brain, skeletal muscle or thymus. This data was verified by Bohm et al. (1996). Discrepancies arise with

the other tissues assayed. While Nystedt and co-workers (1995b) found reasonable levels of expression in the spleen, ovary, peripheral blood leucocytes and stomach, Bohm et al. (1996) found no detectable message in these tissues.

		Cleavage Site ↓	
mPAR-2	(31-53)	SKGR / SLIGRL	ETQPPITGKGVPV
hPAR-2	(33-54)	SKGR / SLIGKV	DGTSHVTGKGVT
hTR	(38-60)	LDPR / SFLLRN	PNDKYEPFWEDEE
		Tethered Ligand Agonist Peptide Domain	

Figure 1.8 *Comparison of protease activated receptor functional domains.* Human PAR-2 (hPAR-2), mouse PAR-2 (mPAR-2) and the human thrombin receptor (hTR).

1.3.3 PAR-2 activating peptides

The use of TRAPs ranging from five to fourteen amino acids on a variety of target cells including neutrophils (Jenkins et al., 1995), some tumour cell-lines (Nierodzik et al., 1994) and venous and arterial endothelial cells (Simonet et al., 1992) suggested the presence of another protease receptor, as thrombin was unable to elicit a response in these tissues even though they responded to TRAP. The apparent differences of TRAP responses in different tissues suggested the possibility of pharmacologically distinct thrombin receptor subtypes (Tay-Uyboco et al., 1995; Laniyonu and Hollenberg et al., 1995).

Responses to TRAP have been used to imply a role for the thrombin receptor in various cellular responses. However, the proposed occurrence of thrombin receptor subtypes could be explained by the cross-reactivity of the thrombin receptor and PAR-2 agonist peptides. *Xenopus* oocytes expressing mouse or human PAR-2 receptor respond to both SFLLRN and SLIGRL agonist peptides with nearly identical EC_{50} values as measured by calcium efflux experiments. In contrast SLIGRL or SLIGKV is unable to activate the human thrombin receptor expressed in oocytes (Blackhart et al., 1995). The *Xenopus*

thrombin receptor tethered ligand sequence TFRIFD (Figure 1.7), which has been shown to activate the human thrombin receptor (Gerszten et al., 1994) is unable to activate PAR-2 (Blackhart et al., 1995) and can be used to distinguish between the thrombin receptor and PAR-2 (Santulli et al., 1995).

This non-selectivity of the receptor activating peptides indicates that they cannot be used to identify cells or tissues expressing a particular receptor. Moreover, even the use of present selective peptides cannot exclude the possibility of the agonist peptide activating yet another undiscovered receptor of this family. Likewise the use of trypsin to demonstrate the presence of PAR-2 on different cells does not exclude the possibility of trypsin cleaving and activating the thrombin receptor (Brass et al., 1992), or another uncloned protease receptor. The development of specific agonists or antagonists of the thrombin receptor and PAR-2, should enable the more precise detection of PAR-2, or other members of this protease receptor family (Hollenberg, 1996).

1.3.4 Comparison of PAR-2 and the thrombin receptor

Amino acid sequence comparisons of hPAR-2 and mPAR-2 with the thrombin receptor show that outside the highly conserved transmembrane regions, there is a region in the second extracellular loop that has a remarkable similarity between all the sequences: the fourteen amino acid sequence PXLNITTCHDXLXE (where X denotes amino acids that vary between the receptors). Studies with chimeric human and *Xenopus* thrombin receptors (Gerszten et al., 1994) have pointed to the importance of the second extracellular loop in determining the specificity of these two receptors for their respective agonist peptides.

1.4 Monoclonal Antibody Technology

1.4.1 Hybridoma technology

The generation of monoclonal antibodies was first described by Kohler and Milstein (1975). Since their pioneering work, hybridoma technology has been used to produce mouse and rat monoclonal antibodies (Mabs) against a diverse array of protein, carbohydrate, nucleic acid and hapten antigens (Galfre et al., 1979; Galfre and Milstein, 1981).

Mammalian B lymphocytes when activated by antigen, produce mono-specific antibodies against that antigen. These cells, however, have a finite lifespan and die after only a few days in culture. In contrast, plasmacytomas, derived from malignant tumours of antibody producing cells, grow indefinitely *in vitro*. Some of the plasmacytomas also no longer secrete antibody. Hybrids between these two cell types can be produced and cloned giving rise to cell lines (hybridomas), which possess both the properties of immortal growth in culture and the ability to produce antibody against a single epitope (Figure 1.9).

The B lymphocytes (splenocytes) from an immunised mouse or rat are fused with plasmacytoma cells using the chemical fusogen polyethylene glycol (PEG). Once fused, the cells are cultured in a medium which only allows fused splenocytes and plasmacytomas (hybridomas) to grow. This overcomes the potential problems of unfused plasmacytoma cells competing for nutrients in the culture medium. The selective medium contains hypoxanthine (H), aminopterin (A), and thymidine (T) and is referred to as HAT medium (Littlefield, 1964). Aminopterin blocks the normal *de novo* pathway of deoxyribonucleic acid synthesis, leaving only the salvage pathways to operate. The salvage pathways rely on the activity of the enzyme hypoxanthine phosphoribosyl transferase (HPRT) to allow thymidine (T) incorporation into deoxyribonucleic acid; the enzyme HPRT uses exogenous hypoxanthine (H) as a substrate.

The plasmacytomas used for fusion are deficient in HPRT, and therefore die in the presence of aminopterin. Unfused splenocytes also die in culture after a few days. The only

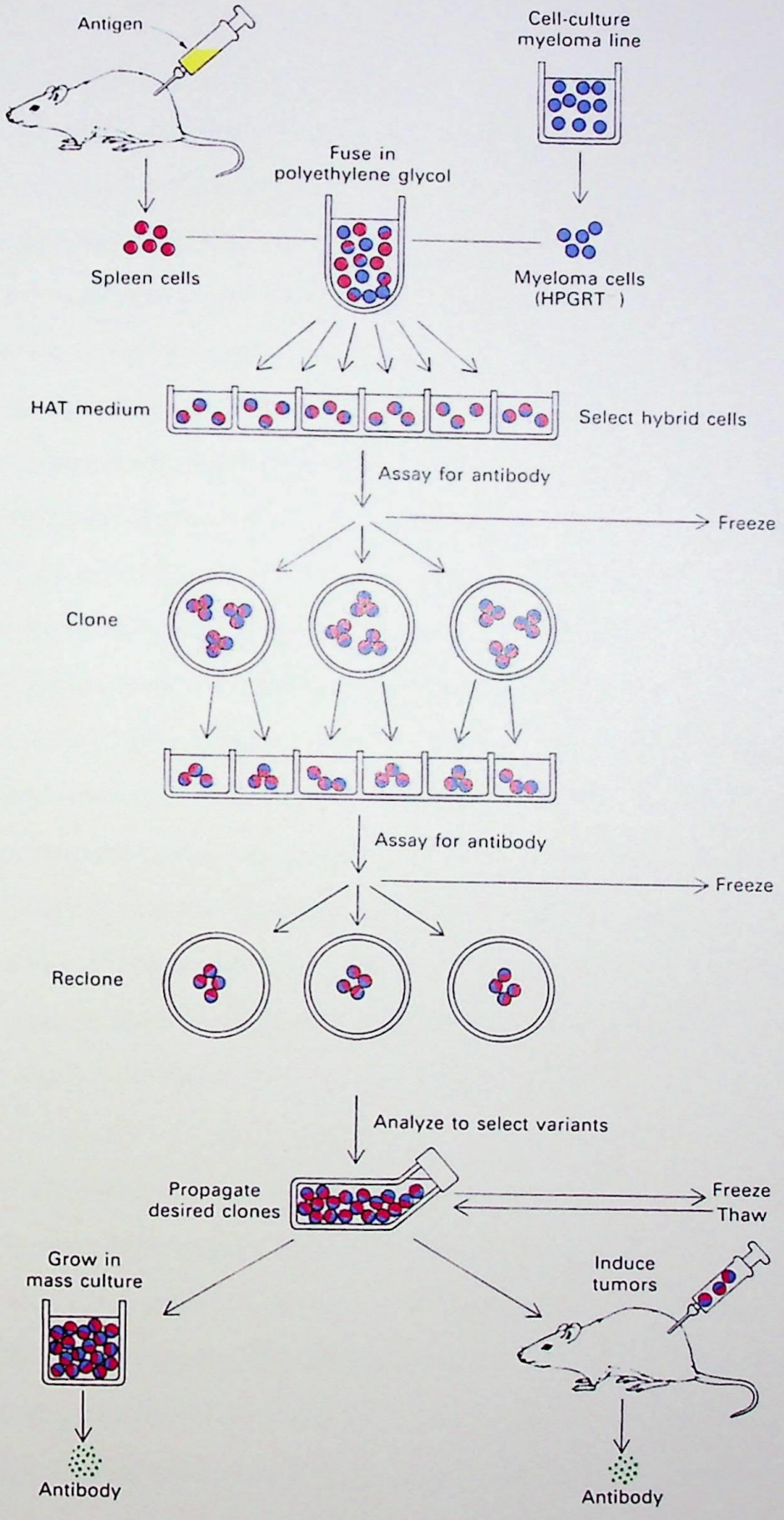


Figure 1.9 *Scheme depicting hybridoma technology*. From Milstein (1980).

cells that can survive in HAT medium are the hybridomas in which the plasmacytoma provides the ability to grow continuously in culture, and the splenocyte the necessary HPRT. Following HAT selection, the hybridomas are screened for production of antibody against the antigen used. The cells positive for the desired antibody are grown in medium without aminopterin for a short period. Because of the high probability of chromosome loss in the hybrids, and to ensure that the monoclonal cell line producing the antibody is derived from a single progenitor and is stable, the hybridomas are cloned.

Hybridoma antibody technology has been improved, particularly by antigen-specific selection of the B-cells to be immortalised (Casali et al., 1986) or by more rapid screening methods (Gheradi et al., 1990). Other animals have been used to produce monoclonal antibodies by hybridoma technology. The development of a chicken B-cell line deficient in thymidine kinase allowed the fusion and selection from immunised chicken spleen cells (Nishinaka et al., 1991). The generation of HAT sensitive rabbit plasmacytoma cell lines through transgenic technology (Spieker-Polet et al., 1995) permitted the fusion of spleen cells from immunised rabbits, to produce stable rabbit monoclonal hybridomas that secreted antibodies specific for the immunogen (Spieker-Polet et al., 1995). The availability of rabbit mAbs is highly desirable, as rabbits are known to produce antibodies to many antigens that are not immunogenic in mice and previous rabbit-rodent hybridomas are generally unstable (Raybould and Takahashi, 1988).

1.4.2 Recombinant antibody technology

There are other ways of tapping the antibody repertoire of immunised animals or humans. Instead of immortalising B-cells for the production of monoclonal antibodies using hybridoma technology, the antibody heavy and light chains can be immortalised by gene technology (reviewed by Winter and Milstein (1991)). The recombinant antibodies or antigen binding fragments can be expressed in mammalian cells (Dorai et al., 1994), insect cells (Putlitz et al., 1990), yeast (Ridder et al., 1995; Bowdish et al., 1991), bacteria (Ward, 1992) or plants (Artsaenko et al., 1995). Human monoclonal antibodies have a huge potential for therapy, but are difficult to make by immortalising human B-

lymphocytes, and it is especially difficult to generate human mAbs directed against human antigens due to immunotolerance mechanisms (reviewed by James and Bell, 1987).

Mouse and human monoclonal antibody fragments have been expressed and selected using phage display antibody technology (McCafferty et al., 1990; Griffiths et al., 1993) (for reviews, see Hoogenboom et al., 1992; Chiswell and McCafferty, 1992).

In the immune system, the B lymphocyte provides a "genetic display package" with antibody displayed on the outside of the cell to encounter and bind to antigen, and the genes encoding the antibody within. The single-stranded, non-lytic bacteriophage M13 has been used to display libraries of peptides as fusions with the N-terminus of the gene III protein, which is located at one tip of the phage (Smith, 1985). Similar fusions have displayed folded and functional antibody fragments (Figure 1.10).

Human monoclonal antibodies have also been isolated using this technique. It involves obtaining rearranged heavy and light chain V-chain genes by reverse transcription-PCR amplification from the μ, κ and λ mRNA of peripheral blood lymphocytes from either an unimmunised healthy human donor, or an individual who has been exposed to a particular antigen. For the isolation of recombinant mouse monoclonal antibodies, the mRNA is isolated from the spleen cells of a hyperimmunised animal, or from the specific monoclonal cell line producing the antibody to be expressed as a recombinant one (Figure 1.11)

In either case after the V-genes are obtained by PCR amplification, they are assembled at random (Clackson et al., 1991a; Huse et al., 1989) to encode repertoires of single chain Fv (scFv) fragments (Bird et al., 1988). These fragments are displayed on the surface of the filamentous bacteriophage M13 (McCafferty et al., 1990) and phage encoding scFv fragments which recognise the antigen are selected by specific binding of the phage to antigen (Figure 1.10). On infection of bacteria with the selected phagemid (Hoogenboom et al., 1991), soluble scFv fragments can be produced and expressed in *E. coli* from individual clones by secretion into the bacterial periplasm (Glockshuber et al., 1990) (Figure 1.12). These recombinant antibodies can be further analysed for their binding characteristics.

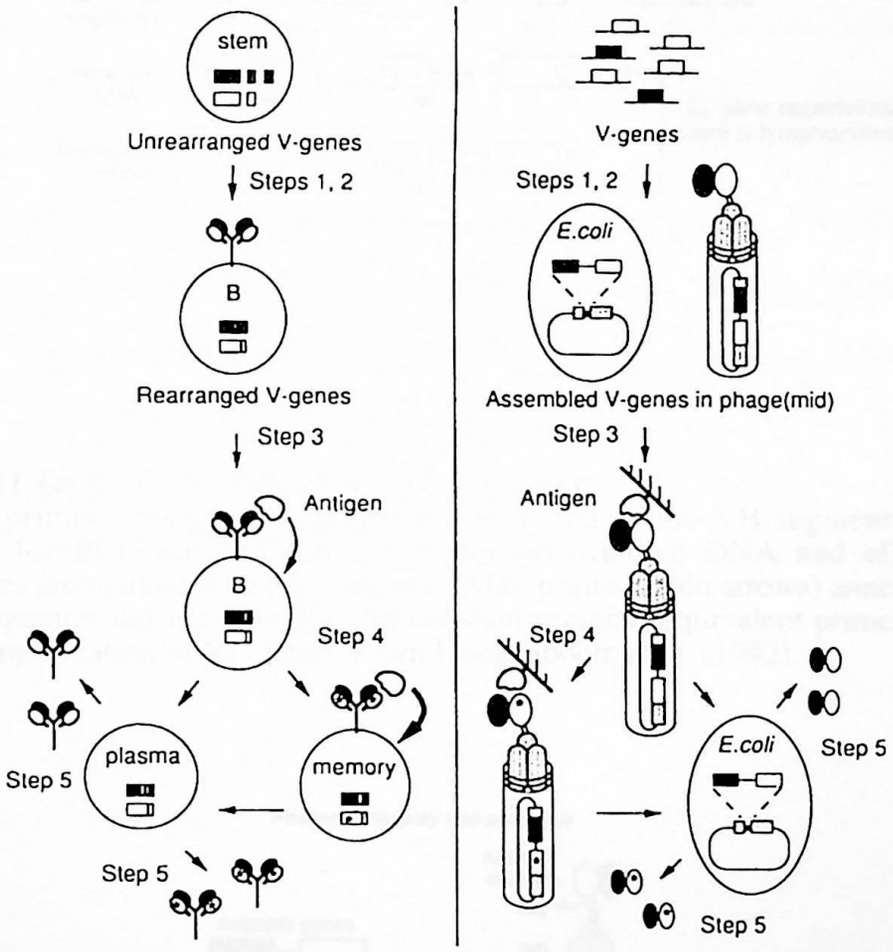


Figure 1.10 *The strategy of the immune system in vivo (left) and using phage (right).* Step 1, rearrangement or assembly of germ line V-genes; step 2, surface display of antibody; step 3, antigen-driven or affinity selection; step 4, affinity maturation; step 5, production of soluble antibody (or antibody fragment). From Hoogenboom et al. (1992).

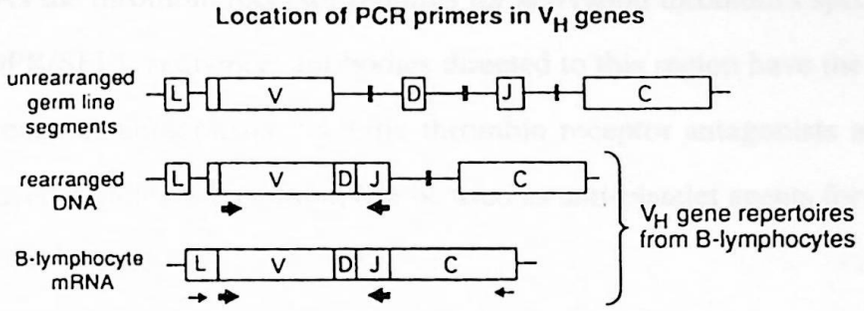


Figure 1.11 *Location of PCR primers in VH-genes.*
Universal primers designed to anneal to the 5' end of the VH segment and in the J-segments, for PCR amplification from both rearranged DNA and cDNA from B-lymphocytes are marked with thick arrows. Other primers (thin arrows) anneal to the leader peptide sequence and immunoglobulin constant regions. Equivalent primer locations are used for amplification of VL genes. From Hoogenboom et al. (1992).

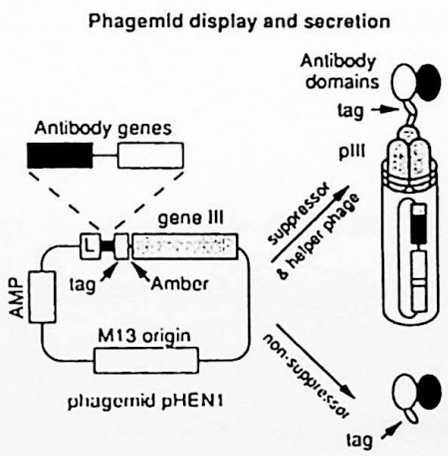


Figure 1.12 *Phagemid vector for display of antibody fragments on the surface of filamentous phage.* Phagemid vector pHEN1 for display of antibody after rescue with helper phage from an *E. coli* suppressor strain. The amber codon allows production of (tagged) soluble antibody fragment in a non-suppressor *E. coli* strain. Key: AMP, ampicillin resistance gene; L, PelB leader secretion peptide sequence; tag, C-myc peptide sequence. From Hoogenboom et al. (1992).

1.5 Aims

Antibodies directed to the human thrombin receptor, generated using hybridoma or recombinant DNA technology, are useful reagents in the detection and characterisation of the receptor. As the thrombin receptor requires for activation thrombin's specific cleavage within the LDPR/SFLL sequence, antibodies directed to this region have the potential use as thrombin receptor antagonists. Specific thrombin receptor antagonists able to inhibit thrombin-induced platelet aggregation, can be used as anti-platelet agents for the treatment of cardiovascular disease.

In order to investigate the role of the recently discovered proteinase activated receptor-2 (PAR-2), antibodies generated to the receptor can be used to study its cellular expression. Identification of proteases that are possible physiological activators of PAR-2 could highlight the possible role of PAR-2. In order to identify potential enzymatic activators of PAR-2, a peptide chloromethylketone inhibitor based on the sequence of the cleavage site, can be used to investigate with enzyme kinetics potential trypsin-like enzymes that may activate PAR-2 *in vivo*.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 *Bacterial media and plates*

The following types of liquid media, agar and agarose plates were prepared by the Laboratory of Molecular Biology central kitchen facility (MRC Centre, Cambridge, UK): 2xTY (containing 16 g bacto-tryptone, 10 g bacto-yeast extract and 5 g NaCl per litre); this medium was supplemented when necessary with ampicillin (100 µg/ml), choramphenicol (34 µg/ml), kanamycin (75 µg/ml) or tetracycline (100 µg/ml), when selection for plasmids or bacteria with an antibiotic resistance gene was required. Agar and agarose plates were prepared by adding 15 g of agar or agarose to 1 litre of liquid media. M9 minimal medium (containing 12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl and 0.4% (w/v) glucose per litre). Modified TYE agar plates (containing 10 g tryptone, 5 g bacto-yeast extract, 8 g NaCl and 15 g agar plus 5% (w/v) glucose per litre), were supplemented with antibiotics as required. H- top agar was prepared as for TYE except that 7 g of agar or agarose was added instead of 15 g and no antibiotic was included.

2.1.2 *Bacterial strains*

E. coli strain XL1-Blue F', [*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ *F' proAB*$^+$ *lacI*^{q} *lacZΔM15 Tn10 (tet*^{r}*)*] (Bullock et al., 1987) was purchased from Stratagene (Cambridge, UK). *E. coli* strain CJ236 [*dut*1 *ung*1 *thi*-1 *relA*1 /pCJ105 (cam^r) F'] (Kunkel, 1985) was purchased from Invitrogen (De Schelp, Netherlands). For long term storage bacterial strains were kept at -70°C in 2xTY and 50% (v/v) glycerol. Short term storage was at 4°C on minimal media agar plates or stabs.

2.1.3 *Media for cell culture*

Dulbecco's modified essential medium (DMEM), Hams-F12 medium, Glutamax-1 (alanyl-glutamine), M199 medium were purchased from Gibco-BRL (Inchinnan, Paisely, UK). Trypsin-EDTA, the antibiotics penicillin and streptomycin sulphate and the antimycotic fungizone were obtained from ICN pharmaceuticals (Thame, Oxfordshire, UK). Roswell Park Memorial Institute medium (RPMI) and L-glutamine was from the Department of Pathology media kitchen (University of Cambridge, UK). Foetal calf serum (FCS) was obtained from Seralab (Crawley Downs, Sussex, UK). Hypoxanthine, aminopterin and thymidine (HAT) supplement were purchased from Sigma (Poole, Dorset, UK).

2.1.4 *Mammalian cell lines*

The following mammalian cell lines were obtained from the European Collection of Cell Cultures (ECACC) (Salisbury, Wiltshire, UK): Sp2/0-Ag14 myeloma cell line, MRC5 fibroblast cell line and Mycl-9E10 mouse monoclonal cell line. The human SV40 transformed keratinocyte cell line and human fibroblast cells were cultured and maintained by Dr. G. L. Howells (Department of Oral Pathology, London Hospital Medical College). All other murine haematopoietic cell lines used were obtained from Dr. A. R. Green (University of Cambridge, UK), were cultured and maintained by Dr. A. M. Murrell (University of Cambridge, UK) and are listed in Appendix 1.

2.1.5 *Proteolytic enzymes*

Human α-thrombin was prepared as described (Stone and Hofsteenge, 1986) and was fully active. Bovine factor Xa was a gift from Dr. B. LeBonniec (University of Cambridge, UK). Human plasmin, bovine pancreatic TPCK- treated trypsin, immobilised trypsin, human tissue plasminogen activator and human plasma kallikrein were from Sigma (Poole, Dorset, UK). Human activated protein C was from Drs J. Stenflo and A. Öhlin (Malmö, Sweden). Porcine acrosin was from Dr. R. Jones (AFRC, Babraham, Cambridge, UK). Human lung tryptase was purchased from Bioass (Diessen, Germany). The activated

complement enzyme C1s was purchased from Enzyme Research Laboratories (Swansea, UK). The proenzyme C1r was obtained from Calbiochem (Nottingham, UK).

2.1.6 *Enzymes for molecular biology*

Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, T4 DNA polynucleotide kinase and calf intestinal phosphatase were purchased from New England Biolabs (Hitchin, Hertfordshire, UK). T7 DNA polymerase (Sequenase version II) was purchased from USB (Cleveland, OH, USA). Recombinant Taq polymerase was from Perkin Elmer Cetus (Warrington, Cheshire, UK), or Promega (Southampton, UK). RNaseA (bovine pancreas) was from Sigma (Poole, Dorset, UK), RNasin (recombinant) was purchased from Promega. M-MLV reverse transcriptase was obtained from Gibco-BRL. Proteinase K was from Boehringer Mannheim (Lewes, Sussex, UK).

2.1.7 *Recombinant DNA materials*

The helper phage M13KO7 and the TA cloning vector pCRII were purchased from Invitrogen. The DNA molecular weight markers, Hae III digested ϕX174 and 1 Kb ladder were from New England Biolabs and Gibco-BRL, respectively. The cloning vectors pUC119 and M13mp19 were purchased from New England Biolabs. The pUC119-D1.3 vector (Ward et al., 1989) was a gift from Dr. G. Winter (Laboratory of Molecular Biology, Cambridge, UK). The mouse mPAR-2 genomic clone (Nystedt et al., 1994) and mouse thrombin receptor cDNA clone both obtained in pBluescript SK+ were generous gifts from Dr. J. Sundelin (Wallenberg Laboratory, Lund, Sweden) and Dr. H. Suidan (Friedrich Meischer Institute, Basel, Switzerland), respectively. The murine GAPDH cDNA probe was a gift from Dr. A. R. Green (University of Cambridge, UK). Mouse adult kidney and heart poly $(A)^+$ purified mRNA were purchased from Clontech (Palo Alto, CA, USA).

§ The generation of a multiple antigenic peptide (MAP) (Posnett et al., 1988; Tam, 1988) allows preparation of a multimeric peptide antigen on a branching lysine core by a single solid phase synthesis. The resulting macromolecules, generally of final molecular weight above 10 kDa, are composed of multiple copies of the peptide antigen, and the lysine core usually represents less than 10% of the total molecular weight.

2.1.8 *Peptides*

The peptides SKGRSLIGRLETQPPITGK-NH_2 corresponding to residues 31 - 49 of mouse PAR-2, SKGRSLIGKVDGTSHVTGK-NH_2 residues 33 - 51 of human PAR-2 and corresponding multiple antigenic peptide § (MAP hPAR-2) as well as the biotinylated peptide chloromethylketone inhibitor (biotin-SKGR-CH_2Cl) were synthesised by Dr. P. Harriot and Dr. B. Walker, (Department of Biochemistry, Queen's University of Belfast, UK). The other peptides LDPRSFLLRNPNDKYEPFWEDEE (TR 38-60), SFLLRNPND (TR 42-50) and EPFWEDEEKNES (TR 53-64) were gifts from Biogen (Cambridge, MA, USA).

2.1.9 *Oligonucleotide primers*

A detailed list of the oligonucleotide primers is given in Appendix 2. Oligonucleotides used for the cDNA cloning and PCR amplification of antibody heavy and light chains were gifts from Biogen. All other oligonucleotides were synthesised at the Department of Biochemistry, (University of Cambridge, UK). The lyophilised products of synthesis were dissolved in sterile distilled water, aliquoted and stored at -20°C until required.

2.1.10 *Radiochemicals*

γ^{32}P-dCTP (specific activity 3000Ci /mM) and α^{35}S-dATP (specific activity 400Ci /mM) were purchased from Amersham International (Buckinghamshire, UK).

2.1.11 *Commonly used buffers and solutions*

The following buffers and solutions were used for many applications throughout the course of this thesis:

TAE: (40 mM Tris-HCl, 50 mM sodium acetate, 0.4 mM EDTA, pH 7.4)

TBE: (134 mM Tris-HCl, 44 mM boric acid, 3 mM EDTA, pH 8.8)

TE: (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

TBS: (50 mM Tris-HCl, 150 mM NaCl, pH 7.5)

PBS: (1.5 mM KH_2PO_4, 8.1 mM Na_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.2)

10 x PCR buffer: (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3)

5 x RT buffer: (0.25 M Tris-HCl, 0.375 M KCl, 15 mM $MgCl_2$, pH 8.3)

10 x T7 POL buffer: (400 mM Tris-HCl, 200 mM $MgCl_2$, 320 mM NaCl, 50 mM DTT, pH 7.4)

2.1.12 *Chemicals and reagents*

Unless otherwise stated all chemicals and reagents were purchased from either Sigma (Poole Dorset, UK), or BDH (Bristol, UK) and were of the highest grade available commercially. Adenosine triphosphate, deoxy-nucleotides (dNTPs) were obtained from Pharmacia LKB Biotechnology (St. Albans, UK). The chromogenic substrates S-2222, S-2288, S-2238 and S-2302 were from Chromogenix (Quadratech, Surrey, UK). Spectrozyme t-PA was from American Diagnostica Inc. (Greenwich, CT, USA). Unless otherwise stated all antibodies for immunological detection studies were obtained from Dako Ltd. (High Wycombe, Buckinghamshire, UK). Sodium deoxycholate, sodium dodecylmaltoside and Keyhole Limpet Haemocyanin (KLH) were obtained from Calbiochem (Nottingham, UK).

2.2 Molecular biology methods

2.2.1 *Preparation of plasmid DNA*

High-quality plasmid or M13 phage replicative form (RF) vector DNA was prepared from small (2 to 10 ml) and large scale (200 ml to 1 litre) bacterial cultures based on the alkaline lysis procedure of Birnboim and Doly (1979) as described in Sambrook et al. (1989). Bacteria were grown at 37°C in 2xTY media supplemented with the appropriate antibiotics (in the case of bacteria harbouring the D1.3 or D1.3Loop expression plasmids it was necessary also to include 5% (w/v) glucose in the medium), harvested by centrifugation and resuspended. Alkali lysis was then performed as described in Sambrook et al. (1989). RNA was removed by treatment with RNAse. Bacterial lysates from the small or large scale plasmid preparations were further purified using the Wizard ™ minipreps or maxipreps

plasmid DNA purification systems from Promega (Southampton, UK) following the instructions given by the manufacturers. The purified plasmid DNA was stored in TE buffer at -20°C.

2.2.2 *DNA digestion and cloning of DNA*

Restriction digestion of plasmid vector DNA or PCR products was performed using conditions recommended by the manufacturers. Cut vectors were generally prepared by digestions of 1-2 µg of DNA overnight. These were then dephosphorylated by incubation with 24 units of calf intestinal phosphatase for 1 hr at 37°C followed by heat inactivation of the enzyme and two phenol/chloroform extractions (Sambrook et al., 1989). Ligation reactions generally contained 10-50 ng of cut vector (100-150 ng for dephosphorylated vectors), 100-200 ng insert DNA and 100-200 units T4 DNA ligase in supplied buffer (12 µl) at 16°C overnight.

2.2.3 *Purification of DNA fragments*

DNA fragments generated by restriction enzyme digestion or by PCR were separated by agarose gel electrophoresis as described in Sambrook et al. (1989), using ultra-pure low melting point agarose (Gibco-BRL). The running buffer used was either 1 x TBE or 1 x TAE. The Wizard ™ PCR Preps DNA purification system was used according to the manufacturer's instructions (Promega, Southampton, UK) to purify DNA fragments in the range of 200-800 bp. Larger fragments were purified using the QIAEX II ™ DNA purification kit according to the manufacturer's instructions (Qiagen, Dorking, Surrey, UK). For fragments smaller than approximately 200 bp, purification was achieved using centrifugal filtration with a SPIN-X ™ filter (Costar, Buckinghamshire, UK) as described by Clackson et al. (1991b).

2.2.4 *Preparation of Escherichia coli for transformation*

Competent cells for transfection were prepared by the method of Hanahan (1983). Cells for electroporation were prepared according to Dower et al. (1988) and stored at -70°C for up to 3 months. These cells were electroporated using a Biorad Gene Pulser Transfection apparatus (Biorad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK), as recommended in the manufacturer's instructions.

2.2.5 *Manipulation and sequencing of DNA*

Single-stranded (ss) phage DNA for sequencing was prepared on a 1.5 ml or 10 ml scale, with a RNase treatment step according to Sambrook et al. (1989). For larger preparations (100 ml), the phage preparation was filtered through a 0.22 µm filtration disc to remove residual bacterial cells (Sambrook et al., 1989). Single stranded phagemid DNA was rescued from bacterial cultures by superinfection with helper phage M13KO7 as described by Vieira and Messing (1987). For mutagenesis, ss phage DNA was prepared using *E. coli* CJ236.

Double-stranded plasmid DNA isolated by the protocol described in section 2.2.1 was used as a template for sequencing. Dideoxy sequencing (Sanger et al., 1977) was performed using the Sequenase Version 2.0 sequencing kit (USB, Cleveland, OH, USA) and α^{35}S-dATP as recommended by the manufacturers. After the sequencing reactions were denatured, aliquots were electrophoresed through denaturing polyacrylamide gels (6% acrylamide, 6 M urea, 1 x TBE) using 1 x TBE as the running buffer. After electrophoresis the gels were fixed in 10% (v/v) glacial acetic acid, 10% (v/v) methanol before being vacuum dried and exposed to Fuji X-ray film (Tokyo, Japan) as described in Sambrook et al. (1989).

Oligonucleotide-mediated site-directed mutagenesis was carried out using the *dut ung* selection system of Kunkel (1985) with two variations. First, template was prepared from *E. coli* CJ236 (*dut*$^-$, *ung*$^-$) and heteroduplexes transformed into *E. coli* strain XL-1 Blue F' (*dut*$^+$, *ung*$^+$). Second, extension was performed using native T7 DNA polymerase. Extension-ligation reactions were performed in 1 x T7 POL buffer; 2.5 units

of T7 DNA polymerase, 5 units of polynucleotide kinase and 100 units of T4 DNA ligase were added and the reaction incubated for 1 hr at room temperature.

2.2.6 *Polymerase chain reaction (PCR)*

All thermal cycling was performed on a Perkin-Elmer thermal cycler (Saiki et al., 1985; Saiki, 1990) in 0.5 ml Eppendorf tubes. Recombinant Taq polymerase (Perkin-Elmer or Promega) was used either in PCR buffer (2.1.11) or in the buffer supplied by the manufacturer. PCR reactions contained the following components in a volume of 50 µl.

	µl
H_2O	to 50 (final volume)
10 x PCR buffer	5
5 mM dNTPs	2
primer 1 (10 pmol/µl)	2.5
primer 2 (10 pmol/µl)	2.5

Template DNA was added, the mix overlaid with 50-100 µl light mineral oil and the tube placed in a PCR block at 94°C. After 5 min at 94°C, 1 - 2.5 units of Taq polymerase were added under the oil and cycling commenced. A typical program involved 30 cycles, each consisting of a denaturation step for 1 min at 94°C, annealing for one min and extension at 72°C for one min per kilobase of DNA. The annealing temperature was selected as detailed below:

Primer length (nucleotides)	**Annealing temperature (°C)**
17-19	55
19-21	60
21-24	65

For primers bearing multiple mismatches to the template, or a 5'-'tag', the above temperatures were dropped by 5-10°C. For primer pairs of different lengths, the lower

temperature was selected. Amplification was performed using both cloned DNA (including bacterial colonies and M13 plaques) and complex DNA sources (cDNA libraries). Adding the enzyme at 94°C avoided the non-specific extension that can occur as the reaction mix is heated through non-stringent temperatures. In some cases specificity was also enhanced by the use of "Touch-Down" PCR (Don et al., 1991; Roux, 1994). The parameters used for the "Touch-Down" PCR were as follows:

Annealing temperature (°C)	**Number of cycles**
66	1
64	1
62	2
60	2
58	2
56	2
54	20

PCR screening on bacterial colonies and M13 plaques was performed according to Güssow and Clackson (1989a).

2.2.7 *RNA extraction from cell lines*

Poly $(A)^+$ RNA was prepared from cell lines by the method described by Gonda et al. (1982). Briefly, 1 x 10^8 cells were washed twice in sterile PBS and snap frozen on dry ice. Frozen cell pellets were stored at -70°C. After partial thawing, the cell pellets were homogenised in 18 ml RNA lysis buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA; 0.5% (w/v) SDS, 300 µg/ml Proteinase-K, pH 7.4) and incubated at 37°C for 1 hr. Thereafter, the mRNA was bound to oligo-dT cellulose (Collaborative Biomedical Products, Bedford, MA, USA) in a high salt binding buffer by adjusting the NaCl concentration of the lysate to 0.5 M and the addition of 2 ml of prepared oligo-dT cellulose. 1 g oligo-dT cellulose was prepared by treatment with 0.5 M NaOH for 5 min, followed by

washing twice in 1 M Tris-HCl, pH 7.4 and equilibration in binding buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.2% (w/v) SDS, pH 7.4). Finally, the oligo-dT cellulose was resuspended in 20 ml binding buffer. The binding reaction was allowed to proceed on a roller mixer. The lysate/cellulose was then washed twice in binding buffer, followed by a wash in 30% (v/v) diluted binding buffer. Poly $(A)^+$ RNA was eluted twice, each time by incubating the oligo-dT in 2 ml H_2O at 55°C for 5 min. The eluent was transferred to a fresh tube and the mRNA was ethanol precipitated (0.1 volume 3 M sodium acetate pH 5.2 and 2.5 volumes of absolute ethanol), RNA was stored in ethanol at -20°C.

2.2.8 *Northern analysis*

Approximately 5 µg of poly $(A)^+$ RNA prepared as described above was precipitated by centrifugation and dried under vacuum. The RNA was resuspended in 12 µl RNA sample buffer (0.02 M 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, pH 7.0, plus 50% (v/v) formamide, 17.5% (v/v) formaldehyde, 10% (w/v) Bromophenol blue gel loading dye) and heated to 65°C for 15 min before being fractionated in a 0.8% agarose/2.2M formaldehyde gel as described in Sambrook et al. (1989). The gels were transferred to nylon membranes (Hybond N^+, Amersham, Buckinghamshire, England) and fixed with 0.05 M NaOH for 10 min. The membranes were hybridised with a 1 x 10^6 cpm/ml $\gamma^{32}P$ -dCTP labelled probe prepared according to Sambrook et al. (1989) using an Amersham labelling kit (Amersham, Buckinghamshire, England) following the manufacturers instructions. The membranes were washed twice at 65°C in 0.2 x SSC (v/v), 0.1% (w/v)SDS before exposing the filter to Fuji X-ray film (Tokyo, Japan).

2.2.9 *"Boilate" method for crude cDNA preparation from hybridomas*

The method for rapid preparation of cDNA for PCR was a modification of that of Ferre and Garduno (1989), in which reverse transcription is performed on cells boiled in diethyl-pyrocarbonate (DEPC) to inactivate RNases. Cultured hybridoma cells (about 1 x 10^6 cells) were washed once in PBS, then suspended in 100 µl 0.1% (v/v) DEPC in sterile water. The DEPC solution was made up immediately before use. The resuspended cells were

immediately transferred to an Eppendorf tube, boiled in a water bath for 5 min, then spun at 10,000 x g for 2 min in a microfuge. Meanwhile the following buffer mix was made up:

	µl
5 mM dNTPs	5
5 x RT buffer	20
0.1M DTT	5
Variable heavy chain cDNA oligonucleotide mix (10 pm/µl)	2
Variable light chain cDNA oligonucleotide mix (10 pm/µl)	2

67 µl supernatant from the boiled cells was added and the mix incubated at 67°C for 5 min, then cooled on the bench for 15 min to anneal the primers. 4 µl RNasin and 5 µl of M-MLV reverse transcriptase (RT) were added and the reaction incubated at 37°C for 1 hr.

2.3 Protein biochemistry methods

2.3.1 *SDS-PAGE*

The mini-PROTEAN II ™ (Biorad Laboratories, Hemel Hempstead, Hertfordshire, UK) gel electrophoresis system was used to cast mini gels (6 cm x 8 cm x 0.75 cm) and perform SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). The stacking gel contained 5% (w/v) acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.05% (w/v) NNN'N'tetramethyl-ethyldiamine. The resolving gel contained 12% (w/v) acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.05 % (w/v) ammonium persulphate, 0.05% (w/v) NNN'N'tetramethyl-ethyldiamine. The running buffer was 0.025 M Tris, pH 8.8, 0.192 M glycine, 0.1% (w/v) SDS. The sample buffer consisted of 0.0025% (w/v) Bromophenol blue, 10% (v/v) glycerol, 2% (w/v) SDS, 0.0625 M Tris-HCl, pH 6.8, and 5% (v/v) β-mercaptoethanol for reducing gels. Membrane protein samples were solubilised in sample buffer with the addition of 2.5 M urea and incubated for 30 min at room temperature before loading according to Vouret-Craviari et al. (1995a). After electrophoresis the gels were stained with

0.025% (w/v) Coomassie blue R-250 in 25% (v/v) methanol and 10% (v/v) acetic acid, or subjected to Western blotting.

2.3.2 *Western blot analysis*

Western blotting was essentially performed according to Towbin et al. (1979). Following electrophoresis, SDS-PAGE gels were placed in a sandwich consisting of 2 layers of 3M filter paper, gel, nitrocellulose, 2 layers of 3M filter paper. Nitrocellulose membranes were from Schleicher and Schuell, (Dassel, Germany) and filter paper from Whatman (Kent, UK). Wet electroblotting was performed using Biorad equipment at 400 mA for 3 hr according to the manufacturers instructions. The transfer buffer used was 10% (v/v) methanol, 0.2 M glycine, 25 mM Tris, pH 8.3. Remaining protein-binding sites on the blot were then blocked by incubation with 4% (w/v) skimmed milk powder (Marvel) or 1% BSA (fraction V) in TBS (blocking buffer), overnight at 4°C. The blot was then incubated with antibodies against the protein of interest in blocking buffer with 0.1% (v/v) Tween-20 (TBSBT) overnight at 4°C with gentle shaking, washed three times for 10 min with washing buffer TBS plus 0.1% (v/v) Tween-20 (TBST) and incubated with an alkaline phosphatase conjugated second antibody directed against the primary antibody (diluted 1:1000) for 1-2 hr at room temperature in TBSBT. Subsequently, unbound second antibodies were removed by washing as previously stated and finally developed using bromochloroindolyl phosphate / nitro-blue tetrazolium (BCIP / NBT) detection according to Harlow and Lane (1988).

Dot blot Western analysis was performed using Pro Blot membranes (Applied Biosystems, CA, USA) onto which 5 µl drops were applied. The incubation procedures were carried out as described above.

2.3.3 *Recombinant E. coli expression of D1.3 and D1.3Loop single chain antibody fragments (scFv)*

2.3.3.1 *Small scale expression of recombinant scFv*

The plasmid containing DNA encoding the scFv anti-lysozyme antibody D1.3 or mutant scFv antibody D1.3Loop was transfected into XL1-Blue F' *E. coli* cells (section 2.2.4). Overnight cultures (10 ml) in 2xTY with 100 µg/ml ampicillin and 5% (w/v) glucose were diluted 1:100 in the same medium and incubated at 37°C for 8 hr. Cells were pelleted, and resuspended in the original volume of medium without glucose but with 1 mM IPTG and incubated overnight at 30°C.

2.3.3.2 *Large scale expression of recombinant scFv*

Cultures (1 litre) were grown according to De Bellis and Schwartz (1990); glucose was included at a low level (0.1% w/v) so that it was exhausted during growth and induction could be effected simply by adding 1 mM IPTG without washing out the catabolite repressor. A 1/100 dilution of an overnight culture was grown at 37°C to an OD of 0.9, IPTG added and growth continued for 16-18 hr at 30°C.

2.3.3.3 *Electrophoresis of cell lysates*

Small scale (10 ml) culture lysates were prepared by resuspending the cell pellet in 50 µl distilled water and 50 µl 2 x electrophoresis sample buffer (section 2.3.1). For the preparation of *E. coli* periplasmic extracts, the cell pellet was resuspended in 5 ml of ice cold 20% (w/v) sucrose, 25 mM Tris-HCl, 1 mM EDTA, pH 8.0 for 15 min. The cellular suspension was spun at 10,000 x g for 5 min. The remaining pellet was then extracted with the addition of 2.5 ml of ice cold water. 50 µl of the supernatant was mixed with 50 µl 2 x sample buffer (section 2.3.1). The mixtures were boiled for 15 min and 50 µl samples were electrophoresed using 12% SDS-polyacrylamide gels. When required, proteins were transferred onto nitrocellulose by electroblotting and recombinant expressed scFv antibody detected with the 9E10 monoclonal antibody which recognises a C-myc decapeptide tag

present on the C-terminus of the single chain fragments (Evan et al., 1985; Ward et al., 1989) and visualised as described in section (2.3.2).

2.3.3.4 *Large scale preparation of soluble scFv fragments*

Cultures that were grown on a large scale according to section (2.3.3.2), were spun at 4°C at 6000 x g to pellet the bacteria. To prepare the lysate, the isolated bacteria pellet was resuspended in ice-cold PBS buffer and sonicated in the presence of protease inhibitors (1 mM benzamidine, 1 mM PMSF, 100 µM TLCK, 100 µM TPCK, 10 mM EDTA) until the bacteria were sufficiently lysed. The bacterial lysate was then clarified by centrifugation at 25,000 x g for 1 hr at 4°C, after which the clarified lysate was filtered through a 0.2 µm filter and stored at 4°C until applied to an affinity column.

2.3.3.5 *Purification of soluble scFv fragments using either a 9E10 or Hen Egg Lysozyme (HEL) affinity column*

All purification steps were performed at 4°C. In brief, the clarified lysate from section (2.3.3.4) was loaded onto a 9E10 affi-gel column (section 2.5.14). The column was washed successively with 10 column volumes of: PBS, PBS plus 0.5 M NaCl and PBS until the absorbance at 280 nm was zero. The bound protein was eluted with 0.2 M glycine-HCl, pH 2.5. The fractions containing antibody were immediately neutralised with 1 M Tris-HCl pH 8.0, dialysed against PBS and stored at -20° C or 4°C with the addition of 0.05% (w/v) sodium azide. The HEL affinity column (section 2.5.13) was loaded, washed and eluted as described for the 9E10 column.

2.4 General cell culture and cell handling

2.4.1 *Cell lines and cell culture*

All the cell lines were cultured in 25 cm^2, 75 cm^2 or 175 cm^2 tissue culture flasks supplied by either Costar (Buckinghamshire, UK) or Falcon (Meylan, France) in 5% CO_2/ 95% air at 37°C. The following culture media were used with the addition of 10% FCS: DMEM, Sp2/0-Ag14 myeloma and Mycl-9E10 monoclonal cell lines; Ham's-F12, MRC5 fibroblasts. Growth media was supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and fungizone (2.5 µg/ml). Culture media was replaced every 3 to 4 days with the exception of the Sp2/0-Ag14 myeloma cell line (section 2.5.4) and cells were subcultured as required. The adherent MRC5 cell line was passaged according to Harlow and Lane (1988). The murine haematopoietic cell lines (Appendix 1) were cultured and maintained by Dr. A. M. Murrell (University of Cambridge, UK) and the conditions and media used are listed in Appendix 1. The SV40 transformed human keratinocyte cell line and human fibroblast cells were cultured and maintained by Dr. G. L. Howells (Department of Oral Pathology, London Hospital Medical College). Human primary culture endothelial cells were isolated from human umbilical cord veins by the procedure of Jaffe et al. (1973) and were cultured and maintained by Dr. C. Plumpton (University of Cambridge, UK) according to Plumpton et al. (1994).

2.4.2 *Determination of cell viability*

Cell viability was determined by visually counting cells which excluded trypan blue dye. Briefly, a 100 µl aliquot of cells was added to 100 µl trypan blue (80% trypan blue vital stain, 0.07 M NaCl, 0.01 M KH_2PO_4) and incubated at room temperature for 5 min. Cells were counted in a haemocytometer.

2.4.3 *Cryopreservation and recovery of cell lines*

Cell lines were washed with Hanks Buffered Saline solution (HBSS) and resuspended at a concentration of approximately 1 x 10^6 cells/ml in sterile freezing medium (90% (v/v) FCS,

10% (v/v) DMSO). The cell suspension was immediately added to 2 ml cyropreservation vials (NUNC), transferred to a polystyrene container and placed at -80°C for 24 hr. The frozen cell suspensions were then placed in cryogenic storage containers containing liquid nitrogen. For recovery of frozen cells, they were removed from liquid nitrogen and thawed rapidly in a 37°C waterbath. The cell suspension was then diluted in pre-warmed culture medium. The cells were then centrifuged, washed twice more with pre-warmed medium, and finally resuspended in the appropriate culture medium.

2.5 Methods for preparation and characterisation of antibodies

2.5.1 *Preparation of the thrombin receptor extracellular domain (TRED) glutathione S-transferase (GST) fusion protein (GST-TRED)*

E. coli strain BL21 (DE3) carrying either the GST-TRED expression vectors pGEX-EK or pGEX-met (Parry, 1995) (obtained from Dr. M. A. Parry, Department of Haematology, University of Cambridge, UK) were grown until saturation in 2xTY media supplemented with ampicillin. After a 1/10 dilution of the culture and further incubation for 1 hr, IPTG was added to induce protein expression. The incubation continued for 4 hr at 37°C. *E. coli* were pelleted at 5400 x g for 30 min at 4°C and resuspended in ice-cold PBS buffer containing a cocktail of protease inhibitors (1 mM benzamidine, 1 mM PMSF, 100 µM TLCK, 100 µM TPCK, 10 mM EDTA). All further steps were carried out without interruption at 4°C. Bacterial lysis was achieved by sonication, after which Triton X-100 was added to 1 % (v/v). Cell debris was removed by centrifugation at 30,000 x g for 30 min, and the supernatant was incubated with glutathione -agarose beads for 30 min. Beads were washed with 50 mM Tris-HCl, pH 8.0, containing 10 mM EDTA and the fusion protein eluted with the same buffer containing 20 mM glutathione and 150 mM NaCl. SDS-PAGE using a 12% gel and Western Blot analysis revealed that the eluted protein had the same apparent molecular mass as the starting material and was essentially pure. The eluted GST-TRED protein sample was dialysed extensively against PBS at 4°C and stored at -70°C.

2.5.2 *Mouse immunisation schedule*

(performed by Dr. C. Plumpton (University of Cambridge, UK)

On day 1, a group of 6 female BALB/c mice (Central Biomedical Services, University of Cambridge, UK), were injected at two sites subcutaneously (SC) with 200 µl antigen (10 µg) in complete Freund's adjuvant. Secondary intraperitoneal injections were repeated at intervals of three to four weeks, with 500 µl antigen (16.5 µg) in incomplete Freund's adjuvant (IFA). Five to seven days after the second and subsequent injections, test bleeds were collected from immunised mice via the tail vein according to Harlow and Lane (1988) and the antibody response analysed. The mice with the best response were injected intraperitoneally with 500 µl of antigen (75 µg) without adjuvant. Five days after the final injection the mice were sacrificed and the spleen cells isolated for fusion.

2.5.3 *Preparation of spleen cells*

(performed by Dr. C. Plumpton (University of Cambridge, UK)

An immunised mouse was killed by cervical dislocation five days after the final boost. The animal was placed in a laminar flow cabinet and the skin on the abdomen soaked in 70% (v/v) ethanol. Using sterile instruments the skin was cut and an incision carefully made in the body wall. The spleen was pulled through the incision and carefully removed from the attached connective tissue. Any remaining pieces of connective tissue were then removed, and the spleen was washed in 10 ml DMEM media pre-warmed to 37°C. A cell suspension in 10 ml DMEM was made by pushing the spleen through a fine metal mesh with a syringe plunger. Large clumps were allowed to settle for 2 min, then the supernatant was pipetted into a fresh tube. At this stage a sample of cells was counted using a dilution of 1:20 (section 2.4.2).

2.5.4 *Preparation of plasmacytoma*

(performed by Dr. C. Plumpton (University of Cambridge, UK)

The mouse plasmacytoma Sp2/0-Ag14 was used for fusion. The plasmacytoma was thawed from liquid nitrogen stocks at least two weeks prior to the fusion experiment and

cultured with fresh media every 2 to 3 days. Every day for four to five days before fusion, the plasmacytoma was fed and the cell density adjusted to 0.5 - 1 x 10^6 /ml to ensure that the cells were in the exponential phase of growth.

2.5.5 *Cell fusion and post-fusion care*

The spleen cell population was divided into two, and about 1 x 10^7 plasmacytoma cells were added to each fraction to achieve a ratio of 5:1 spleen:plasmacytoma cells. The volume of each cell mixture was adjusted to 50 ml with DMEM, mixed well and centrifuged for 5 min at 400 x g. The supernatant was completely removed by aspiration and the cells loosened by tapping the base of the tube gently. PEG-1500 (1 ml) was added over a period of 1 min while stirring continuously with the end of the pipette. The solution was stirred for a further 1 min. With the same pipette, DMEM (1 ml) was added over 1 min to the cell suspension; a further 1 ml of DMEM was added over 30 s, followed by 8 ml DMEM in 1 ml aliquots, each over 15 s. An additional 35 ml DMEM were added slowly, stirring continuously. The PEG-1500 and DMEM solutions were maintained at either 37°C or room temperature for different experiments. The fusion mixture was centrifuged for 5 min at 400 x g. The supernatant was removed by aspiration and the cell pellet gently resuspended in DMEM plus 20% FCS.

The fusion mixture was seeded into either 24 well or 96 well plates. For 24 well plate fusions, four plates were used with each well containing 2 ml. For 96-well plate fusions, 10 plates were used with 200 µl fusion mixture added per well and HAT was introduced at full strength on the day of fusion. The fusion wells were fed every 2 to 3 days with HAT medium and the wells inspected daily for clonal growth. When macroscopic growth of colonies was visible (usually 7 to 14 days after fusion) the medium was not changed for at least 1 day and then the media screened for antibody activity. Wells with positive activity were duplicated and expanded in medium containing HT. The cells from these wells were frozen and cloned as soon as possible. Positive cultures were fed with medium for at least a week to dilute out the residual aminopterin and allow time for *de novo* synthesis of deoxyribonucleic acid to begin. Cells in wells producing the desired antibody

were frozen several times, kept growing during cloning to monitor stability and to provide a reserve of cells if cloning failed. Samples of supernatant from each positive line were stored at 4°C.

2.5.6 *Cloning by limiting dilution*

For cloning, cells were counted and diluted in DMEM plus 20% FCS such that the final dilutions contained 30, 10 and 3 cells per ml. Aliquots (100 µl) of each dilution were then added to 96-well plates containing a feeder layer of cells (MRC5 fibroblasts) plated out at a density of 1 - 4 x 10^4 /ml. Usually 48 wells were used for each of the low dilutions and 96 wells for the highest dilution. The mean values were 3, 1 and 0.3 cells per well. After 7 to 10 days, supernatants from wells showing growth were tested for the desired antibody. Three positive wells from the highest dilution plates were expanded and frozen. The cells were recloned if less than 95% of the wells from the highest dilution tested positive.

2.5.7 *Mouse monoclonal Enzyme-Linked Immunosorbent Assay (ELISA)*

96 well NUNC polystyrene ELISA plates (Gibco) were coated with 50 µl of glutathione S-transferase (GST) and thrombin receptor extracellular domain (TRED) fusion protein (GST-TRED) (1µg/ml) (section 2.5.1) in PBS plus 0.05% (w/v) NaN_3 and left overnight at 4°C in a humidified atmosphere. The plates were washed 3 times with PBS plus 0.1% (v/v) Tween-20, then once with PBS and shaken dry. The plates were then incubated with a solution of 1% (w/v) bovine serum albumin fraction V (BSA) in PBS for 2 hr at room temperature or at 4°C overnight to block non-specific binding sites. Plates were washed as above. Serial dilutions of both mouse pre-immune and anti-GST-TRED test bleeds were made starting at 1/100 using 1% (w/v) BSA in PBS plus 0.1% (v/v) Tween-20 (PBSBT) as the diluent. 100 µl of each serial dilution were added to wells and incubated for 2 hr at room temperature or overnight at 4°C in a humidified atmosphere. When testing hybridoma tissue culture supernatants, 50 µl of undiluted supernatant were added to each coated well. Plates were washed again as described above. Peroxidase-conjugated rabbit anti-mouse immunoglobulin was diluted 1/1000 using PBSBT and 100 µl of this solution were added

to each well and incubated for 1 hr at room temperature in a humidified atmosphere. Plates were washed again as described above. The peroxidase substrate 3',3',5',5'-tetramethylbenzidine (TMB) was dissolved at 1 mg/ml in dimethylsulfoxide (DMSO). 100µl of this TMB solution were added to 9.9 ml of 0.1 M sodium acetate, pH 6.0. Hydrogen peroxide (H_2O_2) was added just before use to a final concentration of 0.01% (v/v). 100 µl of the substrate solution were added per well and incubated for 10 to 30 min at room temperature until suitable colour developed. 100 µl of 1 M sulphuric acid (H_2SO_4) were added to each well. OD 450 nm readings were taken on a Molecular Devices microtitre plate reader and the associated SOFTmax software (Molecular Devices, Alpha Laboratories, Hampshire, UK).

2.5.8 *Monoclonal antibody competition ELISA*

2.5.8.1 *Conjugation of antibodies with biotin*

The purified antibodies were dialysed against 0.1 M $NaHCO_3$, pH 8.0 at 4°C and concentrated by centrifugal filtration using a Centricon-30 (Amicon Ltd, Gloucester, UK) to a final concentration of 2 mg/ml. The conjugation reaction was performed using biotin succinimide ester (Pierce, Chester, UK) according to the manufacturer's instructions. The biotin-conjugated antibody reaction mix was dialysed against PBS at 4°C and stored at 4°C with the addition of 0.05% (w/v) NaN_3.

2.5.8.2 *Chequerboard ELISA*

Each of the eight rows (A-H) of a 96 well NUNC Maxisorp polystyrene plate were coated with serial (1:2) dilutions of GST-TRED antigen diluted in PBS plus 0.05% (w/v) NaN_3, starting from row A with a concentration of 1 µg/ml and leaving column 12 uncoated. Coated plates were left overnight at 4°C in a humidified atmosphere. The plates were blocked and washed as in section 2.5.7. Serial (1:3) dilutions of biotinylated antibody were made with PBSBT, starting from 10 µg/ml (column 1) leaving row H without antibody. The biotinylated antibody was left overnight at 4°C in a humidified atmosphere. Plates were washed as described in section 2.5.7. Streptavidin-HRP conjugate (Vector Laboratories,

VectaStain, Peterborough, UK) was diluted 1/1000 with PBSBT, and 100 µl of this solution were added to each well and left for 1 hr at room temperature in a humidified atmosphere. Detection was performed using the peroxidase substrate TMB as described in section 2.5.7.

2.5.8.3 *Competition ELISA*

For each antibody to be tested, the concentrations of GST-TRED and biotinylated antibody that produced an optimal signal using a chequerboard ELISA as described in section 2.5.8.2 were used. Each biotinylated antibody was mixed with different dilutions of either the same unlabelled monoclonal or a different one. 1:3 dilutions of unlabelled antibody, starting from 0.2 mg/ml were made. Binding of the biotinylated antibody was detected as described in section 2.5.8.2.

2.5.9 *Peptide epitope mapping ELISA*

Epitope mapping was conducted using three peptides derived from TRED; LDPRSFLLRNPNDKYEPFWEDEE (TR 38-60), SFLLRNPND (TR 42-50) and EPFWEDEEKNES (TR 53-64). ELISAs were performed with these peptides as described in section 2.5.7. In brief, a NUNC maxisorp plate was coated overnight with each peptide (5 µg/ml) in PBS at 4°C. Serial dilutions (1:2) of each antibody in PBSBT starting from 5 µg/ml were then incubated overnight at 4°C.

A peptide competition ELISA was also carried out with the same peptides using a GST-TRED coated plate as described in section 2.5.7. Each of the three peptides was incubated at a final concentration of 10 mg/ml with the serial dilutions of antibody overnight at 4°C. The same serial dilutions of antibody in the absence of peptide were used as controls. Antibody bound to GST-TRED in the presence and absence of peptide was then detected as described in section 2.5.7.

2.5.10 *Peptide coupling to carrier proteins with glutaraldehyde*

The peptides SKGRSLIGRLETQPPITGK-NH_2 (linear mPAR-2) and SKGRSLIGKVDGTSHVTGK-NH_2 (linear hPAR-2) were coupled to the carrier protein KLH using glutaraldehyde in a single step as described by Harlow and Lane (1988).

2.5.11 *Rabbit (New Zealand White) immunisation schedule*

A 43 day immunisation schedule was used as shown below. Antibodies were raised using 2-3 rabbits per antigen. After collection, blood was stored overnight at 4°C. Serum was removed, and centrifuged at 1400 x g for 30 min to pellet red blood cells. Serum was stored at -20°C.

	Procedure
Day 1:	Preimmune bleed (10 ml). Rabbits subsequently immunised with 1.25 mg antigen in 0.5 ml PBS mixed 1:1 with complete Freunds adjuvant (4 subcutaneous sites).
Day 15:	Rabbits immunised with 2.5 mg antigen in 0.5 ml PBS mixed 1:1 with incomplete Freunds adjuvant (4 subcutaneous sites).
Day 29:	Test bleed (10ml). Immunisation with 5 mg antigen in 0.5 ml PBS mixed 1:1 with incomplete Freunds adjuvant.
Day 43:	Major bleed (20 ml).

2.5.12 *Rabbit serum ELISA*

ELISAs using rabbit sera were performed as described in section 2.5.7, except that bound antibodies were detected using peroxidase conjugated goat anti-rabbit immunoglobulin.

2.5.13 *Preparation of matrices to affinity purify antibodies*

The affinity columns were prepared using Activated CH Sepharose 4B (Pharmacia LKB Biotechnology, St. Albans, UK) according to the manufacturer's instructions. In brief, 15 mg of the peptide ligand, either mPAR-2 or hPAR-2, were dissolved directly in the coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.0). Lyophilised KLH (30 mg), Hen Egg Lysozyme (HEL) (40 mg) and GST-TRED (20 mg), obtained as described in section 2.5.1, were all dialysed extensively against the coupling buffer at 4°C. Each ligand solution and gel suspension was coupled overnight at 4°C in an end over end mixer. The coupled gel was finally stored in PBS plus 0.05% (w/v) NaN_3 at 4°C.

2.5.14 *Preparation of antibody affinity columns*

(performed by Dr. C. Plumpton (Cambridge, UK)

Affinity purified antibodies, 2/389 (30 mg) and 9E10 (20 mg) were coupled to an Affi-Gel hydrazide (-Hz) gel (Biorad Laboratories, Hemel Hempstead, Hertfordshire, UK) according to the manufacturer's instructions. The coupled gel was finally stored in PBS plus 0.05% (w/v) NaN_3 at 4°C.

2.5.15 *Affinity purification of rabbit polyclonal antibodies*

All purifications were performed at room temperature essentially as described by Harlow and Lane (1988). In brief, the pre-immune sera was precipitated with ammonium sulphate, dialysed extensively against PBS and loaded onto a protein-A Sepharose column. Antibodies were eluted as described in section 2.3.3.5. The rabbit antisera against mPAR-2 and hPAR-2 was either diluted 1:10 with PBS, or precipitated with ammonium sulphate and dialysed against PBS before being applied to their respective affinity matrix. The affinity matrix (mPAR-2 or hPAR-2 sepharose) was washed as described in section 2.3.3.5. After neutralisation of the fractions, the purified hPAR-2 or mPAR-2 antibodies were applied to a KLH affinity column to remove any residual anti-KLH antibodies. An exception was the affinity purified hPAR-2 antibodies generated using the hPAR-2 MAP

peptide where no carrier protein had been used. The affinity purified mPAR-2 and hPAR-2 antibodies were stored at - 20°C or 4°C in PBS plus 0.05% (w/v) NaN_3.

2.5.16 *Purification of mouse monoclonal antibodies*

All purifications were performed at room temperature essentially as described in Harlow and Lane (1988). In brief, mouse monoclonals of the IgG1 subtype were purified on protein A Sepharose with the high salt modification (Harlow and Lane, 1988). The IgA subtype antibody (3/138) was affinity purified on a GST-TRED affinity column (section 2.5.13). Either an ammonium sulphate precipitate of the 3/138 antibody, or undiluted tissue culture supernatant was applied to a GST-TRED affinity column, washed as described in section 2.3.3.5 and finally eluted with 0.2 M glycine-HCl pH 2.5. The affinity purified mouse monoclonal antibodies were stored at 4°C in PBS plus 0.05% (w/v) NaN_3.

2.5.17 *Immunoaffinity purification of the platelet thrombin receptor*

Platelet membrane fractions were prepared from 2.5 l of platelet-rich plasma (Department of Transfusion Medicine, University of Cambridge, UK). The platelets were pelleted at 2000 x g for 30 min at 4°C. All steps were performed at 4°C unless otherwise stated. The pellet was suspended in lysis buffer (7.5 mM NaCl, 2.5 mM Tris-HCl, 1 mM EDTA, 5 mM EGTA, pH 7.2) containing 1 mM phenylmethylsulfonylfluoride (PMSF), 10 mg/ml leupeptin, 10 mg/ml aprotinin and 5 mg/ml soybean trypsin inhibitor. The cells were homogenised for 1 min at maximum speed with a Polytron (Brinkman, PTA-10 rotor) and centrifuged for 45 min at 30,000 x g. The pellet was resuspended in lysis buffer and the homogenisation step was repeated three times. The pellet was resuspended in 30 ml solubilisation buffer (20 mM Tris-HCl, 15 mM EGTA, 10 mM EDTA pH 7.4) plus protease inhibitors as above, together with 1% (w/v) digitonin and 0.1% (w/v) sodium deoxycholate. The suspension was homogenised as above and then stirred for 90 min at 4°C before being centrifuged for 60 min at 30,000 x g. The supernatant was loaded at room temperature onto a column of agarose matrix (5 ml bed volume), to which the monoclonal 2/389 had been coupled. The column had been previously equilibrated with 0.1M Tris-

HCl, pH 7.4, 0.2% (w/v) digitonin (buffer A). The column was then washed with 5 column volumes (25 ml) of buffer A, followed by buffer A containing 0.5 M NaCl and finally 0.1 M Tris-HCl, pH 7.4, 0.2% (w/v) sodium-dodecyl maltoside. The bound protein was eluted with 0.1 M triethylamine, 0.2% (w/v) sodium-dodecyl maltoside, pH 11 and the eluted fractions were immediately neutralised with 1M HEPES, pH 5.2. The eluted thrombin receptor was dialysed extensively against 0.05 M HEPES, pH 7.4, 0.2% (w/v) sodium-dodecyl maltoside, containing 50% (v/v) glycerol and 0.1 M NaCl at 4°C. The final affinity purified thrombin receptor protein was concentrated using a Centricon-10 (Amicon Ltd, Gloucester, UK) and stored at -20°C to minimise protein aggregation. The amount of eluted protein was calculated using a Micro BCA Protein Assay kit using bovine serum albumin as a standard (Pierce, Chester, UK), according to the manufacturer's instructions.

2.5.18 *Immunocytochemistry*

Immunocytochemistry was performed on human umbilical vein endothelial cells (section 2.4.1) which had been grown on glass coverslips to subconfluence. The cells were fixed for 30 min in 4% (v/v) paraformaldehyde in PBS at 4°C. Non specific binding was blocked by incubation with swine or rabbit serum depending on which species was used for the production of the detection antibodies. Primary antibodies (mouse monoclonal or rabbit polyclonal) were diluted in TBS containing swine or rabbit serum (see above) and incubated with the fixed cells overnight at 4°C. The primary antibodies were visualised using swine anti-rabbit antibody, or rabbit anti-mouse secondary antibody using the peroxidase-anti-peroxidase (PAP) procedure according to the manufacturer's instructions (Dako Ltd, High Wycombe, Buckinghamshire, UK). The final detection step used was the peroxidase substrate diaminobenzidine (DAB) which was prepared according to Harlow and Lane (1988). The slides were finally mounted in Depex as described by Harlow and Lane (1988).

2.5.19 *Flow cytometry analysis*

(performed by Dr. G. L. Howells, Dr. M. G. Macey and Dr. M. A. Curtis, Department of Oral Pathology, London Hospital Medical College)

For the analysis of human leucocytes, freshly drawn blood anticoagulated 9:1 with 0.105 M sodium citrate was incubated with control or specific antibodies for 20 min at room temperature. For further analysis the red cells were lysed and the white blood cells fixed using the Q-Prep procedure (Coulter). After washing with HEPES-buffered (10 mM) Hanks balanced salt solution (Gibco-BRL) containing 0.5% (w/v) BSA (HBSS/BSA), the cells were incubated with either fluorescein conjugated swine anti-rabbit antibodies or fluorescein conjugated rabbit anti-mouse antibodies for 20 min at room temperature. After further washing with HBSS/BSA, the cells were resuspended in this solution and analysed on a FACScan (Becton Dickinson) equipped with Consort 32 Lysys version 1.02 software. Lymphocytes, monocytes and neutrophils were gated using standard forward and side scatter profiles.

Platelets were stained in whole blood washed with PBS containing EDTA (9 mM) without lysis of red blood cells and were analysed using log amplification of forward and side scatter.

For the analysis of cell lines, the cells were washed and resuspended in HBSS/BSA, incubated with the primary antibody and analysed as described above.

2.6 *In vitro platelet aggregation assay*

Platelet rich plasma (PRP) was prepared from fresh blood anticoagulated with 0.35% tri-sodium citrate 10:1. The anticoagulated blood (35 ml) was centrifuged at 160 x g for 30 min at room temperature. The supernatant platelet-rich plasma, approximately 20 ml (PRP), was added to 20 ml washing buffer (103 mM NaCl, 5 mM KCl, 36 mM citrate pH 6.5, 0.1% (w/v) glucose, 0.5 mM $CaCl_2$, 0.35% (w/v) BSA, also containing 200 nM prostacyclin (PGI2) and 0.2 units /ml apyrase). After acidification to pH 6.5 with 0.1 M citric acid, the platelet suspension was incubated for 15 min at 37°C and then centrifuged at 2000 x g for 10 min. The platelet pellet was gently resuspended in 20 ml washing buffer,

incubated for 10 min at 37°C and centrifuged at 160 x g for 10 min. The volume of the resultant platelet suspension was adjusted to 40 ml with washing buffer, incubated for 10 min at 37°C and centrifuged as above. The washed platelets were resuspended in 15 ml resuspension buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES pH 7.4, 0.1% (w/v) glucose, 1 mM $CaCl_2$, 0.35% (w/v) BSA, apyrase 0.2 units /ml) and counted in a thrombocounter (Department of Clinical Haematology, University of Cambridge, UK). The final platelet count was adjusted to 330 x 10^6 / ml. Aggregation experiments were performed using a BioData Corporation Platelet Aggregation Profiler (Horsham, PA, USA). The washed platelet suspension (0.460 ml) was kept at 37°C and stirred at 1100 rpm. After addition of antibody diluted in resuspension buffer, the platelets were stirred for at least 2 min before aggregation was induced by the addition of human α-thrombin to a concentration of 1 nM. The increase in light transmission was monitored for at least 2 min after the addition of thrombin.

2.7 Enzyme kinetic analysis

2.7.1 *Methods for kinetic analysis*

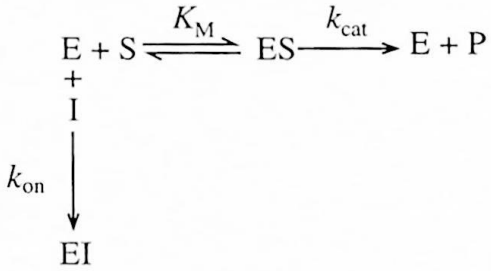
All kinetic experiments were performed at 37°C in 0.05 M Tris-HCl buffer pH 7.8, containing 0.1 M NaCl and 0.1% (w/v) polyethyleneglycol (PEG) Mr 6000 as described by Stone et al. (1991) using a Hewlett-Packard 8452A diode array spectrophotometer. The assays contained an appropriate peptidyl-*p*-nitroanilide substrate for the protease under study and were started by the addition of the protease. The activity of the protease was monitored by following the absorbance over the wavelength range of 400-410 nm. Concentrations of peptidyl-*p*-nitroanilide substrates were determined spectrophotometrically at 342 nm using an absorption coefficient of 8270 M^{-1} cm^{-1} (Lottenberg and Jackson, 1983).

2.7.2 *Determination of K_m values*

The initial rates of hydrolysis of peptidyl *p*-nitroanilide substrates were determined over a ten-fold range of substrate concentrations with at least one concentration above the K_m value and one below. The initial velocities (v) were fitted to the Michaelis-Menten equation by non-linear regression to determine an estimate for the K_m value.

2.7.3 *Kinetic analysis*

The irreversible inhibition of an enzyme (E) in the presence of a substrate (S) with an irreversible peptide chloromethylketone inhibitor (I) can be represented by the following scheme:



EI is the irreversibly inactivated enzyme. The inhibition of various serine proteases with the irreversible biotinylated peptidylchloromethylketone inhibitor biotin-SKGR-CH_2Cl was analysed according to this scheme as described by Stone and Hofsteenge (1985). The progress curve for the production of *p*-nitroaniline (P) due to substrate cleavage will be given by equation (1)

$$P = \frac{v_o}{k'} \left[(1 - \exp^{(-k' t)}\right] \qquad (1)$$

where *vo* is the velocity in the absence of inhibitor and k' is the apparent first-order rate constant for the formation of the inhibited protease. The value of k' will be given by equation (2)

$$k' = \frac{k_{on}\,[I]}{1+\frac{[S]}{K_m}} \qquad (2)$$

Equation 2 was substituted into equation 1 and progress curve data obtained at a number of concentrations of biotin-SKGR-CH_2Cl were simultaneously fitted to the resultant equation to yield estimates for k_{on}.

Chapter 3

Isolation And Characterisation Of Monoclonal Antibodies To The Human Thrombin Receptor

3.1 Introduction

Antibodies directed against the thrombin receptor have been used to study and characterise the receptor. Initial studies utilised polyclonal antibodies directed against peptide fragments of the extracellular domain. Hung et al. (1992a) produced rabbit polyclonal antibodies to an 18 amino acid peptide from the hirudin-like domain (amino acids 52 to 69). These antibodies specifically blocked platelet aggregation and secretion induced by low concentrations of thrombin, but did not affect activation of platelets by the 14 amino acid peptide ligand (TRAP) (amino acids 42 to 55 from the thrombin receptor). Thus, the antibodies prevented receptor activation by blocking the interaction between thrombin and the hirudin-like domain which is essential for cleavage.

Similar antibodies (IgG 9600), inhibited thrombin-induced aggregation of African green monkey platelets (Cook et al., 1995). The effect of thrombin receptor blockade by these antibodies on arterial thrombosis was evaluated with an animal model of platelet -dependent cyclic flow reductions (CFR) in the carotid artery of the monkey. Intravenous administration of IgG 9600 (10 mg/kg) abolished CFR in three monkeys and reduced CFR frequency by 50% in a fourth monkey tested (Cook et al., 1995). *In vitro* platelet aggregation in response to up to 100 nM α-thrombin was completely inhibited. This suggests that direct blockade of the platelet thrombin receptor may be an attractive antithrombotic target *in vivo.*

Mouse monoclonal antibodies have also been produced to peptide fragments of the receptor (Brass et al., 1992; Norton et al., 1993). Monoclonal antibodies directed against the cleavage site or flanking regions also inhibited platelet aggregation induced by thrombin, but failed to inhibit aggregation in response to TRAP (Brass et al., 1992).

Rabbit polyclonal antibodies to the thrombin receptor have also been produced using a fusion protein containing amino acid residues 1-160 of the human thrombin receptor as the immunising antigen (Bahou et al., 1993a). The polyclonal antibodies obtained recognised regions around the thrombin cleavage site and prevented platelet aggregation induced by thrombin. In addition, antibodies specific to amino acid residues 83 to 94 of the extracellular domain, located before the first transmembrane helix, inhibited platelet aggregation induced by TRAP (Bahou et al., 1994). Site directed mutagenesis and the production of receptor chimeras demonstrated that this region was important for the initiation of signal transduction events by TRAP (Gerszten et al., 1994; Nanevicz et al., 1995).

Monoclonal antibodies to peptide fragments have been used to visualise the receptor on Western blots of membranes isolated from platelets, human erythroleukemic (HEL) cells, Children's Hospital Research Foundation cell line number 288 (CHRF-288) cells, human umbilical vein endothelial cells (HUVECs) and selected human tumour cell lines. The apparent molecular mass of the immunoreactive protein was 66 kDa (Brass et al., 1992; Wojtukiewicz et al., 1995). A similar estimation of the size was obtained from platelet membranes using a rabbit polyclonal antibody (Hung et al., 1992a). This apparent molecular mass is about one third greater than that predicted from the cDNA sequence. The higher molecular mass can be attributed to the N-linked glycosylation present on the receptor (Vouret-Craviari et al., 1995a).

In the five years since the identification of the thrombin receptor, there have been no reports of purification of the receptor, or its characterisation at the protein level. Because of the low abundance of the G-protein-coupled receptor, purification procedures usually involve an affinity chromatography step. Thrombin would not be a suitable ligand for affinity chromatography since it cleaves the receptor. Moreover, TRAP possesses a relatively low affinity for the receptor and is subject to degradation by aminopeptidases that are commonly found in biological samples (Coller et al., 1993). Thus affinity chromatography using a monoclonal antibody is a promising approach. In order to investigate such a strategy, monoclonal antibodies were generated against a fusion protein

consisting of glutathione S- transferase fused to amino acid residues 26-99 of the thrombin receptor extracellular domain (GST-TRED). Three antibodies were isolated and these were shown by flow cytometry and immunocytochemistry to recognise the native receptor. The epitopes of two of these antibodies were found to reside within the thrombin cleavage site and these antibodies blocked thrombin-dependent platelet aggregation. One of these monoclonal antibodies was subsequently used to purify the human thrombin receptor from platelet membranes.

3.2 Results

3.2.1 Generation of monoclonal antibodies

The extracellular domain of the thrombin receptor (TRED) was expressed in *E. coli* as a fusion protein at the C-terminus of glutathione S-transferase (GST). The construct used encoded residues from the putative pro-peptidase cleavage site to the beginning of the first transmembrane domain (residues 26-99) of the thrombin receptor. GST was separately expressed in *E. coli* and used in an ELISA to screen for mice producing high levels of antibodies to the TRED portion of the fusion protein. Mice immunised with the GST-TRED fusion protein (section 2.5.1 - 2.5.2) produced a stronger immunogenic response to the GST portion of the protein. Table 3.1 shows the titre to GST and GST-TRED of mice from 4 different immunisation groups. Three mice (B6, C1 and C3) were selected on the basis of their titre to GST-TRED versus GST. The spleens from these mice were removed and used to generate hybridomas (section 2.5.3 - 2.5.5). The culture supernatants of the hybridomas were screened by ELISA against GST-TRED and GST (section 2.5.7). Hybridomas were selected on the basis of production of antibodies that recognised GST-TRED but not GST. A stable hybridoma producing an anti-TRED monoclonal (2/389) was obtained from mouse C1 and two stable anti-TRED monoclonals (3/17 and 3/138) were isolated from mouse B6; mouse C3 did not yield stable positive clones. All three monoclonal cell lines were cloned at least twice by limited dilution (section 2.5.6) and remained stable and fully active. The monoclonal antibodies were isotyped using an ELISA (section 2.5.7) with specific rabbit

anti-isotype antibodies; 2/389 and 3/17 were members of the mouse IgG1 subclass, whereas 3/138 was IgA.

Table 3.1 *Titres of immunised mice to GST and GST-TRED.*
Titre dilution of sera from the different bleeds of the mice that were immunised with GST-TRED antigen were determined by ELISA to both GST and GST-TRED. The lowest titre for which a signal was twice that of the corresponding pre-immune bleeds is listed below. It was observed from the titre dilutions that after bleed three the antibody titres to GST and GST-TRED were equivalent for the majority of the immunised mice. The three mice B6, C1 and C3 were selected to generate hybridomas as they had final titres to GST-TRED at bleed two that were greater than 100,000, and they had a stronger immunogenic response to GST-TRED than GST after bleed 1, indicating that antibodies to TRED were being produced by these mice. The mice group (A1 - A6) were immunised at a later date than the other groups, and were kept in reserve in case any anti-TRED hybridomas could not be obtained using mice already selected from the other groups.

	Anti-GST Titre			Anti-GST-TRED Titre		
Mouse #	Bleed 1	Bleed 2	Bleed 3	Bleed 1	Bleed 2	Bleed 3
A1	1,800	437,400		1,800	437,400	
A2	5,400	145,800		1,800	145,800	
A3	5,400	48,600		16,200	145,800	
A4	5,400	16,200		1,800	48,600	
A5	1,800	437,400		1,800	437,400	
A6	600	145,800		600	437,400	
B1	0	5,400	16,200	5,400	16,200	16,200
B2	16,200	145,800	437,400	16,200	437,400	437,400
B3	0	16,200	16,200	5,400	48,600	48,600
B4	16,200	145,800	145,800	48,600	145,800	145,800
B5	5,400	145,800	48,600	16,200	145,800	48,600
B6	16,200	437,400	437,400	48,600	437,400	437,400
C1	0	145,800		1,800	145,800	
C2	0	16,200	48,600	600	48,600	48,600
C3	1,800	48,600	48,600	5,400	145,800	48,600
C4	0	145,800	145,800	5,400	145,800	145,800
C5	0	48,600	48,600	5,400	48,600	48,600
C6	0	48,600	437,400	1,800	145,600	437,400
D1	0	5,400	48,600	1,800	5,400	145,800
D2	0	5,400	48,600	1,800	1,800	145,800
D3	0	0	437,400	1,800	0	145,800
D4	1,800	16,200	145,800	16,200	16,200	437,400
D5	1,800	16,200	437,400	1,800	16,200	437,800
D6	0	5,400		5,400	5,400	

3.2.2 Epitope mapping

The epitopes recognised by the monoclonal antibodies were mapped using a competition ELISA with biotinylated monoclonals (section 2.5.8). Each monoclonal antibody had a unique epitope in the TRED. The results displayed in Figure 3.1 illustrate that the antibodies did not compete for a binding site, only the identical unlabelled antibody was able to block the binding of the biotinylated antibody to GST-TRED.

Smaller peptides were used to further define the epitopes recognised by the monoclonals. The following peptides were used; LDPRSFLLRNPNDKYEPFWEDEE (TR 38-60), SFLLRNPND (TR 42-50) and EPFWEDEEKNES (TR 53-64). Monoclonal antibody 3/138 did not bind to any of the peptides. The monoclonal 2/389 bound to TR 38-60 both in solution and immobilised to an ELISA plate (Figure 3.2). In contrast, 3/17 only bound to TR 38-60 in solution and did not bind the immobilised peptide. Although the two antibodies 2/389 and 3/17 recognised the TR 38-60 peptide, the smaller peptides TR 42-50 and TR 53-64 did not inhibit the binding of the antibody to GST-TRED (Figure 3.2).

Western blotting was performed with GST-TRED fusion protein that had been cleaved by thrombin. Cleavage of GST-TRED by thrombin removes 57 amino acids from the C-terminus of the fusion protein and the apparent molecular mass of the fusion protein decreases by about 5.5 kDa; a portion of TRED (residues 26-41) remains attached to GST (section 2.3.1 - 2.3.2). After cleavage of GST-TRED by thrombin, a lower molecular mass species could be recognised by 3/138. Thus, the epitope for 3/138 resides between residues 26-41. From Figure 3.3, it is apparent that part of the preparation of GST-TRED was already expressed truncated near this site. The other two antibodies both recognised epitopes that were lost after cleavage (Figure 3.3). These data support the previous observations from the peptide competition experiments; 3/17 and 2/389 recognised TR 38-60 and the majority of this sequence would be lost upon cleavage. In contrast, the 3/138 antibody did not recognise TR 38-60, consistent with the GST-TRED cleavage experiments which suggested that its epitope lies within residues 26-41.

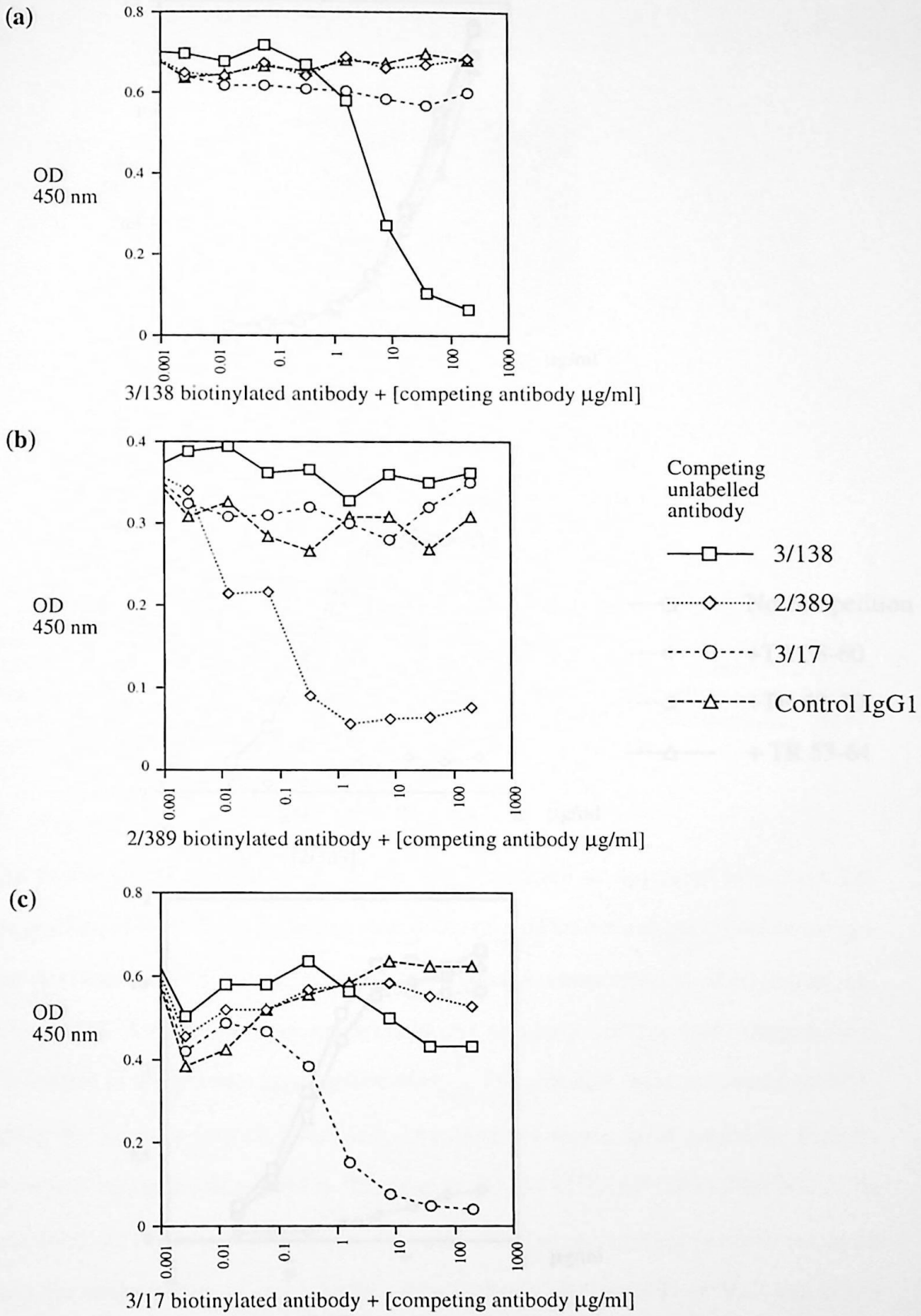


Figure 3.1 *Competition for the binding of biotinylated monoclonal antibody to GST-TRED.* The biotinylated antibody was present at a fixed concentration and competing antibodies were present in varying amounts. **(a)** Biotinylated monoclonal antibody 3/138. **(b)** Biotinylated monoclonal antibody 2/389. **(c)** Biotinylated monoclonal antibody 3/17. The competing antibodies were 3/138, 2/389, 3/17 and control IgG1. Assays were determined as described in section 2.5.8.

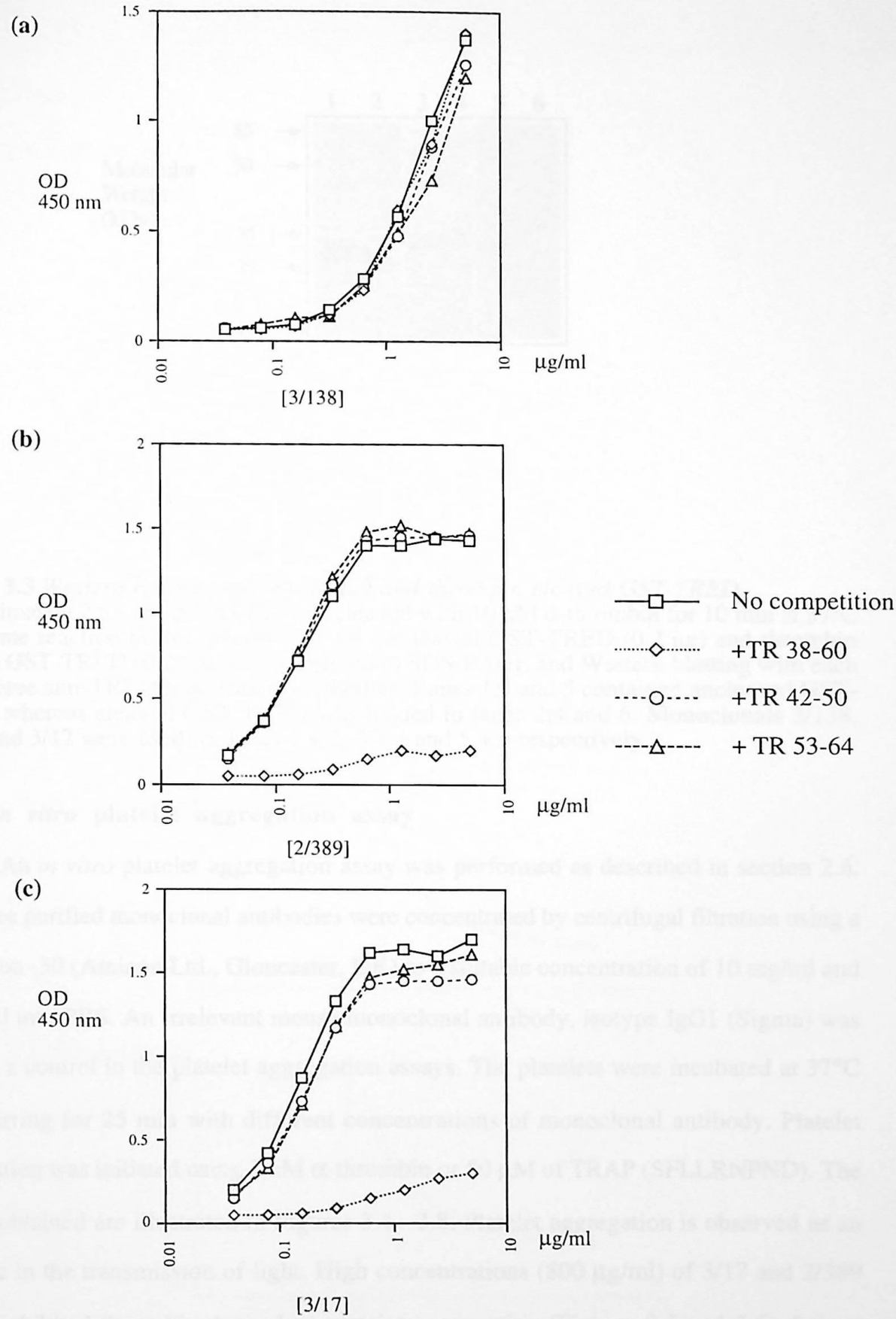


Figure 3.2 *Peptide epitope mapping.*
The binding of the monoclonal antibodies to GST-TRED was determined by ELISA. The antibodies were preincubated with 10 mg/ml of TR 38-60, TR 42-50 or TR 53-64 and assays performed as described in section 2.5.9. (**a**) Monoclonal antibody 3/138. (**b**) Monoclonal antibody 2/389. (**c**) Monoclonal antibody 3/17.

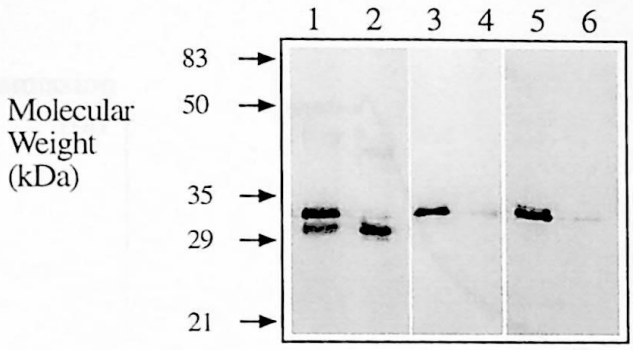


Figure 3.3 *Western blotting of GST-TRED and thrombin cleaved GST-TRED.* Approximately 2 μg of GST-TRED was cleaved with 10 nM α-thrombin for 10 min at 37°C in enzyme reaction buffer (section 2.7.1). Uncleaved GST-TRED (0.2 μg) and thrombin cleaved GST-TRED (0.2 μg) were subjected to SDS-PAGE and Western blotting with each of the three anti-TRED monoclonal antibodies. Lanes 1,3 and 5 contained uncleaved GST-TRED, whereas cleaved GST-TRED was loaded in lanes 2,4 and 6. Monoclonals 3/138, 2/389 and 3/17 were used for lanes 1 + 2, 3 + 4 and 5 + 6 respectively.

3.2.3 *In vitro* platelet aggregation assay

An *in vitro* platelet aggregation assay was performed as described in section 2.6. The three purified monoclonal antibodies were concentrated by centrifugal filtration using a Centricon -30 (Amicon Ltd., Gloucester, UK) to a suitable concentration of 10 mg/ml and dialysed into PBS. An irrelevant mouse monoclonal antibody, isotype IgG1 (Sigma) was used as a control in the platelet aggregation assays. The platelets were incubated at 37°C with stirring for 25 min with different concentrations of monoclonal antibody. Platelet aggregation was initiated using 1 nM α-thrombin or 50 μM of TRAP (SFLLRNPND). The results obtained are illustrated in Figures 3.4 - 3.8. Platelet aggregation is observed as an increase in the transmission of light. High concentrations (800 μg/ml) of 3/17 and 2/389 totally inhibited thrombin-dependent platelet aggregation (Figures 3.5 and 3.6). Lower concentrations (200 and 400 μg/ml) partially inhibited thrombin-induced aggregation. In contrast, 3/138 was unable to block aggregation in response to thrombin (Figure 3.4). Aggregation in response by TRAP was not affected by high concentrations (800 μg/ml) of the antibodies (Figure 3.8).

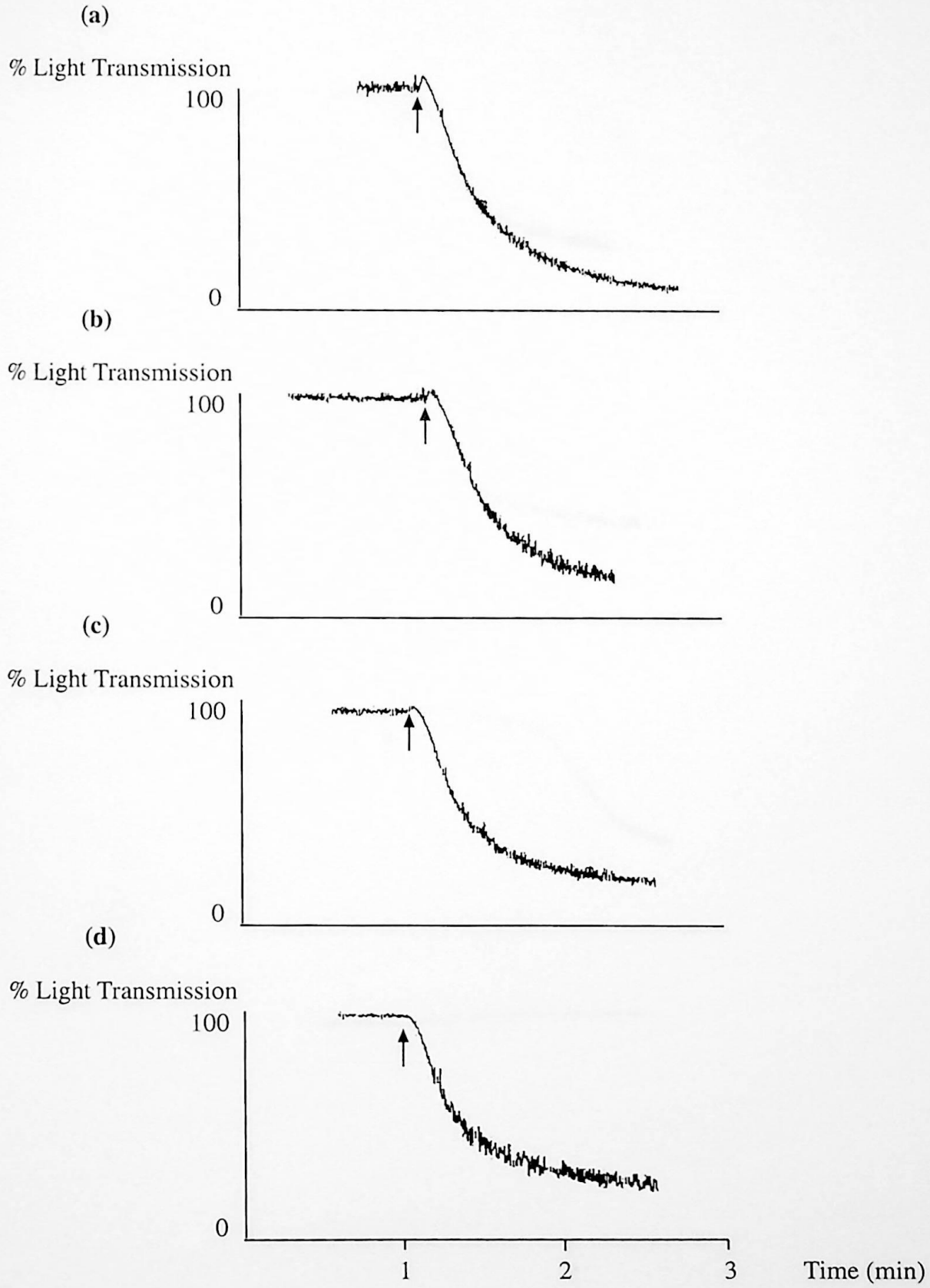


Figure 3.4 *Thrombin-induced platelet aggregation is not inhibited by 3/138 monoclonal antibody.* Platelet aggregation was initiated with 1 nM α-thrombin (indicated by the arrow), after the platelets were incubated with increasing amounts of 3/138 IgA monoclonal antibody for 25 min at 37°C. **(a)** Normal response in the absence of antibody. **(b)** 200 μg/ml. **(c)** 400 μg/ml. **(d)** 800 μg/ml.

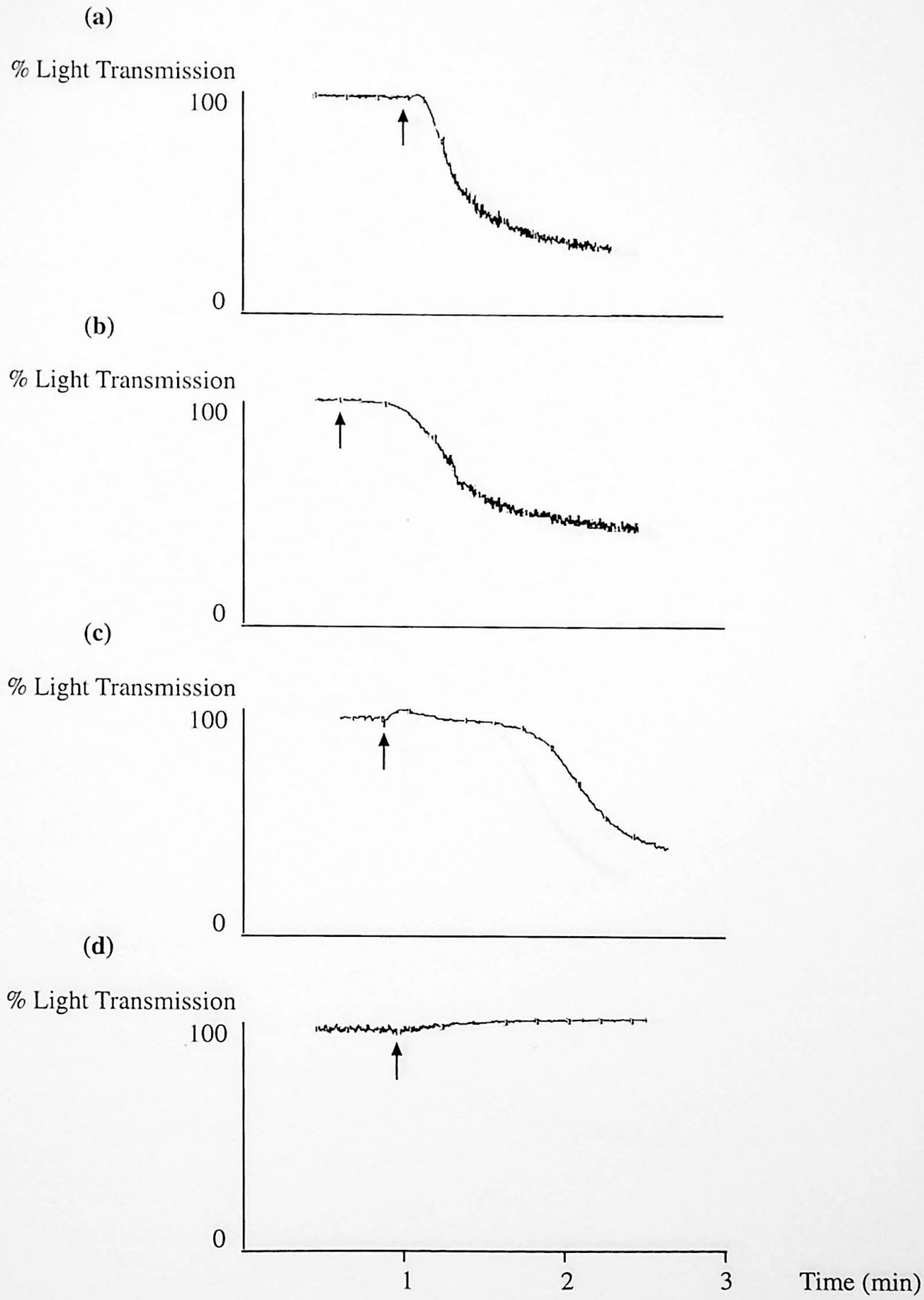


Figure 3.5 *Thrombin-induced platelet aggregation is inhibited by 2/389 monoclonal antibody.* Platelet aggregation was initiated with 1 nM α-thrombin (indicated by the arrow), after the platelets were incubated with increasing amounts of 2/389 IgG1 monoclonal antibody for 25 min at 37°C. (**a**) Normal response in the absence of antibody. (**b**) 200 μg/ml. (**c**) 400 μg/ml. (**d**) 800 μg/ml.

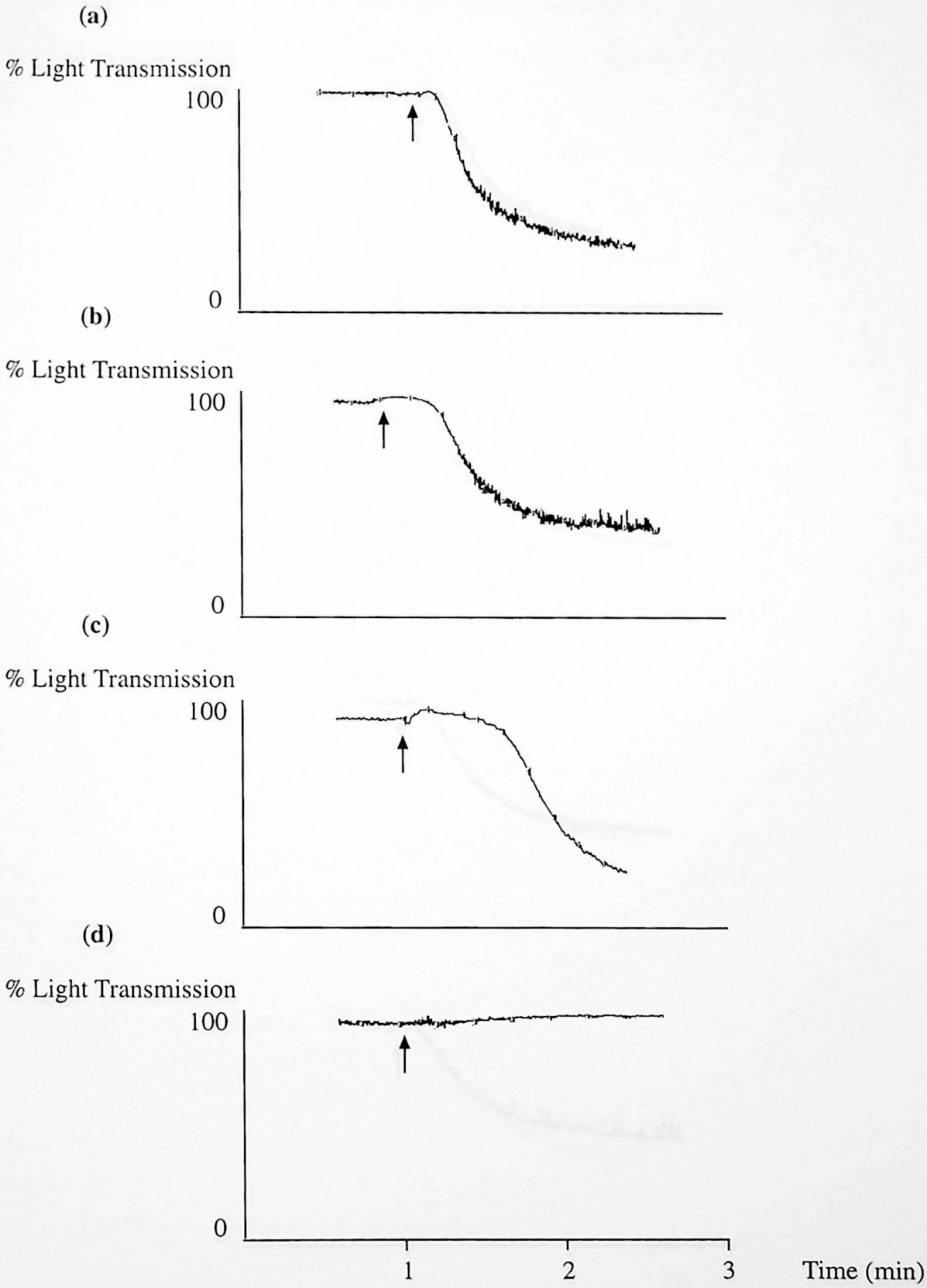


Figure 3.6 *Thrombin-induced platelet aggregation is inhibited by 3/17 monoclonal antibody.* Platelet aggregation was initiated with 1 nM α-thrombin (indicated by the arrow), after the platelets were incubated with increasing amounts of 3/17 IgG1 monoclonal antibody for 25 min at 37°C. **(a)** Normal response in the absence of antibody. **(b)** 200 μg/ml. **(c)** 400 μg/ml. **(d)** 800 μg/ml.

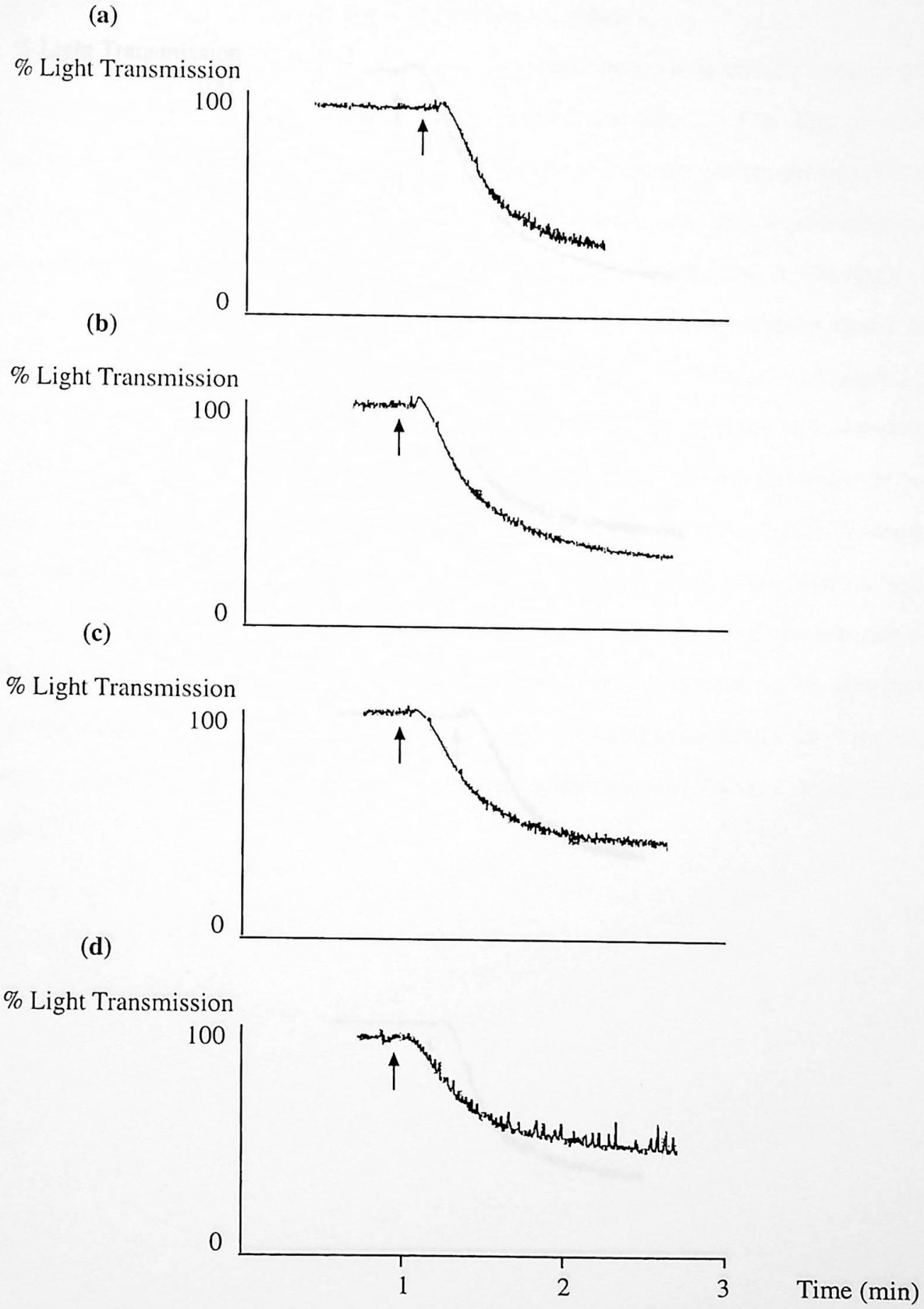


Figure 3.7 *Thrombin-induced platelet aggregation is not inhibited by control IgG1 monoclonal antibody.* Platelet aggregation was initiated with 1 nM α-thrombin (indicated by the arrow), after the platelets were incubated with increasing amounts of control IgG1 monoclonal antibody for 25 min at 37°C. **(a)** Normal response in the absence of antibody. **(b)** 200 μg/ml. **(c)** 400 μg/ml. **(d)** 800 μg/ml.

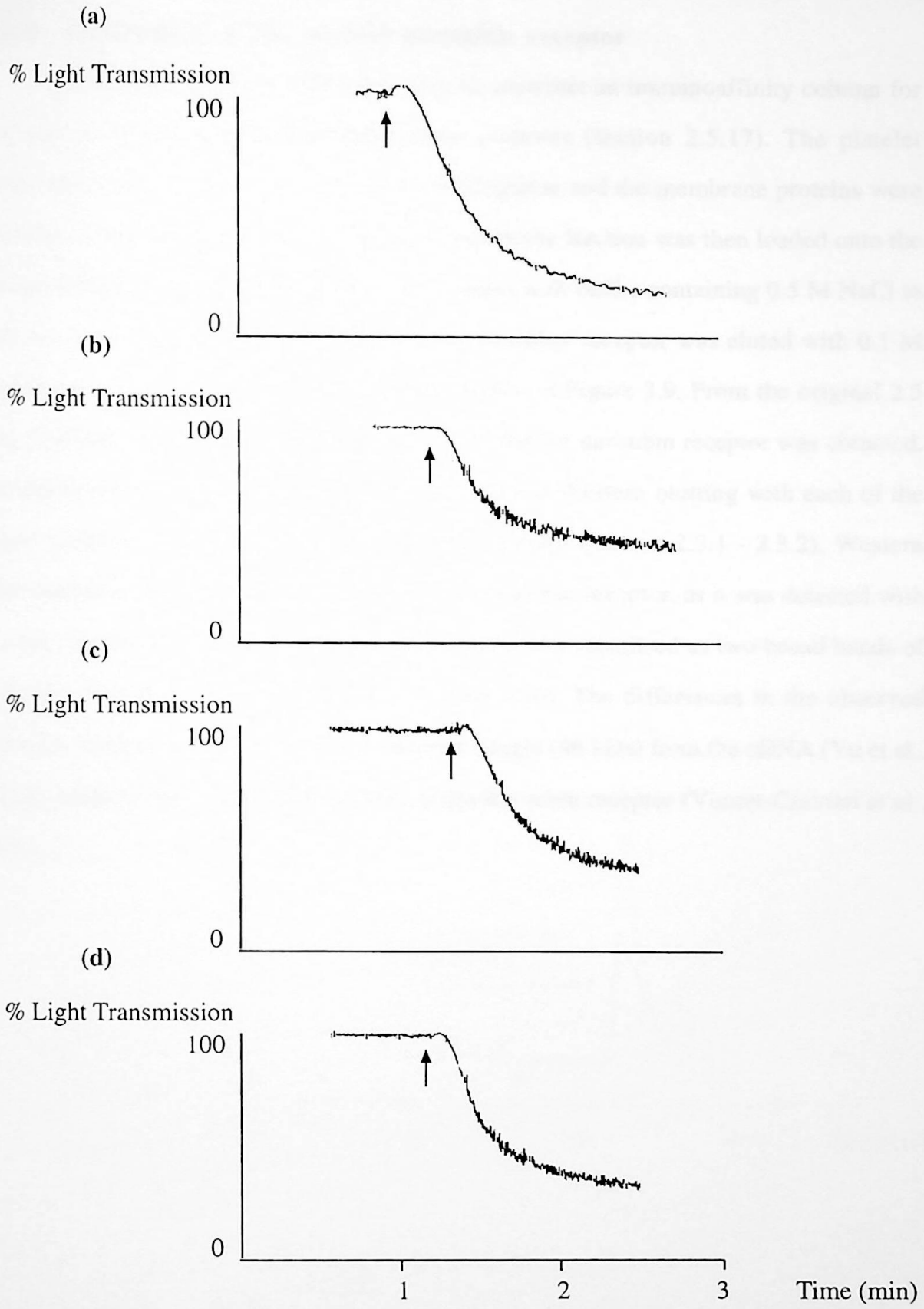


Figure 3.8 *TRAP induced platelet aggregation in the presence of saturating amounts of each monoclonal antibody.* Platelet aggregation was initiated with 50 μM agonist peptide TRAP (SFLLRNPND), (indicated by the arrow) after the platelets were incubated with 800 μg/ml of each monoclonal antibody. **(a)** 3/138 IgA. **(b)** 2/389 IgG1. **(c)** 3/17 IgG1. **(d)** Control IgG1.

3.2.4 Purification of the platelet thrombin receptor

Monoclonal antibody 2/389 was used to construct an immunoaffinity column for purification of the thrombin receptor from platelets (section 2.5.17). The platelet membranes were isolated by differential centrifugation and the membrane proteins were solubilised using detergent. This solubilised membrane fraction was then loaded onto the immunoaffinity column. After washing the column with buffer containing 0.5 M NaCl to elute any proteins bound non-specifically, the thrombin receptor was eluted with 0.1 M triethylamine (pH 11). The elution profile is shown in Figure 3.9. From the original 2.5 litres of platelet rich plasma, 0.11 mg of purified platelet thrombin receptor was obtained. The eluted material was analysed by SDS-PAGE and Western blotting with each of the mouse monoclonal antibodies to the thrombin receptor (section 2.3.1 - 2.3.2). Western blots confirmed that the eluted material was the thrombin receptor, as it was detected with all three monoclonal antibodies. The eluted protein was visualised as two broad bands of molecular weights 46 kDa and 80 kDa (Figure 3.10). The differences in the observed molecular weights and the calculated molecular weight (46 kDa) from the cDNA (Vu et al., 1991a), could be due to the glycosylation of the thrombin receptor (Vouret-Craviari et al., 1995a).

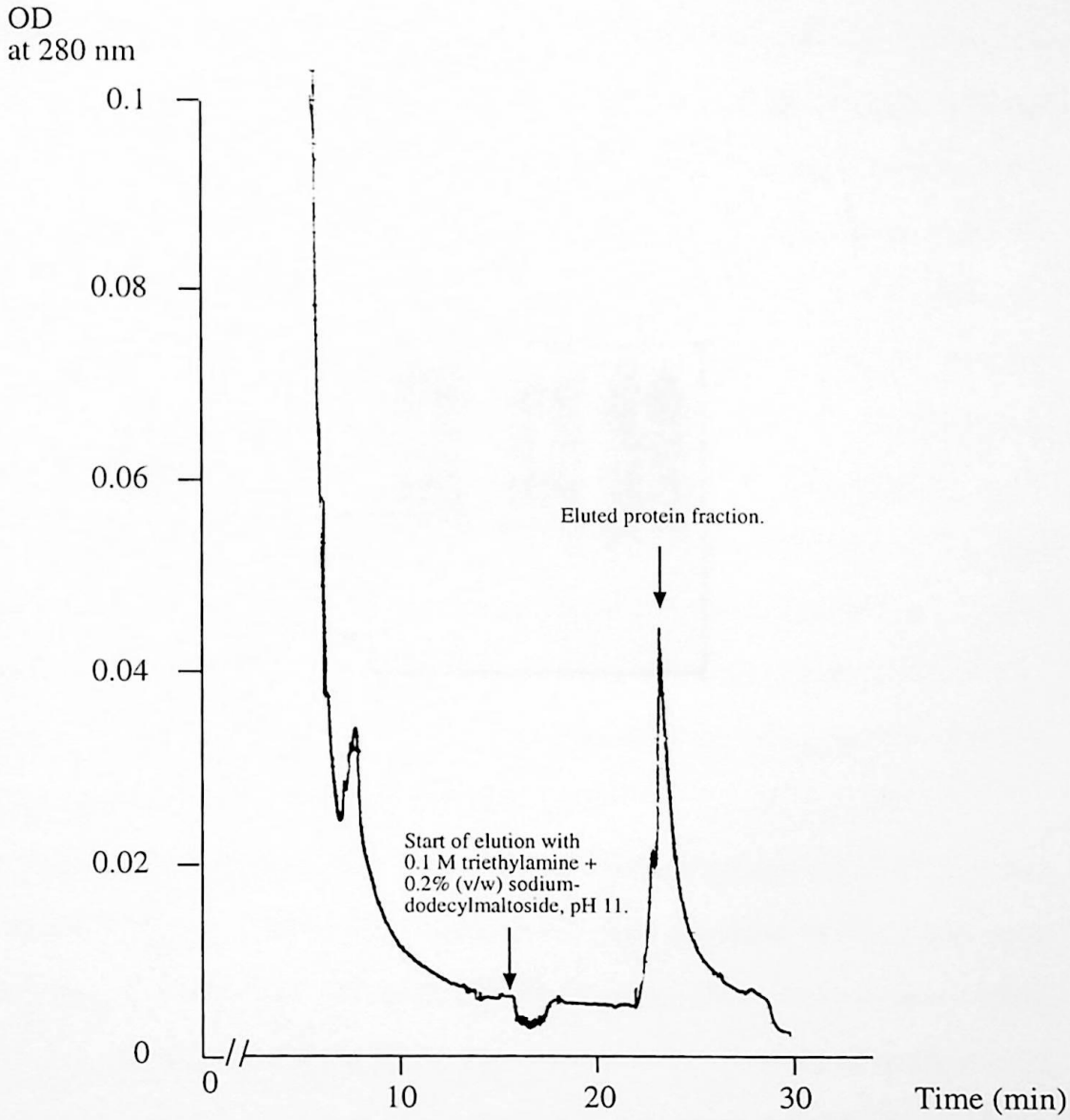


Figure 3.9 *Immunoaffinity purification of the platelet thrombin receptor.* The Affi-gel 2/389 monoclonal column was loaded overnight at room temperature with the solubilised platelet membrane fraction by continuously recycling 20 ml of the extract at 1 ml per min. The column was washed with 0.1 M Tris-HCl, pH 7.4, 0.2% (w/v) digitonin containing 0.5 M NaCl until the absorbance at 280 nm was zero (section 2.5.17). The bound protein was eluted with approximately 20 ml of 0.1 M triethylamine plus 0.2% (w/v) sodium-dodecyl maltoside (pH 11). The eluted fractions were immediately neutralised with 1 M HEPES, pH 5.2.

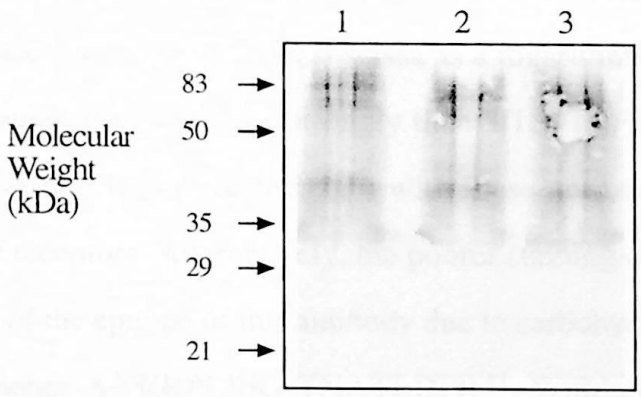


Figure 3.10 *Western blots of the eluted thrombin receptor protein.* SDS-PAGE and Western blotting was performed as described in section 2.3.1- 2.3.2; approximately 100 ng of purified protein were loaded in each lane. Lane 1, Western blot using the 3/138 monoclonal antibody; lane 2, Western blot using the 2/389 monoclonal antibody; lane 3, Western blot using the 3/17 monoclonal antibody.

3.2.5 Flow cytometry and immunocytochemistry with monoclonals against the thrombin receptor

The monoclonal antibodies specifically stained three cell types, platelets, fibroblasts and endothelial cells, that are known to express the thrombin receptor (Figures 3.11 - 3.13). Flow cytometry was performed according to section 2.5.19 using a concentration of 20 µg/ml for each monoclonal antibody. This work was performed by Dr. G. L. Howells, Dr. M. G. Macey and Dr. M. A. Curtis (Department of Oral Pathology, London Hospital Medical College). The results are displayed as fluorescence histograms, obtained on a Becton Dickinson fluorescence activated cell sorter (FACS). Each result is shown with the following axis scales; ordinate (cells/channel) and abscissa (cell fluorescence brightness), with the ordinate scale as linear and the abscissa as a logarithmic scale. The monoclonals 2/389 and 3/17 stained platelets more strongly than 3/138 in Figure 3.12. The frequency histograms were shifted further to the right with these antibodies, indicating a greater binding to platelet receptors. Alternatively, the poorer staining observed with 3/138 could be due to masking of the epitope of this antibody due to carbohydrate. The epitope of 3/138 resides in the sequence A^{26}RRPESKATNATLDPR^{41}. Within this sequence Asn^{35}-Ala-Thr^{37} represents a potential glycosylation site. The data of Vouret-Cravari et al. (1995a) suggest that it is glycosylated in mammalian cells.

The three monoclonal antibodies stained for the human thrombin receptor on human umbilical endothelial cells (HUVECs) (Figure 3.13). The thrombin receptor has been shown to be highly expressed on HUVECs (Woolkalis et al., 1995; Herbert et al., 1994; Molino et al., 1995; Ngaiza and Jaffe, 1991) and consequently, the staining of these cells by the monoclonals was also examined. Monoclonal 3/17 was the most successful antibody for immunocytochemistry, displaying the strongest staining compared to both 3/138 and 2/389. This, however, could reflect that its epitope is more stable using the para-formaldehyde fixative than the respective epitopes of 3/138 and 2/389.

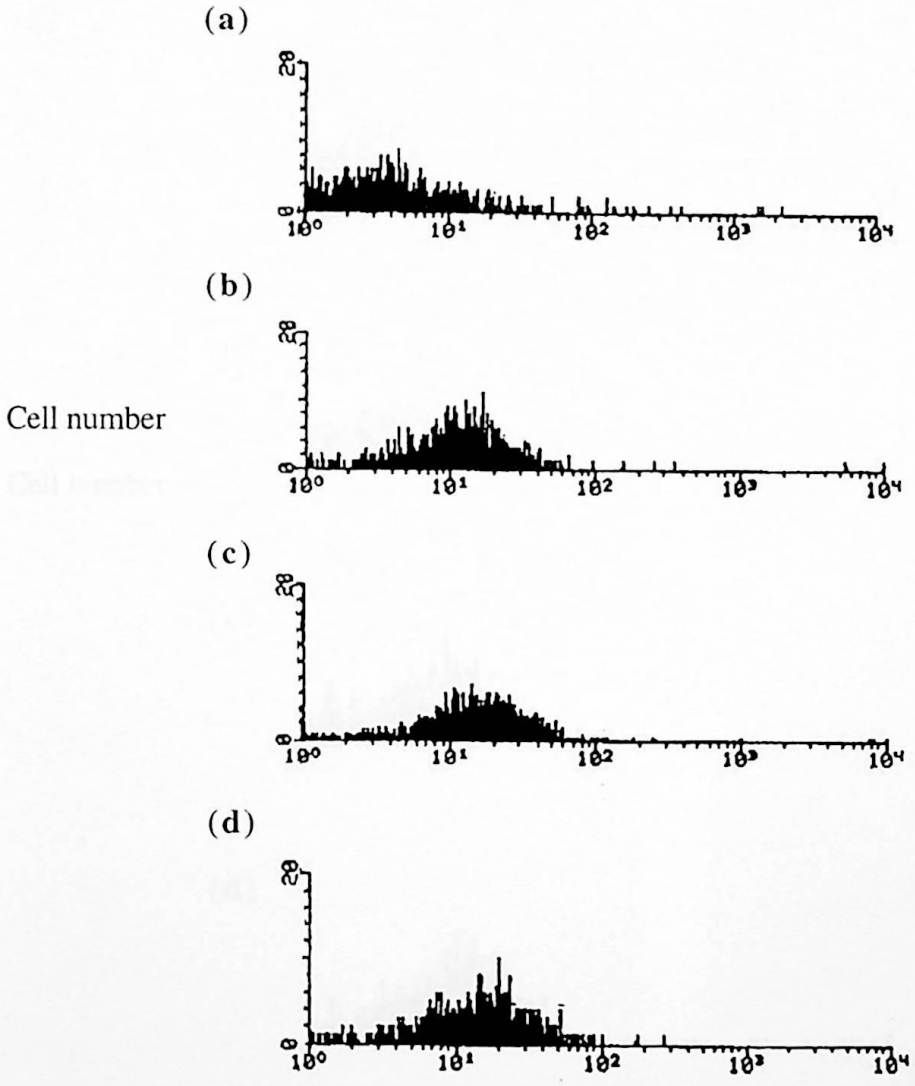


Figure 3.11 *Flow cytometry of human fibroblasts.*
The following monoclonal antibodies, each at a concentration of 20 μg/ml were used. **(a)** Control mouse monoclonal IgG1. **(b)** Monoclonal antibody 3/138. **(c)** Monoclonal antibody 2/389. **(d)** Monoclonal antibody 3/17.

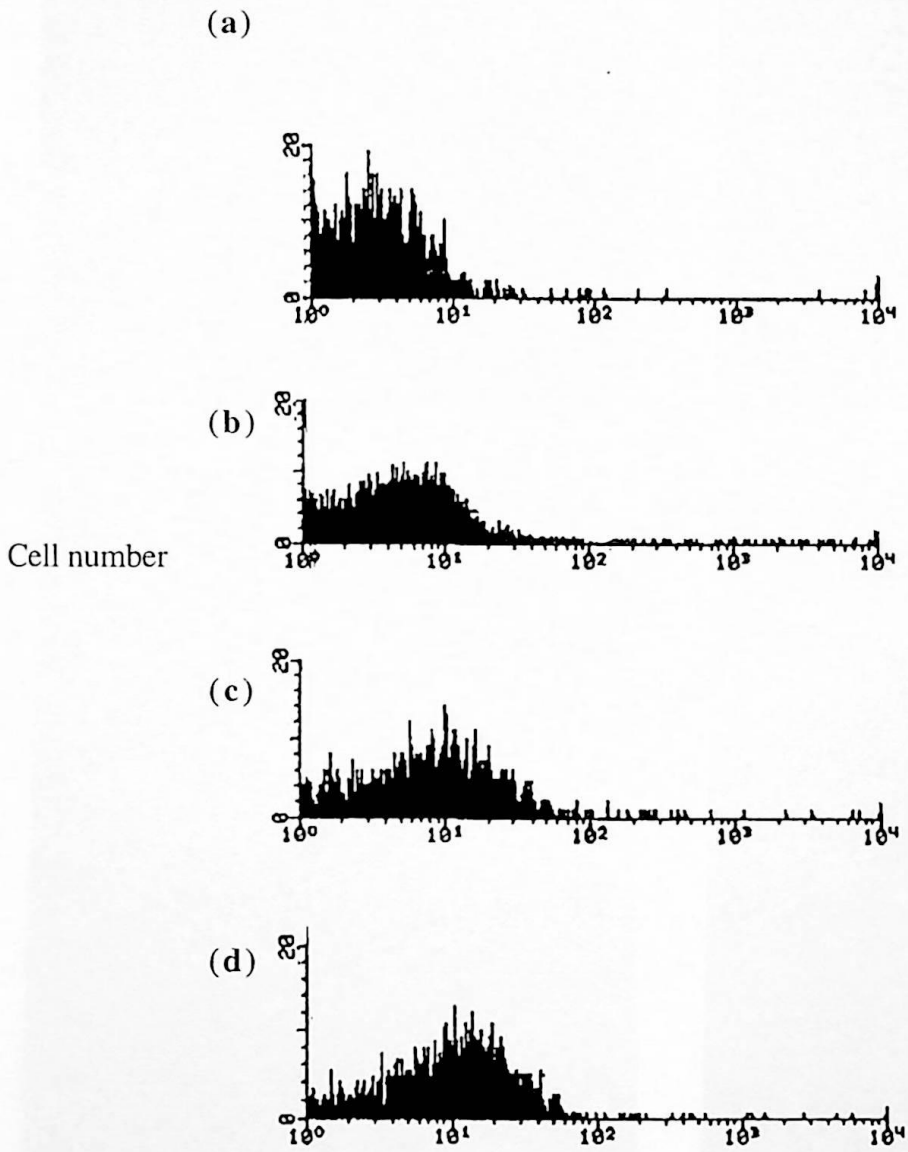


Figure 3.12 *Flow cytometry of human platelets.*
The following monoclonal antibodies, each at a concentration of 20 μg/ml were used. **(a)** Control mouse monoclonal IgG1. **(b)** Monoclonal antibody 3/138. **(c)** Monoclonal antibody 2/389. **(d)** Monoclonal antibody 3/17.

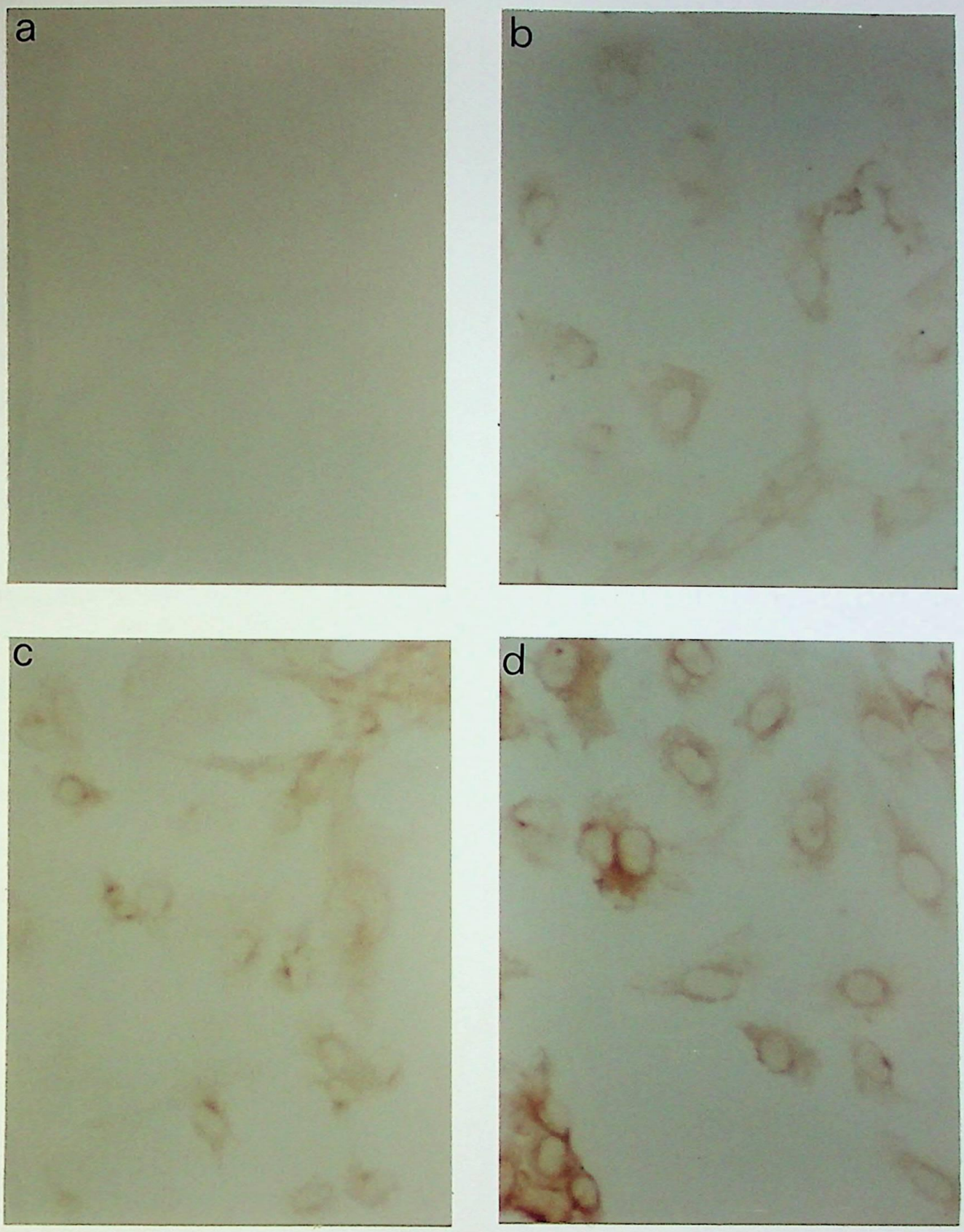


Figure 3.13 *Immunological detection of the thrombin receptor on HUVECs.*
The following monoclonal antibodies were used at a concentration of 40 μg/ml.
(**a**) Control mouse monoclonal IgG1. (**b**) Monoclonal antibody 3/138. (**c**) Monoclonal antibody 2/389. (**d**) Monoclonal antibody 3/17. The binding of the monoclonals was visualised using the peroxidase-anti-peroxidase procedure as described in section (2.5.18).

3.3 Discussion

3.3.1 Generation of anti-TRED antibodies

The commercially available pGEX plasmid expression vectors (Pharmacia) can be used to express foreign polypeptides in *E. coli* as fusions at the C-terminus of glutathione S-transferase (GST), a 26 kDa cytoplasmic protein from eukaryotes. The GST gene used to generate these vectors was originally isolated from the parasitic helminth *Schistosoma japonicum*. The fusion protein typically remains soluble within the bacteria and can be rapidly and efficiently purified under non-denaturing conditions using a glutathione affinity column (Smith and Johnson, 1988).

The GST-TRED fusion protein has been used to generate rabbit polyclonal antibodies to the TRED domain. In the generation of polyclonal antibodies the contaminating anti-GST antibodies are absorbed with a GST protein column (Bahou et al., 1993a; Bouton et al., 1995). The GST-TRED fusion protein has been previously expressed in our research group (Parry, 1995). The GST and TRED portions of the protein were separated by either an enterokinase cleavage site or a methionine for cleavage with cyanogen bromide (CNBr). Incubation of GST-TRED with enterokinase led to degradation of TRED, possibly due to contaminating trypsin. CNBr cleavage generated a complex peptide mixture due to many partial cleavage reactions and TRED could not be successfully isolated from the mixture (Parry, 1995).

In the initial ELISA screen, a combination of GST and GST-TRED in adjacent wells was used, as purified TRED was not available. Other procedures may be more successful in producing full length TRED. More recently, TRED has been obtained from a GST-TRED fusion protein using an engineered Factor Xa cleavage site between the GST and TRED (Bouton et al., 1995). TRED has also been expressed solely in *E. coli* with a combination of a C-terminal poly-histidine tail and a N-terminal monoclonal antibody epitope tag. This allowed its rapid purification using a combination of immobilised metal affinity (IMAC) and immunoaffinity chromatography (Ishii et al., 1995).

From the initial ELISA screening a higher proportion of hybridomas producing anti-GST monoclonal antibodies was detected compared with hybridomas producing anti-TRED antibodies. The nature of the ELISA screen probably missed some specific anti-TRED monoclonal antibodies, because a population (well) of hybridomas producing anti-TRED antibodies was only further investigated when no GST signal was observed. If the well contained both anti-GST and anti-TRED secreting hybridomas, the anti-TRED hybridoma would have been discarded, thus reducing the final total number of monoclonal antibodies isolated to TRED.

The low numbers of anti-TRED producing hybridomas isolated could be due to the relative conservation of amino acid sequence between the human and mouse thrombin receptors (Parry, 1995), leading to some immunotolerance to the immunising human TRED antigen by the mouse. In contrast, the high proportion of anti-GST antibodies is probably due to its high immunogenicity. The GST protein used in the construction of the fusion protein vector was initially isolated on the basis of the observation that WEHI 129/J mice infected with the parasitic helminth *Schistosoma japonicum* generated neutralising antibodies to the parasite and these were mostly directed against the native parasite's glutathione S-transferase (GST) protein (Smith et al., 1986).

3.3.2 Epitope mapping

Competition ELISA experiments indicated that the three monoclonals recognise different epitopes on the thrombin receptor. Western blot analysis of GST-TRED after cleavage by thrombin suggested that 3/138 recognises an epitope between residues 26-41, i.e. on the N-terminal side of the thrombin cleavage site. The antibodies 3/17 and 2/389 bound to TR 38-60. However, the antibodies did not bind to smaller peptides (TR 42-50 and TR 53-64) from this region, either using the peptides as antigens directly coated on ELISA plates or as competing agents in solution. In addition it was observed that monoclonal antibody 3/17 bound to TR 38-60 only in solution and did not recognise this peptide immobilised on an ELISA plate. It seems possible that the failure of 2/389 and 3/17 to recognise fragments of TR 38-60 and the inability of 3/17 to bind immobilised TR 38-60

may be due to the fact that these antibodies only recognise a particular conformation. There is evidence by Smith et al. (1994) that a peptide analogous to TR 38-60 adopts a different secondary conformation in solution compared to the smaller peptide TR 42-53. The structure of the TRAP peptide (S^{42}FLLRNPNDKYEPF^{55}) determined by NMR contained two overlapping turns Arg 46 - Asn 49 and Asn 47 - Asp 50; the five N-terminal residues which are important for biological activity were unstructured. A longer peptide TR 33-55 was found to be more highly structured. The seven residues from Pro 40 to Arg 46 formed a partial 3^{10} helix, which was not present in the shorter tethered ligand peptide. Moreover, the overlapping turns observed in the shorter TRAP could not be distinguished in the longer peptide molecule. This NMR structural evidence highlights that even in small peptides, differences in secondary structure may occur. Therefore, the possibility arises that smaller peptides derived from TR 38-60, adopt different conformations that are unrecognised by the monoclonal antibodies 3/17 or 2/389 in solution.

A further way to map the epitopes of the three monoclonal antibodies would be to use a series of overlapping peptides from the thrombin receptor extracellular domain, as demonstrated by Bahou et al. (1994). Another possibility would be the use of randomly displayed peptide libraries on M13 bacteriophage (Smith, 1985; Parmley and Smith, 1988). This technique has been successfully used to map the epitopes of other monoclonal antibodies (Roberts et al., 1993; Pinilla et al., 1993).

3.3.3 Platelet aggregation assay

The aggregation traces demonstrate that the monoclonal antibodies, 2/389 and 3/17, generated to the GST-TRED fusion protein can bind to the native platelet thrombin receptor and block activation of the receptor by thrombin. Partial inhibition at 200 - 400 µg/ml and total inhibition at 800 µg/ml were observed. In contrast, monoclonal antibody 3/138 showed no inhibition at these concentrations. At the high concentrations (800 µg/ml) of 2/389 and 3/17 which were capable of completely blocking the effect of thrombin, the platelets were still responsive to TRAP. These results are consistent with the proposal that the epitopes of both 2/389 and 3/17 are situated within the thrombin binding domain. Thus,

the antibodies would block thrombin binding, but not inhibit the agonist peptide from activating the receptor, since the binding site for the tethered ligand is distinct from that of thrombin. Similar results have been obtained with other antibodies directed to thrombin binding sites (Hung et al., 1992a; Bahou et al., 1993a; Bahou et al., 1994; Norton et al., 1993; Brass et al., 1992).

The concentrations (200 - 800 μg/ml) at which 2/389 and 3/17 were effective in inhibiting thrombin-dependent aggregation are similar to those previously observed with other blocking antibodies. Partial inhibition of platelet aggregation induced by 1 nM α-thrombin was observed using mouse monoclonal antibodies at a concentration of 25 to 100 μg/ml, while the same concentration caused total inhibition with 0.5 nM α-thrombin (Brass et al., 1994). Rabbit polyclonal antibodies at concentrations of 30 - 150 μg/ml and 310 - 500 μg/ml caused respectively partial and total inhibition of gel filtered human platelets in response to 1 nM α-thrombin (Bahou et al., 1993a; Norton et al., 1993). The aggregation of platelet rich plasma from African green monkeys induced by 1 nM α-thrombin, was totally inhibited using rabbit polyclonal antibodies at a concentration of 30 - 60 μg/ml (Cook et al., 1995).

3.3.4 Purification of the platelet thrombin receptor

The use of a monoclonal affinity column provided a convenient one step procedure for purification of the native thrombin receptor. The tight binding of the 2/389 monoclonal antibody to the thrombin receptor was still maintained in the presence of the two detergents digitonin and dodecyl maltoside. Digitonin and dodecyl maltoside were chosen as detergents in the solubilisation and purification of the thrombin receptor, since they have been used effectively for other seven transmembrane G-protein coupled receptors. Examples are endorphin receptors (Demoliou-Mason and Barnard, 1984) and the β-adrenergic receptor (Caron and Lefkowitz, 1976). Elution was performed under alkaline conditions because of the empirical observation that membrane proteins are less prone to aggregation at high pH (Parham, 1979; Schneider et al., 1982).

Western blotting of the affinity purified platelet thrombin receptor yielded two broad bands of apparent molecular mass 46 kDa and 80 kDa. This is in contrast to a narrow band with a molecular mass of 66 kDa observed by others (Brass et al., 1992; Hung et al., 1992a). These observed patterns on Western blotting of the native purified platelet thrombin receptor correlate with that of purified recombinant thrombin receptor from Sf9 cells (Chinni et al., 1996 submitted for publication). The differences between the masses observed in Figure 3.10 and those obtained by Brass et al. (1992) and Hung et al. (1992a), cannot be attributed to glycosylation of the platelet thrombin receptor. Removal of the oligosaccharide side chains with peptide N-glycosidase (PNGase F) results in only a decrease of about 5 kDa in the apparent molecular mass of both bands (C. Chinni unpublished results). The bands obtained by Western blotting of the affinity-purified native platelet receptor correspond with those observed by Vouret-Craviari et al. (1995a) using a recombinant thrombin receptor with a C-terminal epitope tag. The high molecular weight band was attributed to post-translational modifications of the thrombin receptor such as polyubiquitination or phosphorylation (Vouret-Craviari et al., 1995a)

As the monoclonal antibody 2/389 was effective in purifying the platelet thrombin receptor, the other two anti-TRED monoclonal antibodies could be employed for immunoaffinity chromatography. Less severe conditions may be required to elute the thrombin receptor from the column, and potentially permit the further study of the purified thrombin receptor reconstituted in phospholipid vesicles for biochemical studies.

3.3.5 Flow cytometry

The monoclonal antibodies isolated in the present study should also be useful in the characterisation of the expression of the thrombin receptor on leucocytes. For instance rabbit polyclonal antibodies have successfully been used with mouse monoclonal antibodies to specific leucocyte cell surface (CD) markers, to demonstrate that natural killer (NK) cells and T-lymphocytes, predominantly positive for CD8, CD16 and either CD56 or CD57 expressed the thrombin receptor (Howells et al., 1993; 1994). In this technique of double labelling, two antibodies were labelled with different fluorophores. Problems arise with the

use of commercially available anti-CD marker monoclonal antibodies with a mouse monoclonal antibody to a potential cell surface antigen. Labelled second antibodies cannot differentiate the two primary monoclonal antibodies. The availability of fully active biotinylated anti-thrombin receptor antibodies generated in this thesis allows a double labelling approach to be successfully applied. The cell suspension could be incubated with both a mouse monoclonal antibody to a specific CD marker and the biotinylated anti-thrombin receptor antibody. Subsequently, the cells could be incubated with a fluorescein-conjugated rabbit-anti-mouse-IgG antibody and avidin conjugated phycoerythrin allowing both red (phycoerythrin) and green (fluorescein) to be measured simultaneously by flow cytometry.

3.4 Conclusion

A recombinant GST-TRED fusion protein expressed in *E. coli* was used successfully to raise mouse monoclonal antibodies capable of recognising the native thrombin receptor. The isolation of anti-TRED monoclonal antibodies would have been greatly simplified by the availability of purified full length TRED protein, as the use of the highly immunogenic GST as a carrier protein resulted in a greater immunogenic response to GST than to TRED. Two of the monoclonal antibodies were capable of blocking thrombin-induced platelet aggregation. One of the antibodies was used for the immunoaffinity purification of the platelet thrombin receptor. Such a purification has not previously been reported and should facilitate further characterisation of the receptor at the protein level.

Chapter 4

Cloning and DNA Sequencing of the Anti-Thrombin Receptor Monoclonal Antibodies

4.1 Introduction

The antibody molecule consists of two heavy (H) and two light (L) immunoglobulin (Ig) polypeptide chains, that are disulphide-linked to form a bivalent H2L2 molecule (Figure 4.1). The amino-terminal domain of each chain is the variable (V) region and the carboxy-terminal end is the constant region, termed Fc. The variable region sequences vary extensively from one molecule to another, but the Fc regions are constant and highly conserved across the species.

There are five classes of immunoglobulins (IgG, IgM, IgA, IgD and IgE) which differ by virtue of their heavy chains (γ, μ, α, δ, ε) respectively. Difference in the heavy chains are found mainly within the Fc region. These differences lead to variations in the polymerisation state of the basic Ig four chain monomer unit (Figure 4.1). For example, IgM occurs as a pentamer in serum whereas IgA exists mainly as a monomer in serum, but as a dimer in seromucous secretions. The IgG class can be further divided into subclasses, termed as isotypes (e.g. in mice; IgG1, IgG2a, IgG2b and IgG3). All these heavy chain variants are linked by a single disulphide bond to light chains, formed from either the kappa (κ) or lambda (λ) types. Compared with humans, λ chains in mice are rare.

Studies on immunoglobulin sequences identified three hypervariable or complementarity determining regions (CDRs) in the amino-terminal domain of both the H and L chains. The variable antigen-binding immunoglobulin domain, consists of a conserved core structure formed by a β-barrel, topped by three loops of highly variable length and sequence, the so called complementarity-determining regions (CDRs) Figure 4.2 and Figure 4.3 reviewed in Alzari et al. (1988); Davies et al. (1990).

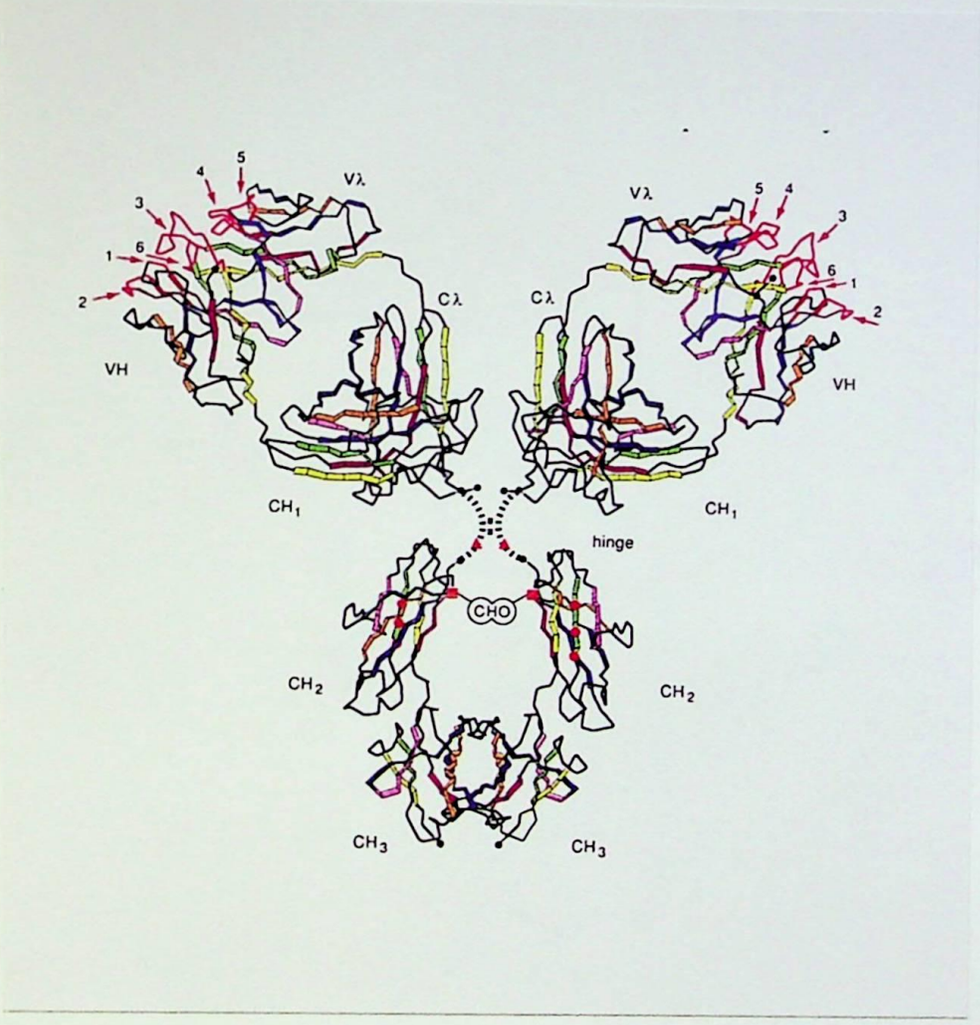


Figure 4.1 *Structure of an IgG antibody.*
The model is taken from the solved X-ray crystallographic structure of the myeloma protein KOL (Marquart et al., 1980). The antibody (IgG) consists of two heavy and light chains in a highly conserved framework structure. The antibody can be proteolytically cleaved at the flexible hinge region, yielding Fab fragments (comprising heavy chain VH and CH1 domain, the hinge and the entire light chain Cλ) and the lower Fc fragment containing the heavy chain domains (CH2 and CH3). Attached carbohydrate is shown as CHO in the Fc domain. From Winter and Milstein, (1991).

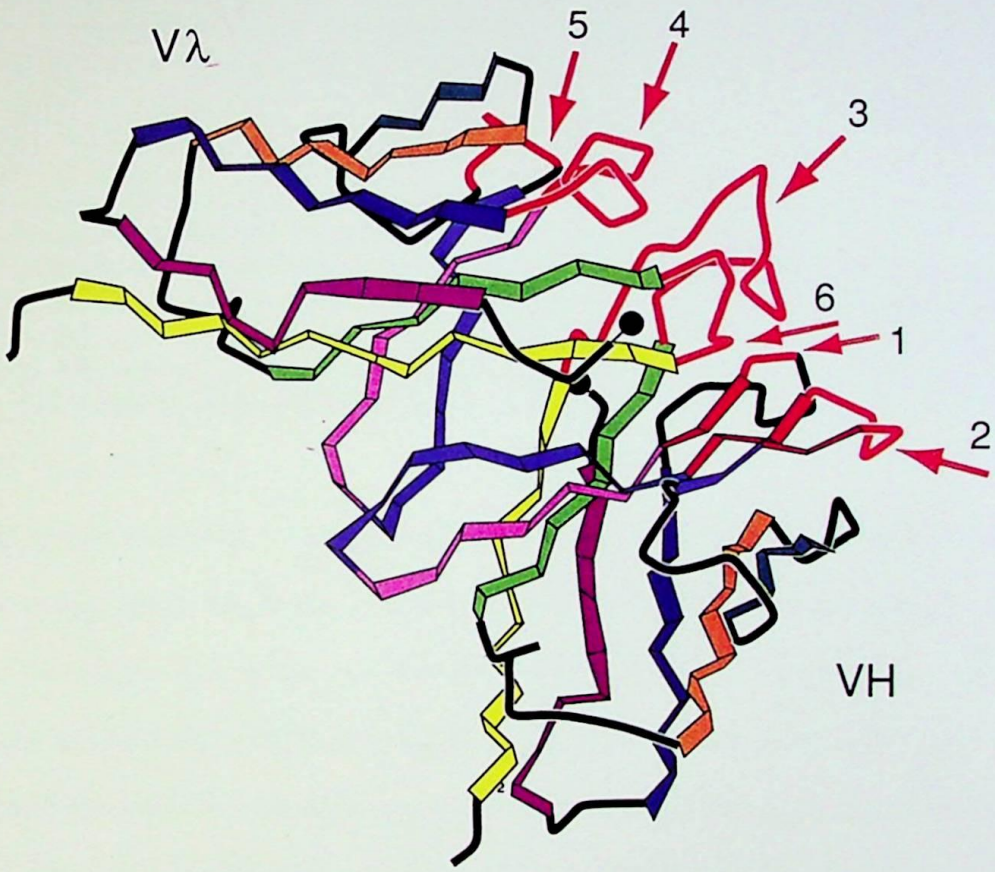


Figure 4.2 *Structure of the antigen binding site of an antibody.*
The CDR loops (VH 1-3; VL 4-6) from (Figure 4.1) are labelled in red. From Winter and Milstein, (1991).

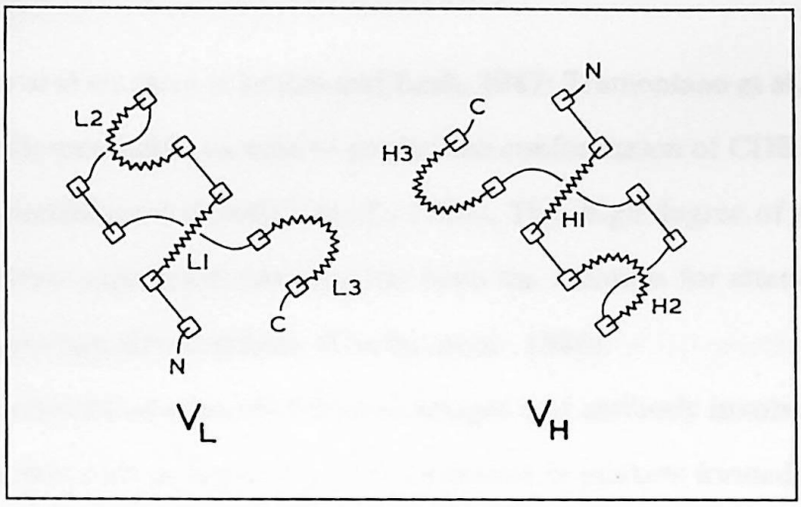


Figure 4.3 *Outline schematic view of the antigen-binding site.*
The relevant positions of the CDR loops in relation to the antibody framework structure are shown. The site is formed by 6 loops of polypeptide (/\/) linked to strands in β sheets (❑). The amino and carboxy terminus of each protein domain is indicated by (N) and (C) respectively. The complementarity determining regions (CDRs) from either the VH and VL domain are labelled as (H1, H2, H3, L1, L2 and L3). From Tramontano et al. (1990).

Although the generation of V domains of immunoglobulins involves multiple and complex molecular events at the DNA level (Alt et al., 1987; Alt and Baltimore, 1982), the tertiary structures of these domains are similar. In recent years, considerable three-dimensional information has become available using X-ray crystallography. Comparison of the three-dimensional structures of variable domains of different antibodies reveals that the non-hypervariable or framework regions (FRs) of these domains are essentially superimposable. The structural variations which ultimately define antibody specificity are mainly confined to the hypervariable segments. The antibody combining site can be viewed as being formed by a relatively small number of segments of variable structure (CDRs) grafted onto an essentially invariant scaffolding (FRs) (reviewed in Padlan and Kabat, 1991).

Comparisons of antibody structures reveal that many CDRs of the same length but of diverse sequences assume a similar main chain conformation (Padlan and Davies, 1975; de la Paz et al., 1986). For all the CDRs except VH-CDR3 (H3), these can be grouped into a small number of discrete structural classes or "canonical forms", dictated by the presence

of key amino acid residues (Chothia and Lesk, 1987; Tramontano et al., 1990). These have been used with reasonable success to predict the conformation of CDR loops in advance of structural determination (Chothia et al., 1986). This high degree of structural similarity among the immunoglobulin domains has been the stimulus for attempts at modelling of antibody combining site structures (Chothia et al., 1986).

The majority of contacts between antigen and antibody involves the CDR regions. Small molecules such as haptens tend to be bound in pockets formed by the CDRs which often contain several tyrosine and tryptophan residues (Alzari et al., 1990; Herron et al., 1989). In contrast, interactions with proteins are characterised by a large surface area of interaction, leading to a striking complementarity of the two protein surfaces. This is a general feature of multi-subunit protein interfaces (Miller et al., 1987). There is an almost total exclusion of water from the interface, although in the most refined X-ray structures, water molecules can be seen mediating contacts between antibody and antigen (Bhat et al., 1990; Davies et al., 1990).

Antibodies against protein antigens also have a large number of tyrosine and tryptophan amino acids in their combining sites. Their preponderance has been attributed to their size, flexibility and amphipathic nature that allows them to participate in a wide variety of electrostatic and van der Waals interactions and to tolerate a change from a hydrophilic to a hydrophobic environment. Their side chain mobility might also facilitate structural adjustments to optimise shape complementarity (de la Paz et al., 1986; Padlan, 1990; Mian et al., 1991), this is a feature of some antibody-antigen interactions (Colman et al., 1987; Stanfield et al., 1990).

Initially the amino acid sequences of the antibody variable regions were determined by standard protein chemistry sequencing techniques. This has been superseded by the advent of molecular biology, which allows the protein sequence to be analysed at the cDNA level. Orlandi and co-workers (1989) undertook a detailed analysis of the large database of rearranged mouse VH and VL sequences assembled by Kabat (1987). This allowed the design of "universal" primers (one, VH1BACK was 32-fold degenerate; the others were unique) that would be expected to prime most murine V-genes in these regions. Using these

primers the VH and VL genes of five mouse hybridomas were successfully amplified from purified mRNA after reverse transcription (Orlandi et al., 1989). Thus PCR could be used to clone V-genes directly from hybridomas without any prior knowledge of the protein sequence, obviating the need to construct an initial cDNA library. Later the primers were used to amplify repertoires of VH genes from immunised mice and subsequently to express recombinant immunoglobulins in *E. coli* (Ward et al., 1989). The complete sequencing of clones amplified from genomic DNA demonstrated that the VH primers were able to generate a diverse repertoire (Güssow et al., 1989b). This isolation and cloning of the antibodies' variable cDNA regions allows recombinant manipulation, expression and characterisation of the recombinant antibodies.

This chapter describes the cloning and sequencing of the three mouse monoclonal antibodies isolated and characterised in the previous chapter. The cDNA of the mouse monoclonal antibodies VH and VL chains, were cloned using a combination of reverse transcription (RT) and PCR, using oligonucleotide primers designed to amplify antibody variable regions (Orlandi et al., 1989; Clackson et al., 1991a; Hoogenboom et al., 1991). This allowed the amino acid sequences of the CDR loops involved in binding to the human thrombin receptor to be determined. The CDR loops of the antibodies were then compared to the CDR loops of other antibodies in an attempt to identify key residues that may be important in binding to the thrombin receptor extracellular domain (TRED).

4.2 Results

4.2.1 cDNA reverse transcription

The cDNA was prepared from the three mouse monoclonal cell lines (section 2.2.9). The reverse transcription (RT) oligonucleotides used for both monoclonals 3/17 and 2/389 were MCG12FOR and MCKFOR (Appendix 2), as these two monoclonal antibodies were isotyped to be members of the IgG1 subclass. For monoclonal 3/138 which was IgA, oligo dT18 (Appendix 2) was also included in the RT reaction, since the RT primers were principally designed to prime on the constant regions of the murine IgG1, IgG2, IgG3, IgM and kappa subclasses (Orlandi et al., 1989). As a negative control, a RT reaction was performed using no RT enzyme; this was used to assay possible genomic DNA contamination in the sample, which may be amplified in the subsequent PCR.

4.2.2 PCR of antibody cDNA

The oligonucleotides used for the separate PCR reactions of the VH and VL chains are listed in Appendix 2. "Touchdown PCR" (section 2.2.6) was used to amplify the VH and VL DNA, using 5 µl of the cDNA mixture obtained in section 4.2.1. This resulted in the amplification of DNA fragments of approximately 310 and 300 bp for the VH and VL domains respectively (Figure 4.4). PCR products were obtained, except for the VH domain of 3/138, the IgA antibody. This lack of a PCR product was due to either inefficient primary cDNA synthesis, or a failure of the PCR primers to amplify that particular VH DNA sequence, as the oligonucleotide primers had been primarily designed using the Kabat database, which mostly contains murine antibodies of the IgM and IgG subclasses (Orlandi et al., 1989).

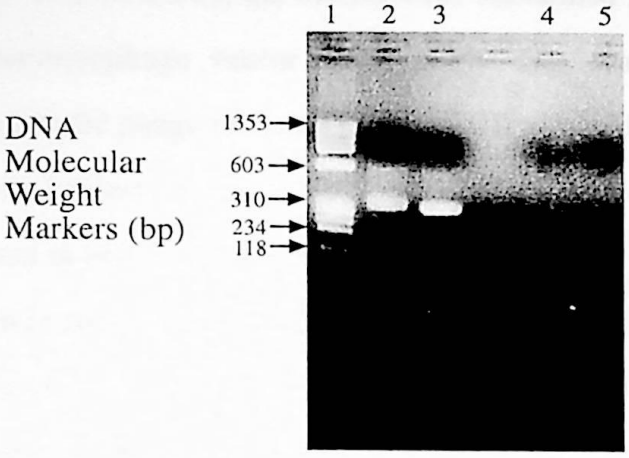


Figure 4.4 *1.5% agarose gel showing the VH and VL PCR fragments amplified from the cDNA prepared from monoclonal antibody 2/389.* Lane 1, ΦX174-Hae III digested double stranded DNA molecular weight markers (bp); 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. Lane 2, VH PCR fragment (310 bp). Lane 3, VL PCR fragment (300 bp). Lane 4, VH RT-PCR negative control. Lane 5, VL RT-PCR negative control.

4.2.3 Cloning of the antibody cDNA

The PCR products from section (4.2.2) were gel purified and subcloned (section 2.2.1 -2.2.4) into pCRII, a commercial "T-plasmid vector" (see Appendix 5 for the DNA sequence of pCRII). Linear pCRII contains a single thymine 5'-overhang which permits cloning of PCR products using the 3' A-overhangs generated by amplitaq. These 3' adenine overhangs are produced in a non-template dependent fashion during the PCR process (Holton and Graham, 1991; Marchuk et al., 1991). The presence of recombinant inserts was screened using PCR using Promega Taq (section 2.2.6) with oligonucleotide primers MF10 and MF20 (Appendix 2).

4.2.4 Sequencing of the antibody cDNA

In order to generate high quality single-stranded DNA (ssDNA) for dideoxy sequencing (section 2.2.5), the inserts were subcloned from pCRII, as EcoR I fragments into the bacteriophage vector M13 mp19. This was performed as problems were encountered in the phage-rescuing of the pCRII plasmid clones. This was attributed to the presence of a kanamycin resistance gene (Kan^r) in the pCRII plasmid (Appendix 5) which is not present in pUC119 or pBluescript based phagemid vectors. Normally kanamycin is used to select for M13KO7, a helper-phage which bears Kan^r. Therefore, uninfected bacteria already containing a plasmid with Kan^r will outgrow the slower M13KO7 infected bacteria, resulting in a final poor phagemid yield.

The presence of recombinant inserts in M13 mp19 plaques was identified using PCR. Sequences obtained from the ssDNA and the deduced amino acid sequences of the antibodies' variable regions are listed in Tables 4.1 - 4.5. The CDRs are in bold type and the framework regions (FRs) are noted above the sequences.

Table 4.1 *VH sequence of 3/17 variable heavy region.*

```
                                                FR1
GTC CAG CTG CAG GAG TCT GGA GCT GAG TTG GTA AGG CCT GGG ACT TCA GTG AAG GTG TCC
Val Gln Leu Gln Glu Ser Gly Ala Glu Leu Val Arg Pro Gly Thr Ser Val Lys Val Ser

                                          H1                                FR2
TGC AAG GCT TCT GGA TAC GCC TTC ACT AAT TAC TTG ATA GAG TGG GTA AAG CAG AGG CCT
Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Tyr Leu Ile Glu Trp Val Lys Gln Arg Pro

                                             H2
GGA CAG GGC CTT GAG TGG ATT GGA ATG ATT AAT CCT GGA AGT GGT GGT ACT ACC TAC AAT
Gly Gln Gly Leu Glu Trp Ile Gly Met Ile Asn Pro Gly Ser Gly Gly Thr Thr Tyr Asn

                                      FR3
GAG AAG TTC AAG GGC AAG GCA ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT GCC TAC ATT
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Ile


CAG CTC AGC AGC CTG ACA TCT GAT GAC TCT GCG GTT TAT TTT TGT TCC TAT TAC TAC GGT
Gln Leu Ser Ser Leu Thr Ser Asp Asp Ser Ala Val Tyr Phe Cys Ser Tyr Tyr Tyr Gly

             H3                                 FR4
AGT AGC TCG TAC TAC TTT GAC TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC
Ser Ser Ser Tyr Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser
```

Table 4.2 *VL sequence of 3/17 variable light region.*

```
                                                FR1
GAT GTT TTG ATG ACC CAA ACT CCA CTC TCT CTG CCT GTC AAT ATT GGA GAT CAA GCC TCC
Asp Val Leu Met Thr Gln Thr Pro Leu Ser Leu Pro Val Asn Ile Gly Asp Gln Ala Ser

                                             L1
ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CAT TGG
Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr Tyr Leu His Trp

                                  FR2                          L2
TAC CTG CAG AAG CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT
Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe

                                      FR3
TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile

                                                                        L3
AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA ACT ACA CAT GTT CCA
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Thr Thr His Val Pro

                          FR4
TTC ACG TTC GGT GCT GGG ACA AAG TTG GAA ATA AAA CGG
Phe Thr Phe Gly Ala Gly Thr Lys Leu Glu Ile Lys Arg
```

Table 4.3 *VH sequence of 2/389 variable heavy region.*

```
                                                FR1
GTC CAG CTG CAG CAG TCA GGG GCA GAG CTT GTG AAG CCA GGG GCC TCA GTC AAG TTG TCC
Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Lys Pro Gly Ala Ser Val Lys Leu Ser

                                H1                                      FR2
TGC ACA GCT TCT GGC TTC AAC ATT AAA GAC ACC TAT ATA CAC TGG GTG AAA CAG AGG CCT
Cys Thr Ala Ser Gly Phe Asn Ile Lys Asp Thr Tyr Ile His Trp Val Lys Gln Arg Pro

                                                        H2
GAA CAG GGC CTG GAG TGG ATT GGA AGG ATT GAT CCT GCG AAT GGT AAT ACT AAA TAT GAC
Glu Gln Gly Leu Glu Trp Ile Gly Arg Ile Asp Pro Ala Asn Gly Asn Thr Lys Tyr Asp

                                        FR3
CCG AAG TTC CGG GGC AAG GCC ACT ATA ACA GCA GAC ACA TCC TCC AAC ACA GCC TAC CTG
Pro Lys Phe Arg Gly Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser Asn Thr Ala Tyr Leu


CAG CTC AGC AGC CTG ACA TCT GAG GTC ACT GCC GTC TAT TAC TGT GCC CGA TGG TTA CGG
Gln Leu Ser Ser Leu Thr Ser Glu Val Thr Ala Val Tyr Tyr Cys Ala Arg Trp Leu Arg

      H3                                        FR4
TAC TAC ATT GAC TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC
Tyr Tyr Ile Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser
```

Table 4.4 *VL sequence of 2/389 variable light region.*

```
                                                FR1
GAT GTT TTG ATG ACC CAA ACT CCA CTC TCT CTG CCT GTC AAT ATT GGA GAT CAA GCC TCT
Asp Val Leu Met Thr Gln Thr Pro Leu Ser Leu Pro Val Asn Ile Gly Asp Gln Ala Ser

                                        L1
ATC TCT TGC AAG TCT ACT AAG AGT CTT CTG AAT AGT GAT GGA TTC ACT TAT TTG GAC TGG
Ile Ser Cys Lys Ser Thr Lys Ser Leu Leu Asn Ser Asp Gly Phe Thr Tyr Leu Asp Trp

                        FR2                                     L2
TAC CTG CAG AAG CCA GGC CAG TCT CCA CAG CTC CTA ATA TAT TTG GTT TCT AAT CGA TTT
Tyr Leu Gln Lys Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Leu Val Ser Asn Arg Phe

                                        FR3
TCT GGA GTT CCA GAC AGG TTC AGT GGC AGT GGG TCA GGA ACA GAT TTC ACA CTC AAG ATC
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile

                                                                L3
AGC AGA GTG GAG GCT GAG GAT TTG GGA GTT TAT TAT TGC TTC CAG AGT AAC TAT CTT CCT
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Ser Asn Tyr Leu Pro

                        FR4
CTC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA CGG
Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
```

Table 4.5 *VH sequence of 3/138 variable light region.*

```
                                                FR1
CAA ATT GTT CTC ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG GAG AAG GTC ACC
Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly Glu Lys Val Thr

                                L1                                      FR2
ATG ACC TGC AGT GCC AGC TCA AGT GTA AAT TAC ATG CAC TGG TAC CAG CAG AAG TCA GGC
Met Thr Cys Ser Ala Ser Ser Ser Val Asn Tyr Met His Trp Tyr Gln Gln Lys Ser Gly

                                    L2
ACC TCC CCC AAA AGA TGG ATT TAT GAC ACA TCC AAA CTG GCT TCT GGA GTC CCT GCT CGC
Thr Ser Pro Lys Arg Trp Ile Tyr Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala Arg

                                    FR3
TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AGC ATG GAG GCT GAA
Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Met Glu Ala Glu

                                        L3                              FR4
GAT TCT GCC ACT TAT TAC TGC CAT CAG TGG AGT AGT AAC CCA CTC ACG TTC GGT GCT GGG
Asp Ser Ala Thr Tyr Tyr Cys His Gln Trp Ser Ser Asn Pro Leu Thr Phe Gly Ala Gly


ACC AAG CTG GAG CTG AAA CGG
Thr Lys Leu Glu Leu Lys Arg
```

4.2.5 Comparison of the amino acid sequence of the anti-TRED monoclonal CDRs with those of antibodies of known tertiary structure

Canonical structures exist for most of the hypervariable loops of similar size with key amino acid residues (Chothia et al., 1986; Tramontano et al., 1990). Consequently, the CDRs of anti-TRED monoclonal antibodies were compared with those of antibodies of known structure in order to obtain some data of the tertiary structure of anti-TRED CDRs. The results of these structural sequence alignments are shown in the following (Tables 4.6 - 4.10). Spaces are included for the best homology fit and amino acid residues that are identical are shown in bold type.

The following antibody sequences were compared with their respective antigens given in brackets: McPC603 (phosphocholine; Satow et al., 1986); DB3 (progesterone; Arevalo et al., 1994); B13I2 (myohemerythrin peptide; Stanfield et al., 1990); R19.9 (*p*-azobenzenenarsenate; Lascombe et al., 1989); 4-4-20 (fluorescein; Herron et al., 1989);

BV04-01 (single-stranded DNA; Herron et al., 1991); D1.3 (hen egg-white lysozyme; Amit et al., 1986); HY5 (hen egg-white lysozyme; Sheriff et al., 1987); NQ10 (2-phenyloxalone; Alzari et al., 1990); Jel103 (RNA; Pokkuluri et al., 1994).

Table 4.6 *Sequence alignment of the VH CDRs of 3/17.*

FR1	**H1**	**FR2**	
CKASG	YAFTNYLIE	WVK	3/17
CKASG	**YAFTNY**GVN	**WVK**	DB3
CKASG	**Y**T**F**SD**Y**W**IE**	**WVK**	HY5
FR2	**H2**	**FR3**	
LEWIG	MINPGSGGT	TYNE	3/17
LEWIG	E**I**L**PGSG**S**T**	N**Y**H**E**	HY5
LEWIG	**M**GWINTNTGEP	**TY**VD	DB3
FR3	**H3**	**FR4**	
YFC	SYYYGSSS YYFD	YWG	3/17
YFC	ARSF**YGG**S**D**LAV **YYFD**	S**WG**	R19.9
YY**C**	TGS**YYG**MDY	**WG**	4-4-20

Table 4.7 *Sequence alignment of the VL CDRs of 3/17.*

FR1	**L1**	**FR2**	
ISCR	SSQSLVHSNGNTY	LHWYL	3/17
ISCR	**SSQSLVHSNGNTY**	**LHWYL**	BV04-01
FR2	**L2**	**FR3**	
LLIY	KVS	NRFSG	3/17
LLIY	**KVS**	**NRFSG**	BV04-01
FR3	**L3**	**FR4**	
YFC	SQTTHVPF	TFGGG	3/17
YFC	**SQ**S**THVP**L	**TFG**A**G**	BV04-01

Table 4.8 *Sequence alignment of the VH CDRs of 2/389.*

FR1	**H1**	**FR2**	
SCT	ASGFNIKDTYIH	WVK	2/389
SCA	T**SGF**TFS**D**F**Y**ME	**WV**R	McPC603
SCT	V**SGF**SLTGYGVN	**WVR**	D1.3

FR2	**H2**	**FR3**	
EWIG	RIDPANGNTK	YDPKFR	2/389
EWIG	E**I**L**P**GS**G**S**T**N	**Y**HER**F**K	HY5

FR3	**H3**	**FR4**	
YYC	ARWLRYYI	DYW	2/389
YYC	**AR**N **YY**GST	**WY**	McPC603
YYC	T**R**YSSDPFYF	**DYW**	B13I2

Table 4.9 *Sequence alignment of the VL CDRs of 2/389.*

FR1	**L1**	**FR2**	
SCK	STKSLLNSDGFTYLD	WYL	2/389
SCK	**S**SQ**SLLNS**GNQKNFLA	**WY**Q	McPC603
SCR	**S**SQ**SL**IH**S**N**G**N**TYL**H	**WYL**	DB3

FR2	**L2**	**FR3**	
LLIY	LVS	NRF	2/389
LLIY	K**VS**	**NRF**	BV04-01:4-4-20:DB3

FR3	**L3**	**FR4**	
YYCFQ	SNYLPL	TF	2/389
YYCFQ	GSHV**P**P	**TF**	B13I2

Table 4.10 *Sequence alignment of the VH CDRs of 3/138.*

FR1	**L1**	**FR2**	
MTC	SASSSVNYMH	WYQQK	3/138
MTC	**SASSSVNYM**Y	**WYQQK**	HY5
MTC	**SASSSV**R**YM**N	**WF**Q**QK**	NQ10
FR2	**L2**	**FR3**	
RWIY	DTS	KLASGVP	3/138
RWIY	**DTS**	**KLSSGVP**	HY5
RWIY	**DTS**	**KLSSGVP**	NQ10
FR3	**L3**	**FR4**	
YYC	HQWSSNPL	TFG	3/138
YYC	Q**QWSSNPL**	**TFG**	NQ10

4.3 Discussion

4.3.1 Comparison of the antibody CDRs

Although the sequence of hypervariable regions is largely responsible for determining the specificity of a given antibody, X-ray data suggests that gross specificity (protein versus small molecule antigen) is largely determined by the lengths of the various CDR loops. Antibodies specific for small haptens often have concave combining sites, frequently characterised as a "deep pocket" or "groove". Such binding sites are achieved with relatively long L1 and/or H3 loops. In contrast, antibodies that bind larger molecules, such as proteins, tend to have flat combining sites such that the surface of antibody-antigen contact is quite large (Amit et al., 1986). The comparison of sequences of antibodies of known structure with those of 3/17, 2/389 and 3/138 appears to show a close fit in the length of both L1 and H3 CDRs with the following antibodies: McPC603 and DB3 with 2/389, R19.9, 4-4-20 and BV04-01 with 3/17, NQ10 and HY5 with the light chain of 3/138. Thus, the length of the L1 and L3 loops of 3/17 and 2/389 are consistent with the

proposal that parts of the TRED polypeptide sequence, which comprise the epitopes for 3/17 and 2/389, bind into a deep hydrophobic pocket or groove such as those present in McPC603, DB3, R19.9, 4-4-20 or BV04-01.

Both 3/17 and 2/389 bind to a polypeptide (TR 38-60) that contains two important regions that are involved in the contact with thrombin: the cleavage site LDPR/SFLL and the hirudin-like sequence KYEFP (section 3.2.2). The thrombin molecule makes extensive interactions with the cleavage site and the hirudin-like sequence, through its active site and its anion-binding-exosite, respectively. The X-ray crystal structure of thrombin bound to the thrombin receptor peptide has been solved (Mathews et al., 1994) and reveals the exact detail of the surface loops and molecular interactions of the thrombin receptor peptide with thrombin.

The analysis of the CDR loops of the two antibodies that bind to the same peptide region does not show any direct sequence homology to any of the important surface loops of thrombin that are involved in binding to either its substrates or inhibitors. Thrombin has important insertion loops that protrude over the active site and loops consisting of basic charged amino acids present in the anion binding exosite. The amino acid CDR sequences of the two antibodies, even though both of their epitopes lie within these thrombin binding domains, do not resemble the same loops present in thrombin.

Within the polypeptide region recognised by both antibodies lies the thrombin receptor tethered ligand SFLLRNP. Monoclonal antibodies raised against peptide hormone receptor agonists have been shown to display some sequence identity between CDR and surface loops present within the cognate seven transmembrane receptor (Amati et al., 1995). In this regard, it is interesting to note that important amino acid residues in the surface loops of the human thrombin receptor are hydrophobic residues, mostly consisting of tyrosine and phenylalanine residues (Nanevicz et al., 1995). Thus, it seems possible that the monoclonal antibodies are making analogous hydrophobic interactions. However, the epitopes of the monoclonals need to be further refined before further speculation could be justified. Sequence alignment did not reveal any significant homology between the anti-TRED monoclonals and the extracellular loops of the receptor. As noted above, however,

the lengths of the L1 and H3 loops of 3/17 and 2/389 suggest that the binding sites of these monoclonals may possess hydrophobic pockets.

4.3.2 Comparison of 3/17 VL sequence with anti-single stranded nucleic acid antibodies

A striking resemblance of sequence was noted with the VL domain of 3/17, with both the VL sequences of BV04-01, an anti single-stranded DNA antibody (Herron et al., 1991) and Jel103, an anti-RNA antibody (Pokkuluri et al., 1994). This is illustrated in the following amino acid sequence alignment (Table 4.11), with the corresponding antibody CDRs shown in bold type and critical amino acid residues involved in antigen binding in both of the anti-nucleic acid antibodies underlined.

Table 4.11 *Comparison of the VL sequence of the 3/17 monoclonal antibody with BV04-01 and Jel103.*

```
          10                  20                  30  L1              40                  50
DVLMTQTPLS LPVNIGDQAS ISCRSSQSLV HSNGNTYLHW YLQKPGQSPK 3/17
DVVMTQTPLS LPVSLGDQAS ISCRSSQSLV HSNGNTYLHW YLQKPGQSPK BV04-01
DVVMTQTPLS LPVSLGDQAS ISCRSSQSLV HSNGNTYLHW YLQKPGQSPK Jel103

      L2    60                  70                  80                  90          L3    100
LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQTTHVP 3/17
LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQSTHVP BV04-01
LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQSTHVP Jel103

          110
FTFGAGTKLE IK 3/17
LTFGAGTKLE LK BV04-01
RTFGGGTKLE IK Jel103
```

The crystal structure of the anti-single stranded DNA antibody (BV04-01) has been determined in the presence and absence of a tri-nucleotide of deoxythymidine $(dT)_3$ (Herron et al., 1991) and this antibody shares a nearly identical VL sequence with Jel 103. Jel103 binds to single-stranded poly-inosine (rIDP). Surprisingly the VL sequence of the 3/17 antibody shares an almost complete homology to both BV04-01 and Jel 103, yet the monoclonal 3/17 was raised against TRED.

The reason why the VL domain of a monoclonal antibody raised to TRED resembles an anti-single stranded DNA antibody, could be explained by the ability of single-stranded DNA molecules to bind human thrombin (Bock et al., 1992). Aptamers are double stranded DNA or single-stranded RNA molecules that bind with specific molecular targets (Ellington and Szostak, 1990). Certain single-stranded DNA oligonucleotides have been shown to dramatically inhibit thrombin-catalysed fibrin-clot formation. A 15-mer nucleotide consensus sequence, ($G^{1}GTTGGTGTGG^{15}$), inhibits thrombin clotting activity at nanomolar concentrations, whereas a scrambled sequence of the same composition does not, indicating the inhibition is DNA sequence specific (Bock et al., 1992).

The structure of thrombin inhibited by the 15-mer single-stranded DNA aptamer has been determined to 2.9 Å resolution (Padmanabhan et al., 1993a). The single stranded 15-mer has a folded structure in the complex with two stacked G-quartets (Padmanabhan et al., 1993a). The aptamer in the crystal structure interacts through salt bridges with two distinct regions of two different thrombin molecules. One is the fibrinogen exosite (anion-binding exosite-1), while the other is the heparin binding site (anion-binding exosite-2). Three residues of the fibrinogen exosite make ion pair interactions with the 15-mer. The specific interactions are ion pairs between His 71, Arg 75 and Arg 77 of thrombin and the phosphodiester backbone of nucleotides G^{8} and G^{10}. In addition, T^{7} and T^{9} are involved in binding within a hydrophobic cluster near the anion binding exosite (Ile 24, His 71, Ile 79, Tyr 117 of thrombin).

In the interaction of BV04-01 with $(dT)_3$, the light chain residues involved are His 97, His 31, Asn 33 and Tyr 37. With Jel103, binding is provided by stacking of the nucleotide base and the Tyr 37 side chain and by interaction of the nucleic acid's α-phosphates with an anionic binding site of the antibody. In both cases, most of the amino acid side-chains interacting with the nucleotides come from the light chain. In Jel103 the phosphate groups are involved in hydrogen bonds. Tyr 37 hydrogen bonds to the α-phosphate, while Asn 33 and Lys 55 contact both phosphates making a hydrogen bond and an ion pair respectively. Comparison of the $(dT)_3$-BV04-01 and rIDP-Jel 103 complexes, shows that the antigen binding site is canyon shaped. Tetin (1993) using fluorescence

quenching methods found that BV04-01 binds $(dG)_6$ and $(dT)_6$ with equal affinity. This is relevant with regard to the thrombin-aptamer structure, as the nucleotide bases involved in binding within thrombin are either guanines or thymines. In the $(dT)_3$-BV04-01 complex, the thymidine bases of the DNA penetrate into a deep cleft and stack against the side chains of aromatic amino acids. This is analogous to the 15 mer DNA aptamer in which the nucleotide bases T^7 and T^9 bind via their non-polar pyrimidine rings into a hydrophobic cluster near the fibrinogen exosite.

The sequences of the light chains of both BV04-01 and Jel103 are virtually identical to the CDRs and the framework region of the light chain of 3/17. Thus it is likely that the CDRs within the 3/17 light chain are presented in a canonical structure identical to those of the light chain CDRs in BV04-01 and Jel103. As a corollary, it follows that the key residues identified in the crystal structures of BV04-01 and Jel103 are also likely candidate residues in 3/17 that are involved in the interaction with the thrombin receptor.

It has been proposed that antibody combining sites are enriched in relatively solvent-exposed aromatic residues and that the burying of these large rigid side chains by antigen contributes to increased binding constants (Padlan, 1990). DNA-binding antibodies are also rich in aromatic amino acids (Mol et al., 1994a; Mol et al., 1994b; Barry et al., 1994), some of which no doubt interact with DNA. Of the six single strand specific nucleic acid binding antibodies examined by Barry et al. (1994), two are specific for single-stranded nucleic acids; Hed 10 (Lee et al., 1982; Cygler et al., 1987) is an autoimmune antibody which shows considerable specificity for single-stranded poly(dT) DNA and Jel 201 (Sibley et al., 1988) is specific for poly ADP ribose. The combining site of Hed 10 has been modelled (Barry et al., 1994) and forms a long cleft roughly parallel to the VH - VL domain interface. The interior of this cleft is particularly enriched in tyrosines, mostly from the H3 CDR loop. This is comparable to the same loop present in the anti-TRED monoclonal 3/17, which is particularly rich in tyrosine residues. The binding cleft in Hed 10 is bordered on the VL side by a large ridge of positive electrostatic potential created by lysine and arginine residues in CDR-L1 and CDR-H2. The model proposed for Hed10 by Barry et al. (1994) places the sugar phosphate backbone of the DNA along this positively

charged ridge while the thymine bases penetrate into a hydrophobic cleft and stack with the side chains of the tyrosine residues in the H3 loop. This proposed interaction is homologous to that of hirudin with the thrombin anion-binding exosite. The binding of hirudin to the anion-binding exosite of thrombin is stabilised by a general complementarity of electrostatic interactions of the two surfaces, combined with the burial of the hydrophobic amino acid residues of hirudin in apolar crevices that line the thrombin exosite (Karshikov et al., 1992).

4.4 Conclusion

This high homology of the light chain of the 3/17 monoclonal antibody with the anti-single stranded DNA antibodies suggests that it is mimicking the thrombin exosite and is making important interactions with the hirudin-like domain of the thrombin receptor. A comparison of binding of single stranded DNA to 3/17 could be explored using the same methodology outlined by Bock (1992) and suitable DNA aptamers selected against the 3/17 antibody combining site from a randomly generated library. The amino acid residues His 97, His 31, Tyr 37, Asn 32 and Lys 55 that play a role in nucleotide binding are also found in the light chain of 3/17, therefore these key residues could interact with the thrombin receptor. Confirmation of this hypothesis must await the crystallization of the anti-TRED antibody with thrombin receptor peptides.

Chapter 5

Characterisation Of The Proteinase Activated Receptor-2

5.1 Introduction

Since the initial cloning of the thrombin receptor (Vu et al., 1991a), interest in G protein-coupled receptors that are activated by proteolytic cleavage has increased with the recent isolation of a second protease activated receptor, PAR-2 (Nystedt et al., 1994). The LDPR/SFLLRN activation sequence of the thrombin receptor can be cleaved by sufficiently high concentrations of other serine proteases, such as trypsin (Brass et al., 1992) and granzyme A (Suidan et al., 1994). Conversely, a protease such as cathepsin G by cleaving the thrombin receptor C-terminal to the thrombin cleavage site can render the receptor insensitive to thrombin (Molino et al., 1995). Trypsin can cleave PAR-2 within the N-terminal region at SKGR/SLIGRL, forming a tethered ligand analogous to the thrombin receptor (Nystedt et al., 1994; Bohm et al., 1996). With the widespread distribution of PAR-2 in tissues not normally exposed to pancreatic trypsin (Nystedt et al., 1994; Nystedt et al., 1995b; Bohm et al., 1996), the question arises as to the physiological activator of PAR-2. In addition, it is of interest which proteases may inactivate PAR-2 *in vivo*.

The expression of PAR-2 in various tissues and cell lines has been examined by Northern analysis, RT-PCR and *in situ* hybridisation. In addition, functional expression of PAR-2 has been demonstrated in some cells, using either trypsin or receptor-derived agonist peptides (Nystedt et al., 1995b; Santulli et al., 1995; Bohm et al., 1996). Immunological techniques have not been used to study the tissue distribution of PAR-2. Immunohistochemical detection of receptors offers some advantages over Northern analysis and *in situ* hybridisation for tissue distribution studies, in that it can be more sensitive and allows a more precise identification of the cell types expressing the protein.

Antibodies to the thrombin receptor have been successfully used to characterise its function on a variety of different cells (Hoxie et al., 1993; Woolkalis et al., 1995; Brass et

al., 1994) and to verify mRNA detection (Nelken et al., 1992). The availability of antibodies for the detection of PAR-2 would facilitate further investigation of the function and distribution of PAR-2. For instance low levels of PAR-2 mRNA were detected in heart compared to kidney by Northern analysis (Nystedt et al., 1995b; Bohm et al., 1996). In heart, however, PAR-2 expression is largely confined to endothelial cells where it is highly expressed (Al-Ani et al., 1995). Such problems are best resolved by immunohistochemistry. In addition, antibodies produced to PAR-2 and capable of blocking enzymatic cleavage of the receptor would have a useful role in determining the potential protease agonist.

Identification of proteases that activate PAR-2 *in vivo* could shed some light on the possible role of the receptor. The thrombin receptor was identified by expression cloning, while PAR-2 was isolated by homology cloning, and its activator is therefore unknown. Moreover, the sequence around the thrombin receptor cleavage site strongly suggest that thrombin is the activator (Coughlin, 1994). The LDPR sequence of the cleavage site is identical to the thrombin activation sequence in protein C and the regions homologous to the KYPEF 'hirudin-like' domain are present in other thrombin substrates. Although trypsin activates PAR-2, this enzyme is not found outside the gastrointestinal tract, where it could act on PAR-2 expressed on intestinal epithelial cells (Bohm et al., 1996). In other tissues, the activator must be a trypsin-like enzyme capable of cleaving at the Arg-Ser bond in the sequence SKGR/SLIGRL. The physiological activator would have to display a high level of selectivity , as it must discriminate between the other arginine or lysine residues present in the PAR-2 extracellular domain. Circulating trypsin-like serine proteinases involved in the processes of blood coagulation, fibrinolysis, or the complement cascade could provide candidates for activating PAR-2. However, transmembrane-linked cell surface trypsin-like serine proteases (Kazama et al., 1995; Tsuchiya et al., 1994) suggest other possible receptor cleavage mechanisms. Certain serine proteinases exert specific requirements in their target substrates for optimal cleavage, primarily dictated by the primary specificity pocket and other subsites (section 1.1.1). In particular, the physiological activator of PAR-

2 should have binding sites for the SKGR/S sequence. Thus, synthetic peptide substrates and inhibitors based on this sequence could be used to identify possible activators.

This chapter demonstrates the immunological characterisation of PAR-2 using rabbit polyclonal antibodies directed against synthetic peptides derived from the PAR-2 sequence. Using these antibodies PAR-2 was investigated on both primary culture human umbilical vein endothelial cells (HUVECs) and a transformed human keratinocyte cell line. Subsequently, they were used to detect PAR-2 expressed on specific peripheral blood leucocytes. To further define a role of PAR-2 in haematopoietic cells, Northern blot analysis was undertaken on a wide variety of murine haematopoietic cell lines. It was noted that while the murine thrombin receptor was strongly expressed in several of the cell lines tested, no expression of PAR-2 could be detected. A peptide chloromethylketone inhibitor incorporating the PAR-2 cleavage recognition sequence (biotin-SKGR-CH_2Cl) was tested with a variety of trypsin-like serine proteases *in vitro*. The results highlighted three candidate enzymes with favourable kinetic profiles that could cleave the receptor *in vivo*, namely pancreatic trypsin, mast cell tryptase and acrosin. The possible roles and physiological relevance of these three enzymes are discussed.

5.2 Results

5.2.1 Generation of rabbit anti-PAR-2 polyclonal antibodies

Rabbit polyclonal antibodies were raised to synthetic peptides corresponding to regions around the putative enzyme cleavage site of both mouse and human PAR-2 (section 2.5.10 - 2.5.15). A multiple antigenic peptide (MAP) of the hPAR-2 peptide sequence was used along with linear mPAR-2 and hPAR-2 peptides coupled to keyhole limpet haemacyanin (KLH). Antibodies were obtained to the mPAR-2 peptide as determined by ELISA (section 2.5.12). In contrast, no antibodies were generated to the KLH coupled hPAR-2 peptide after repeated immunisations. Excellent titres were however generated in rabbits that had been immunised with the corresponding hPAR-2-MAP. Since the MAP-hPAR-2 antigen was generating a high titre, anti-hPAR-2 antibodies were affinity purified from the pooled rabbit MAP-hPAR-2 serum and used subsequently for the immunological detection of native hPAR-2. After affinity purification of the mPAR-2 antibodies, they proved cross-reactive to the hPAR-2 peptide by ELISA. This was attributed to the similar peptide sequence at the N-terminus of each immunising peptide SKGRSLIG. Additionally, rabbit polyclonal antibodies that were specific for this short epitope were prepared by passing the affinity purified rabbit polyclonal mPAR-2 over a human peptide column and these were also used to detect the native human PAR-2 receptor.

5.2.2 Detection of PAR-2 on human umbilical vein endothelial cells (HUVECs) using immunocytochemistry

The affinity purified hPAR-2 antibodies were used for the immunological detection of the native receptor on HUVECs (section 2.5.18), (Figure 5.1).

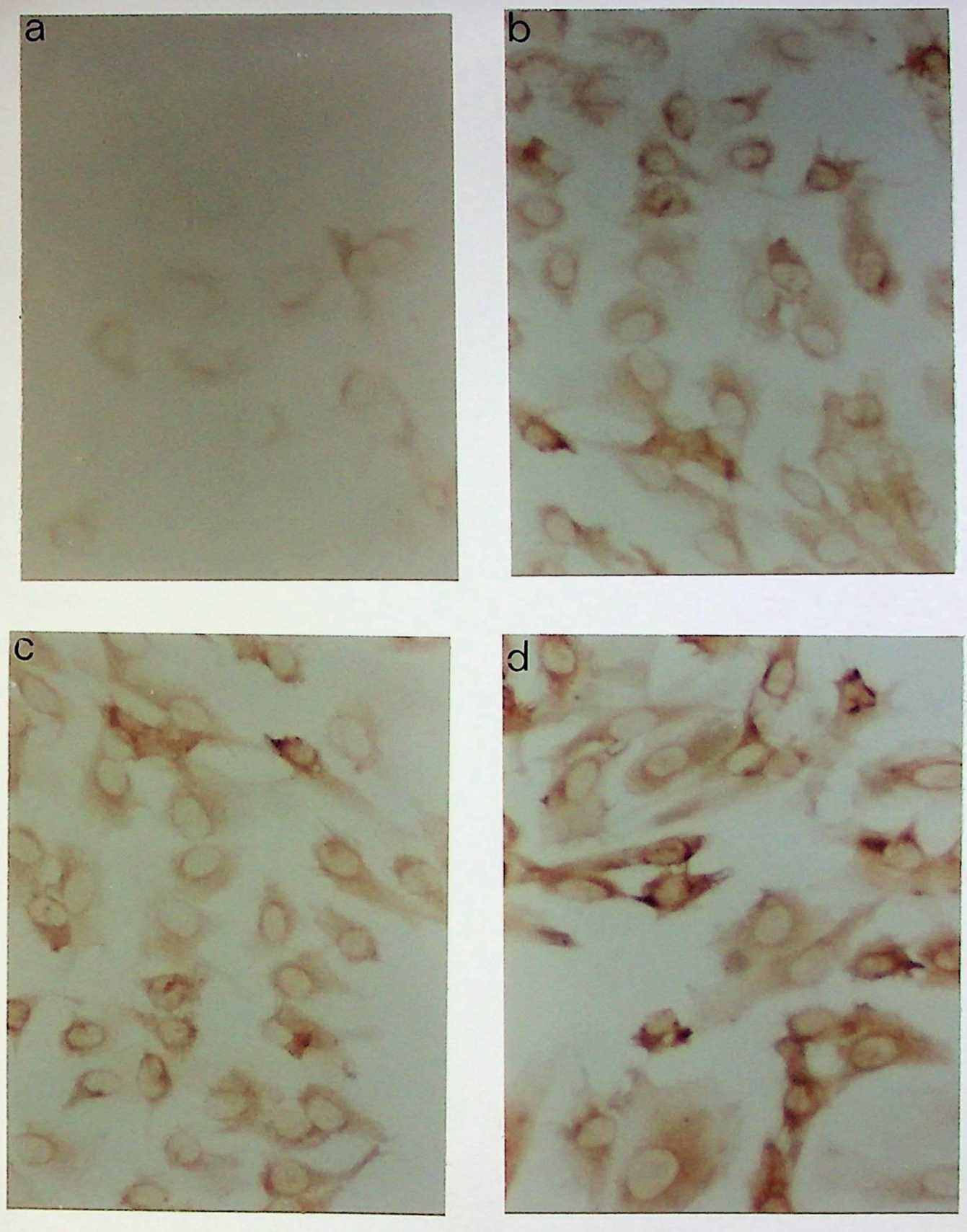


Figure 5.1 *Immunological detection of PAR-2 on HUVECs.*
The following preparations of affinity purified rabbit polyclonal antibodies, each at a concentration of 40 μg/ml were used. (**a**) Protein A-purified pre-immune control human MAP-hPAR-2. (**b**) Affinity purified mouse PAR-2 antibodies to the human PAR-2 peptide (anti-SKGRSLIG). (**c**) Affinity purified human PAR-2 MAP antibodies (bleed 2). (**d**) Affinity purified human PAR-2 MAP antibodies (bleed 3).

5.2.3 Analysis of hPAR-2 using flow cytometry

(performed by Dr. G. L. Howells, Dr. M. G. Macey and Dr. M. A. Curtis, Department of Oral Pathology, London Hospital Medical College)

Flow cytometry analysis was performed (section 2.5.19), with whole blood, human fibroblasts and SV40 transformed human keratinocytes. The results are displayed as fluorescence histograms obtained on a Becton Dickinson fluorescence activated cell sorter (FACS). The results are plotted with ordinate as cells/channel and the abscissa as cell fluorescence brightness; the ordinate scale is linear and the abscissa is a logarithmic scale (Figures 5.2 - 5.4). A discernible population shift was observed with both the affinity purified human PAR-2 MAP and anti- SKGRSLIG antibodies on the SV40 transformed human keratinocyte cells Figure 5.2 and human fibroblast cells Figure 5.3. A larger population of cells in both cases was recognised by the human PAR-2 MAP antibodies reflecting the presence of more immunodominant epitopes compared to the anti-SKGRSLIG antibodies. As Northern Blot analysis revealed the presence of the PAR-2 receptor in human peripheral blood leucocytes (Nystedt et al., 1995b), the affinity purified anti-PAR-2 MAP antibody was used to detect the particular cell-types that express PAR-2. Using whole blood samples, it was found that the receptor was expressed predominantly by granulocytes (Figure 5.4).

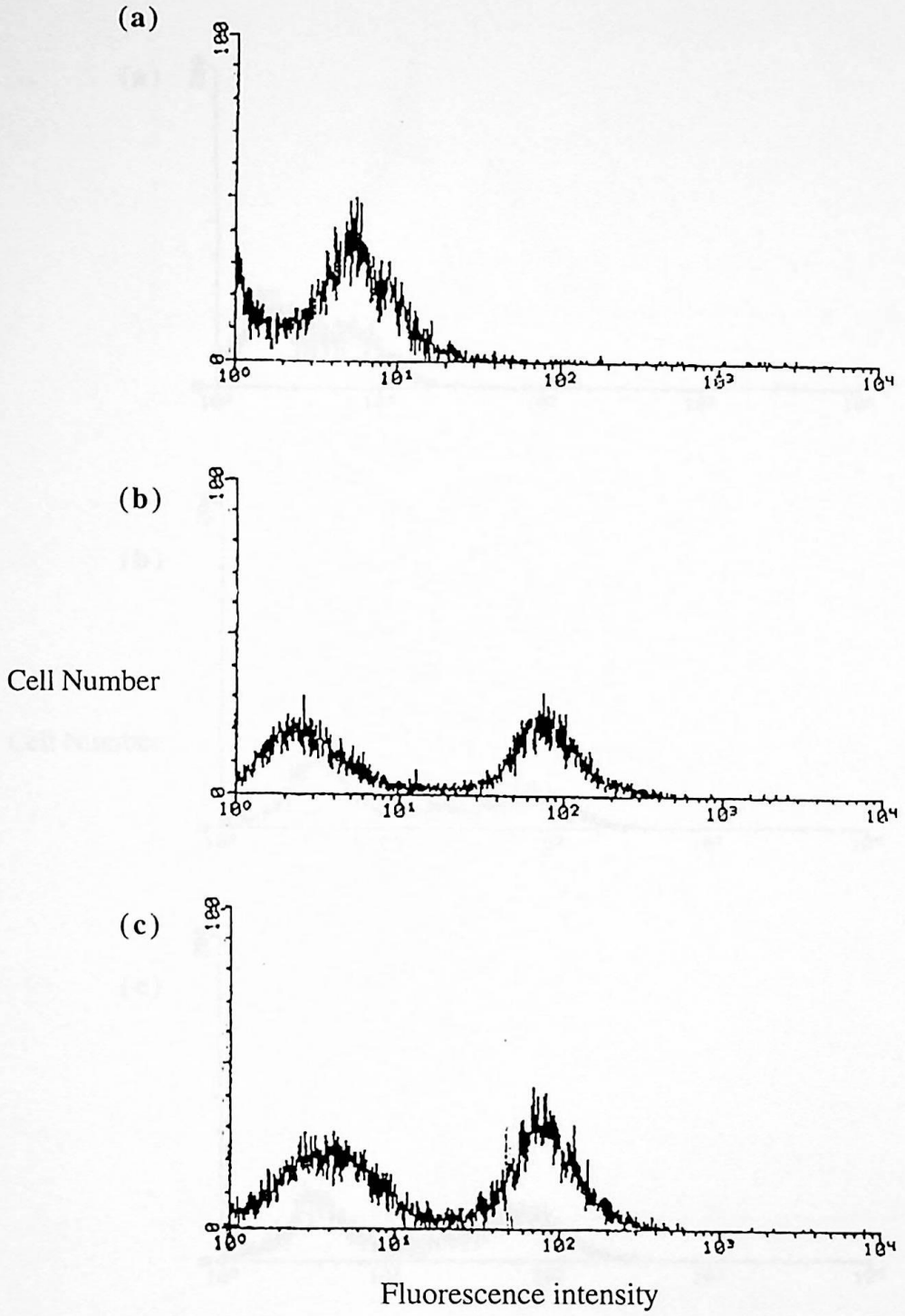


Figure 5.2 *Flow cytometry of SV40 transformed human keratinocytes with anti-PAR-2 polyclonal antibodies.* The following preparations of affinity purified rabbit polyclonal antibodies, each at a concentration of 20 μg/ml were used. **(a)** Protein A-purified pre-immune control human MAP-hPAR-2. **(b)** Affinity purified mouse PAR-2 antibodies to the human PAR-2 peptide (anti-SKGRSLIG). **(c)** Affinity purified human PAR-2 MAP antibodies (bleed 3).

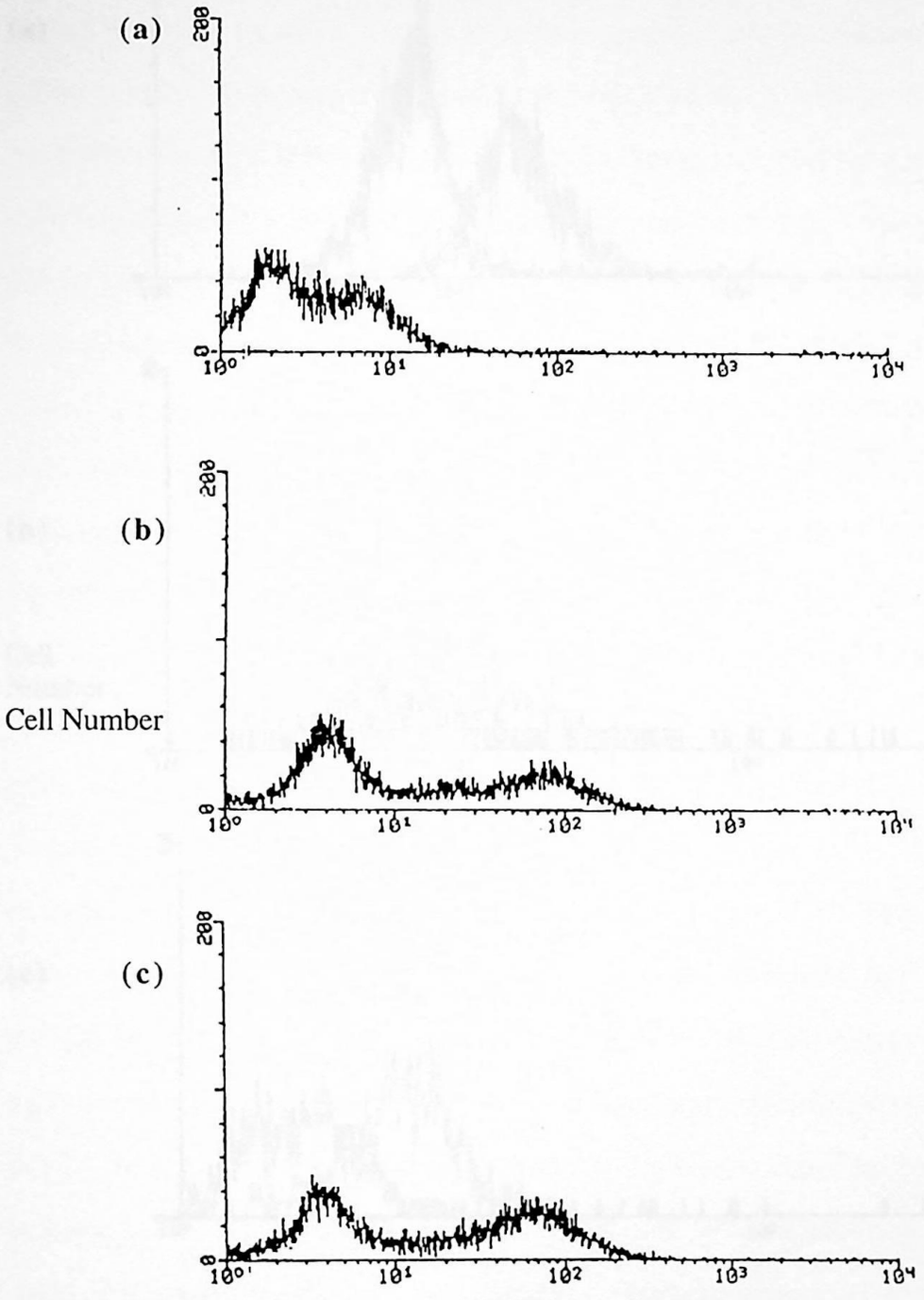


Figure 5.3 *Flow cytometry of human fibroblasts with anti-PAR-2 polyclonal antibodies.* The following preparations of affinity purified rabbit polyclonal antibodies, each at a concentration of 20 μg/ml were used. **(a)** Protein A-purified pre-immune control human MAP-hPAR-2. **(b)** Affinity purified mouse PAR-2 antibodies to the human PAR-2 peptide (anti-SKGRSLIG). **(c)** Affinity purified human PAR-2 MAP antibodies (bleed 3).

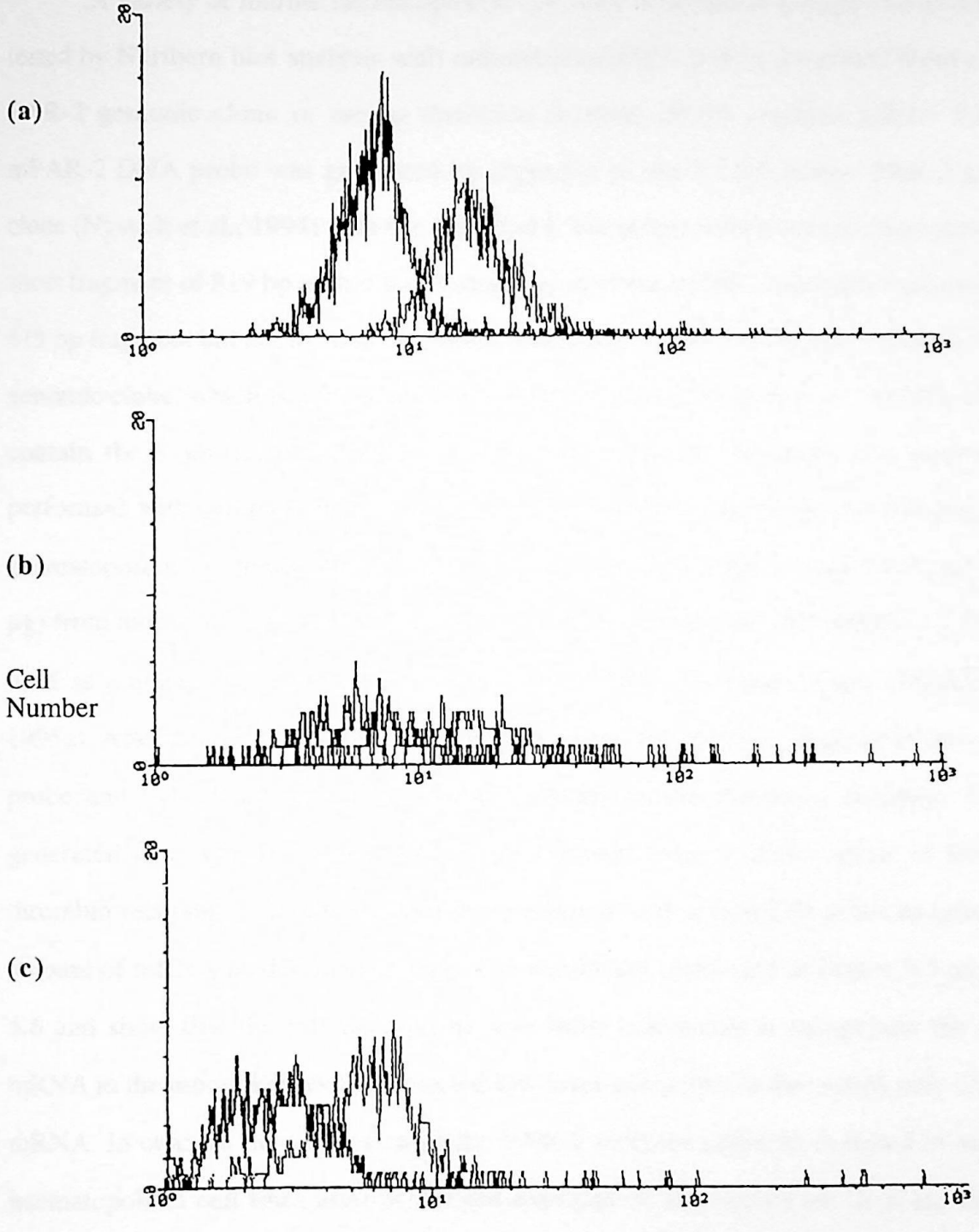


Figure 5.4 *Flow cytometry of whole blood with anti-PAR-2 polyclonal antibodies.* The protein A-purified pre-immune control human MAP-hPAR-2 and affinity purified human PAR-2 MAP antibodies (bleed 3), at a concentration of 20 μg/ml were used. **(a)**; Granulocytes. **(b)** Monocytes. **(c)** Lymphocytes.

5.2.4 Analysis of PAR-2 expression and thrombin receptor expression in murine haematopoietic cell lines

A variety of murine haematopoietic cell lines of different lineage (Table 5.1) were tested by Northern blot analysis with radiolabelled DNA probes generated from a mouse PAR-2 genomic clone or mouse thrombin receptor cDNA (section 2.2.7 - 2.2.8). A mPAR-2 DNA probe was generated by digestion of the 3.7 Kb mouse PAR-2 genomic clone (Nystedt et al., 1994) with Cla I and Sal I. These two restriction enzymes generated a short fragment of 819 bp within the coding region of the mPAR-2 receptor main exon. The 819 bp fragment did not include the region containing the putative signal sequence from the genomic clone, which is not encoded by the cDNA clone (Nystedt et al., 1995a), nor did it contain the 3'-untranslated region of the genomic clone. Northern blot analysis was performed with samples of purified poly $(A)^+$ mRNA (5 µg) from the different mouse haematopoietic cell lines. As a positive control for mouse PAR-2, poly $(A)^+$ mRNA (2.5 µg) from mouse kidney was used. In addition, mouse heart poly $(A)^+$ mRNA (2.5 µg) was used as a negative control, since expression of PAR-2 in heart is low (Nystedt et al., 1995a). After analysis with the mouse PAR-2 probe, the blot was stripped of the mPAR-2 probe and hybridised to a 2 Kb probe from the mouse thrombin receptor. This was generated as a Xba I - Xho I restriction fragment from a cDNA clone of the mouse thrombin receptor. The blot was finally hybridised with a GAPDH probe to estimate the amount of mRNA loaded in each lane. The results are illustrated in Figure 5.5 and Figure 5.6 and show that the mPAR-2 probe was fully functional; it recognised the receptor mRNA in the mouse kidney and detected low level transcripts in the mouse poly $(A)^+$ heart mRNA. In contrast no expression of the PAR-2 receptor could be detected in any of the haematopoietic cell lines after prolonged exposure of the membrane to X-ray film for 1 week. The thrombin receptor was however, strongly expressed in many of the mouse haematopoietic cell lines. The strongest expression was observed in the interleukin-3 (IL-3) dependent cell lines Baf-3 and A4, the murine mast/basophilic cell lines Mst and P815 and the megakaryocytic CD34 progenitor cell lines, 416 B and 416 BMEG. Low level thrombin

receptor mRNA transcripts were also detected in the murine erythroid cell line F4N and the pre-monocytic cell line M1.

Table 5.1 *Murine haematopoetic cell lines.*

Lane Number	Cell line	Phenotype
3	F4N	Erythroid
4	J2E	Erythroid
5	Mst	Myeloid/Basophil
6	W231	B-cell
7	P815	Myeloid/Basophil
8	WeHi 274	Myeloid/Macrophage
9	416 B	Myeloid/Megakaryocyte
10	416 BMEG	Myeloid/Megakaryocyte
11	M1	Myeloid/Monocyte
12	J774	Myeloid/Macrophage
13	WeHi 3BD+	Myeloid/Monocyte
14	BW 5174	T-cell
15	BWN	T-cell
16	NSO	B-cell
17	Baf-3	Interleukin-3 dependent cell line
18	A4	Interleukin-3 dependent cell line

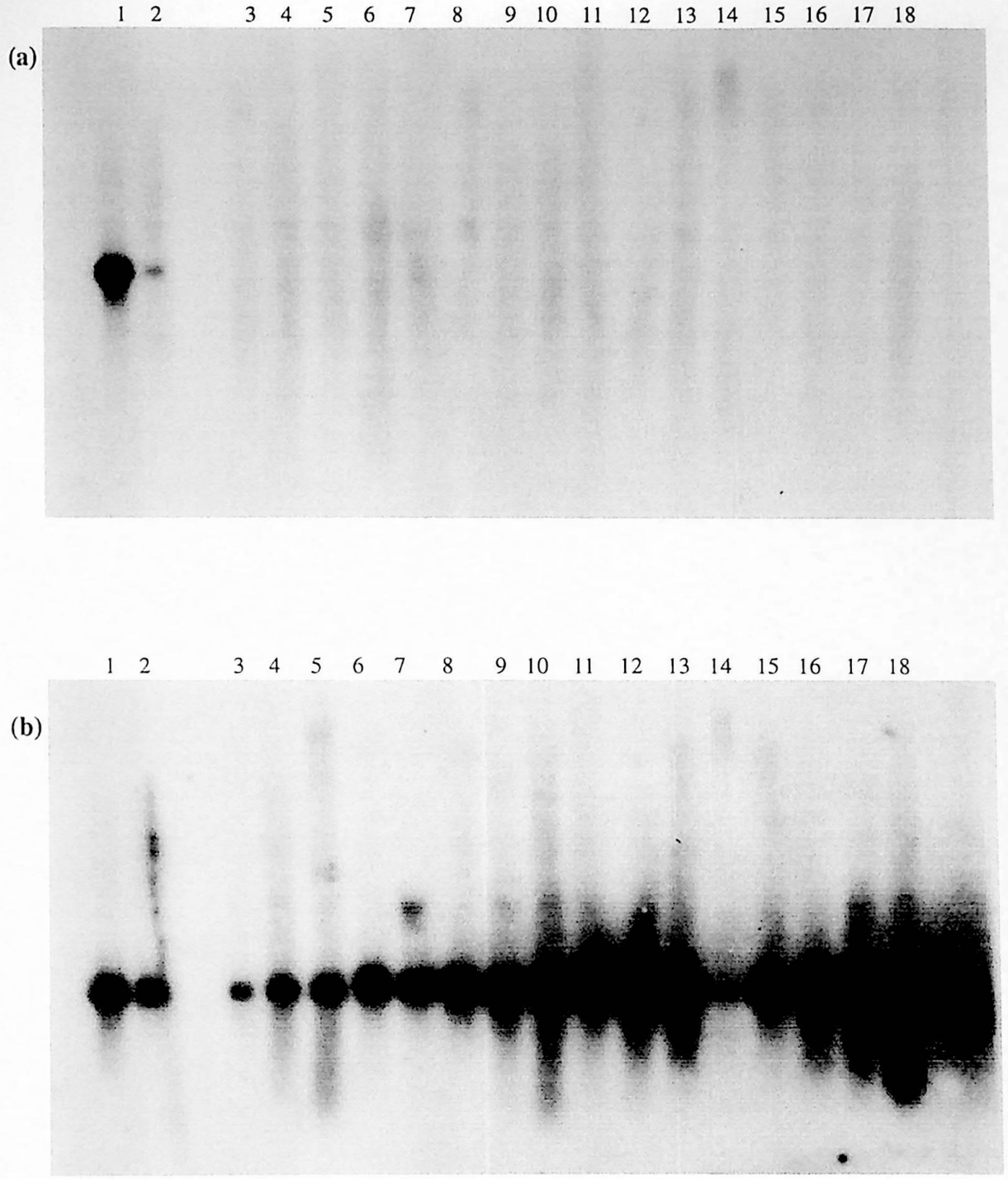


Figure 5.5 *Northern blot analysis of PAR-2 expression in murine haematopoietic cell lines.*
(**a**) 1 week exposure of a blot hybridised with a mPAR-2 probe.
(**b**) Overnight exposure of the identical blot normalised with a murine GAPDH probe.
The following murine mRNA samples have been loaded:
Lane 1, adult mouse kidney; lane 2, adult mouse heart; lane 3, F4N; lane 4, J2E; lane 5, Mst; lane 6, W231; lane 7, P815; lane 8, WeHi 274; lane 9, 416 B; lane 10, 416 BMEG; lane 11, M1; lane 12, J774; lane 13, WeHi 3BD+; lane 14, BW 5174; lane 15, BWN; lane 16, NSO; lane 17, Baf-3; lane 18, A4.

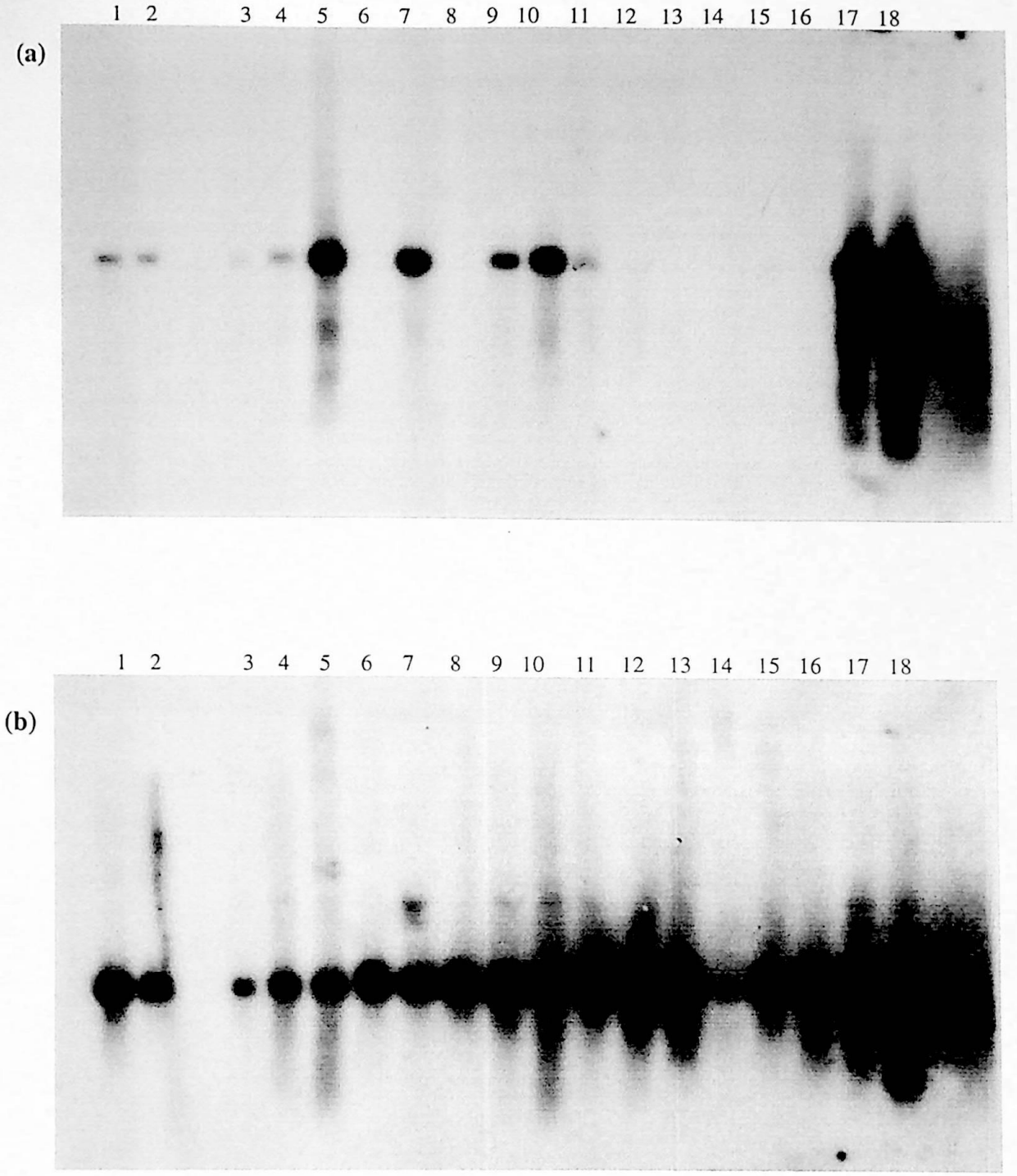


Figure 5.6 *Northern blot analysis of thrombin receptor expression in murine haematopoietic cell lines.*
(**a**) 3 day exposure of a blot hybridised with a murine thrombin receptor probe.
(**b**) Overnight exposure of the identical blot normalised with a murine GAPDH probe.
The following murine mRNA samples have been loaded:
Lane 1, adult mouse kidney; lane 2, adult mouse heart; lane 3, F4N; lane 4, J2E; lane 5, Mst; lane 6, W231; lane 7, P815; lane 8, WeHi 274; lane 9, 416 B; lane 10, 416 BMEG; lane 11, M1; lane 12, J774; lane 13, WeHi 3BD+; lane 14, BW 5174; lane 15, BWN; lane 16, NSO; lane 17, Baf-3; lane 18, A4.

Table 5.2 *Association rate constant k_{on} of biotin-SKGR-CH_2Cl with different serine proteases.*

Protease	k_{on} ($M^{-1}s^{-1}$)	K_m (μM)	Substrate
Trypsin	$(1.11 \pm 0.01) \times 10^5$	28	S-2288
Acrosin	$(3.09 \pm 0.01) \times 10^5$	12 ± 2	S-2288
Tryptase	$(1.48 \pm 0.02) \times 10^5$	188 ± 28	S-2288
Factor Xa	$(2.00 \pm 0.02) \times 10^4$	47	S-2222
Thrombin	$(1.84 \pm 0.02) \times 10^3$	3.6	S-2238
Plasmin	$(6.08 \pm 0.12) \times 10^2$	1110 ± 150	S-2288
Plasma kallikrein	$(3.99 \pm 0.04) \times 10^2$	272	S-2302
Tissue Plasminogen Activator (tPA)	$(4.34 \pm 0.01) \times 10^2$	345	Spec-tPA
Activated C1r (C1r~)	$(6.33 \pm 0.18) \times 10^2$	114 ± 28	S-2288
Activated C1s (C1s~)	$(4.22 \pm 0.08) \times 10^1$	1800 ± 700	S-2288
Activated protein C (APC)	$(2.15 \pm 0.01) \times 10^1$	222	S-2266
Granzyme A	$(1.77 \pm 0.04) \times 10^1$	1100	S-2302

5.2.5 Investigation of potential PAR-2 activating proteases using a synthetic peptide chloromethylketone inhibitor

The kinetics of the irreversible inhibition of a variety of serine proteases involved in blood clotting, fibrinolysis and inflammation by a biotinylated peptide chloromethylketone inhibitor (biotin-SKGR-CH_2Cl) that represents the P1 - P4 residues from the cleavage site in PAR-2 were determined as described in section (2.7).

In experiments to calculate the substrate K_m values for each enzyme, the substrate was varied over a ten fold range (section 2.7.2). Five different initial substrate concentrations were used, and each point was measured in duplicate. In experiments that examined the inhibitory effects of biotin-SKGR-CH_2Cl, for each assay the substrate used was present at a concentration of 400 μM. The inhibitor was added immediately before the assay was started by the addition of the enzyme. In the absence of inhibitors, the rate of product formation was linear up to a product concentration of greater than 10 μM, which represents 2.5 % substrate utilization. In the presence of inhibitor, the reaction was allowed to continue until the amount of product formed was 10 μM. Progress curves were obtained with at least five different concentrations of inhibitor. The values of k' obtained from these analyses were weighted according to the squared inverse of their standard errors and fitted to equation 1 (section 2.7.3). The value of K_m obtained from initial-velocity studies of the substrate with the enzyme was used with the apparent first-order rate constant k' to obtain values of the rate constant k_{on} ($M^{-1}s^{-1}$) for the formation of the enzyme-inhibitor complex. The rate constant k_{on} is a measure of the reactivity of the inhibitor with a particular protease. The kinetic parameters obtained and the substrates used for each enzyme in each assay are detailed in Table 5.2.

The biotinylated SKGR-CH_2Cl displayed a range of k_{on} values from (3.09 ± 0.01) x 10^5 M^{-1} s^{-1} with acrosin to (1.77 ± 0.04) x 10^1 M^{-1} s^{-1} with granzyme A. The results demonstrate that as well as trypsin which has been postulated to cleave and activate PAR-2 (Bohm et al., 1996), the trypsin-like enzyme acrosin from the male reproductive tract and tryptase from human mast-cells are possible activators of PAR-2. Both these proteases displayed k_{on} values that were equivalent to trypsin.

5.3 Discussion

5.3.1 Generation of the rabbit polyclonal antibodies

Although anti-mouse PAR-2 antibodies could be generated with a conjugated KLH-peptide antigen, no response was observed using a similar method of conjugation with the human PAR-2 peptide. This method for the production of anti-peptide antibodies involved coupling of the synthetic peptide to a large carrier protein KLH. The conjugation may have modified the antigenic epitopes of the peptide (Posnett et al., 1988). The presence of an internal lysine residue in the hPAR-2 peptide, instead of the corresponding arginine in the mouse peptide (section 2.5.10), may have compromised the conjugation using glutaraldehyde, as it cross-links free amino groups. This may have resulted in the internal lysine being modified, and/or cross-linked to KLH. The use of a peptide with an extra cysteine residue present on either the N or C-terminus would facilitate controlled conjugation to a carrier protein. The use of a multiple antigenic peptide (MAP) (Posnett et al., 1988; Tam, 1988) for the production of anti-hPAR-2 antibodies avoided the problems encountered with the KLH-hPAR-2 antigen, by eliminating the need for conjugation to a carrier protein. This method allows preparation of a multimeric peptide antigen on a branching lysine core by a single solid phase peptide synthesis. The resulting macromolecules, generally of final molecular weight above 10 kDa, are composed of multiple copies of the peptide antigen, and the lysine core usually represents less than 10% of the total molecular weight. These structures are highly immunogenic in mice and rabbits (Posnett et al., 1988; Tam, 1988) resulting in high titres of antibodies which often react

with the native protein in addition to the peptide immunogen. The antibodies produced react primarily with the peptide antigen and not with the lysine core. The amino terminal residues of the peptide have been found to be the most immunogenic region of the MAP peptide, as they are likely to be exposed at the surface of the MAP structure and consequently are highly mobile (Posnett et al., 1988).

5.3.2 Immunocytochemistry

The affinity purified hPAR-2 MAP antibodies specifically recognised the native hPAR-2 receptor on HUVECs. The affinity purified mouse PAR-2 antibodies to the common SKGRSLIG epitope present on the human PAR-2 receptor also identified the native receptor. This immunological evidence supports the results determined with Northern analysis and PAR-2 activating peptides that HUVECs express PAR-2 (Mirza et al., 1996). The presence of the SKGRSLIG epitope suggests that the uncleaved receptor is abundant. HUVECs also express the thrombin receptor (Mirza et al., 1996). Affinity purified anti-hPAR-2 polyclonal antibodies could be used to study the protein expression levels of PAR-2 on HUVECs in response to extracellular agonists. The vasoactive peptide endothelin-1 has been shown to down regulate thrombin receptor expression on HUVECs (Chen et al., 1995b). The down regulation of PAR-2 by thrombin on HUVECs and the presence of a possible cross-talk between these two protease-activated receptors (Mirza et al., 1996) could be investigated further with these specific PAR-2 antibodies. Ultimately, specific polyclonal antibodies to the activation site could be used to test possible protease activators of PAR-2 on HUVECs.

Specific staining of rat tissues was not observed using affinity purified anti-mPAR-2 antibodies. Cloning and sequencing of rat PAR-2, revealed that the corresponding epitope on the rat receptor was different from that used to generate anti-mPAR-2 antibodies (Al-Ani et al., 1995). The amino acid sequence of rat PAR-2 is N^{34}SKGRSLIGRLDTPP^{48} (Al-Ani et al., 1995). The corresponding peptide region from mouse PAR-2 which was incorporated in the immunising peptide was N^{34}SKGRSLIGRLETQP^{48}. In this short amino acid sequence there are two amino acid modifications. Glu 45 in the mouse sequence

is replaced by Asp in the rat sequence and Gln 47 is changed to a proline. These differences may have contributed to the problems observed in staining rat sections. Comparing the human PAR-2 and mouse PAR-2 sequences (Nystedt et al., 1995b), there is a common 8 amino acid sequence (SKGR/SLIG) around the cleavage site. Affinity purification of the mouse antibodies with a human peptide affinity matrix produced mouse antibodies that were selective to this epitope. These antibodies recognised the human receptor on HUVECs and they could be employed for the further analysis of the mouse or rat PAR-2 receptor, as both share this epitope.

5.3.3 Flow Cytometry

The anti-hPAR-2 antibodies stained keratinocytes which have been showed to express PAR-2 as well as the thrombin receptor (Santulli et al., 1995). The cell line used was a SV40 transformed human keratinocyte cell line which exhibited an increase in intracellular calcium $[Ca^{2+}]_i$ in response to PAR-2 activating peptides (C. Chinni , S. R. Stone and G. L. Howells, unpublished observations). Human gingival fibroblasts also showed specific expression of PAR-2. Interestingly, human skin fibroblasts showed no PAR-2 expression by Northern blot analysis but low level expression was detectable by RT-PCR (Santulli et al., 1995).

On subsequent analysis of whole blood by FACS analysis, the granulocyte fraction, which is primarily 95% neutrophils, was positive for PAR-2. It has been demonstrated that neutrophils have a $[Ca^{2+}]_i$ response to the thrombin receptor agonist peptide (TRAP), but not to thrombin itself (Jenkins et al., 1995). The presence of PAR-2 on neutrophils suggests that the TRAP response was due to its activation of PAR-2 (Jenkins et al., 1995).

The data of Figure 5.4 suggest that PAR-2 may be present on lymphocytes. This observation is interesting in relation to the previous reports that trypsin is a B lymphocyte activator, inducing the expression of IgG and IgM antibodies in mouse spleen cells at levels comparable to those obtained after stimulation with the bacterial lipopolysaccharide (LPS). The effects of trypsin on B-lymphocytes are not mediated by T-cells, as mouse spleen cells isolated from congenitally athymic nude mice, which are unable to produce T-cells, respond

to trypsin (Gisler et al., 1976). Moreover, proteolytic activity of trypsin was required, as the response was blocked by soybean trypsin inhibitor (Vischer et al., 1976).

5.3.4 Northern blot analysis

Northern analysis failed to demonstrate the expression of PAR-2 on any of the haematopoietic cell lines examined. These results suggest PAR-2 may only be expressed on terminally differentiated cells, such as neutrophils which were stained with anti-hPAR-2 antibodies. In contrast, the thrombin receptor was highly expressed on a wide variety of haematopoietic cells (Figure 5.6).

Maturation of human platelet megakaryocytes is inhibited by active thrombin and TRAP, suggesting that megakaryoctyes express functional thrombin receptors at a very early stage of maturation (Vittet et al., 1992) Northern analysis of the different murine haematopoietic cell lines supports this observation. The thrombin receptor was strongly expressed in the two murine megakaryocytic cell lines 416 B and 416 BMEG. Thrombin has been shown to exert an inhibitory effect on proliferation of the human megakaryocytic MEG-01 cell line (Vittet et al., 1992). Thus, thrombin generation during vascular injury may be involved in the regulation of megakaryocytic development.

The thrombin receptor was also expressed in the two interleukin-3 dependent cell lines Baf-3 and A4, which are initial progenitors for all of the haematopoietic lineages. The presence of the thrombin receptor on such an early lineage could be an indication of a thrombin-specific differentiation signal.

The murine mast/basophilic-like cell line Mst expressed high levels of the thrombin receptor. Mouse bone marrow-derived mast cells are activated and degranulate in response to thrombin, suggesting that this response is mediated through activation of the thrombin receptor (Razin et al., 1985). Other results suggest that the thrombin receptor is involved in mast-cell degranulation; a subplantar injection of TRAP led to the degranulation of mast cells *in vivo* (Cirino et al., 1996). Thus, thrombin generated at the sites of vascular-injury can act as a chemotactic agent for polymorphonuclear cells and also stimulate the release of

bioactive amines from mast cells; both responses are consistent with a major role of thrombin in the inflammatory response.

The J774 murine macrophage-like cell line has been extensively studied for the presence of a putative thrombin receptor. Native α-thrombin or catalytically inactive diisopropylfluorophosphate (DIP)-α-thrombin stimulated proliferation of quiescent J774 cells (Bar-Shavit et al., 1986a,b). Binding studies using [^{125}I]-α-thrombin have revealed the presence of approximately 14,000 binding sites on J774 cells with an apparent Kd of 7.5 x 10^{-9} M (Bar-Shavit et al., 1983b). Northern analysis with the murine thrombin receptor probe, however, showed no visible expression of the thrombin receptor in the J774 cell line. This result is consistent with observed responses of J774 cells to inactive thrombin and suggests the presence of a different thrombin binding receptor on the cell surface. As J774 cells display a chemotactic response to catalytic inactive thrombin (Bar-Shavit et al., 1986a,b), this stable cell line could be used in future studies to clone the putative thrombin chemotactic receptor.

The results of Mari et al. (1996) suggest that PAR-2 is present on some human T-cell lines Jurkat and HPB.ALL. However, Northern analysis has not detected PAR-2 in other human haematopoietic cell lines, including the pro-myelocytic leukaemia cell line HL-60 and the chronic myelogenous leukaemia cell line K562 (Bohm et al., 1996). The human HL-60 cell line is a pre-granulocyte cell line that can be induced to differentiate along a pathway to neutrophils or monocytes by the addition of either 1.25% DMSO or TPA respectively (Bunce et al., 1983). The demonstration of the absence of PAR-2 expression in this pre-granulocyte cell line supports the argument that the PAR-2 receptor is specifically expressed at certain times in the lifetime and development of the haematopoietic cell. The HL-60 cell line has been shown to express neither the thrombin receptor nor PAR-2 (Howells et al., 1993; Bohm et al., 1996). Karotype analysis has shown that this leukaemia cell line loses one of the pair of chromosomes 5, 8 and X after passage number 35 (Gallagher et al., 1979). Since it has been observed that both the thrombin receptor and PAR-2 genes reside within chromosome 5 (Nystedt et al., 1995b; Bahou et al., 1993b), loss of this chromosome could explain the lack of transcription of these genes. A possible

experiment would be to differentiate the HL-60 cells along the neutrophil pathway and test for the expression of PAR-2 using flow cytometry with the anti-hPAR-2 antibodies; and to correspondingly differentiate them along the monocytic pathway performing similar experiments with the monoclonal antibodies to the thrombin receptor. The exposure of monocyte differentiated HL-60 cells to thrombin elicited an increase in $[Ca^{2+}]_i$ (Bar-Shavit et al., 1987), suggesting the expression of the platelet thrombin receptor.

On certain human T-cell lines the thrombin receptor has been shown to be present and functional (Tordai et al., 1993; Mari et al., 1994) and its activation has been shown to enhance CD69 expression and interleukin-2 production induced by T-cell receptor cross-linking (Mari et al., 1994). Using flow-cytometric analysis with antibodies to the human thrombin receptor on terminally differentiated peripheral blood leucocytes, the thrombin receptor has been demonstrated on T-cells but not B-cells (Tordai et al., 1993; Howells et al., 1993). The presence of the thrombin receptor on granulocytes, namely human neutrophils has not been demonstrated using flow cytometry (Howells et al., 1993), but low level expression of the thrombin receptor has been detected on monocytes, using a combination of *in situ* hybridisation and immunohistochemistry. Although the expression in peripheral blood monocytes appeared to be very low, macrophages, the tissue form of monocytes, clearly expressed more thrombin receptor (Nelken et al., 1992). Monocytes, like neutrophils, are chemotactic to thrombin and also catalytically inactive thrombin (Crago et al., 1995; Jenkins et al., 1995), while the TRAP peptide which is capable of activating PAR-2, is not chemotactic. These results suggest the existence of another thrombin binding protein on the surface of these granulocyte cells which is responsible for the chemotactic response.

5.3.5 Characterisation of the PAR-2 activating proteases

The high expression of PAR-2 in the gastrointestinal tract, combined with the co-localisation of pancreatic trypsin, strongly suggests that trypsin is the physiological activator of PAR-2 (Bohm et al., 1996). Moreover, trypsin and PAR-2 activating peptides cause increases in $[Ca^{2+}]_i$ in rat intestinal epithelial cells and stimulate amylase secretion in

isolated pancreatic acini (Bohm et al., 1996). Trypsin was rapidly inactivated by biotin-SKGR-CH_2Cl with a k_{on} value of (1.11 ± 0.01) x 10^5 M^{-1} s^{-1}. This rapid inactivation of trypsin by biotin-SKGR-CH_2Cl indicates that the SKGR sequence binds productively to the active site of trypsin and the high k_{on} value correlates with the potent ability of trypsin to activate PAR-2 *in vivo*.

Acrosin is a trypsin-like serine protease that is found in large quantities within the acrosomal vesicle of mammalian spermatozoa (Müller-Esterl and Fritz, 1981). Release of the acrosomal contents, via a calcium-dependent exocytotic mechanism, is necessary for the spermatozoa to penetrate the zona pellucidia and for fertilisation (Rubinstein and Breitbart, 1991; Saling, 1981). The target substrate for acrosin *in vivo* has still not been elucidated. It was thought that acrosin's activity was necessary for sperm penetration of the zona pellucida, but an acrosin null mouse displayed no differences in fertilisation ability compared to the wild-type phenotype (Baba et al., 1994). Like trypsin, acrosin preferentially cleaves after Arg or Lys residues. It has a preference for Arg over Lys at P1 and a mild preference for proline at P2 (Skoog et al., 1989). Acrosin was rapidly inactivated with biotin-SKGR-CH_2Cl with a k_{on} value of (3.09 ± 0.01) x 10^5 M^{-1} s^{-1}; this value is slightly higher than that observed for trypsin Table 5.2, implying that under appropriate conditions acrosin is a potential activator of PAR-2 and in this respect it is interesting to note that Northern analysis demonstrated PAR-2 expression in both the male and female reproductive tract; PAR-2 was expressed in prostate and ovary but not in the testis (Nystedt et al., 1995b). It should be noted however that acrosin will only be released in the prostate from damaged sperm. Activation of PAR-2 in the prostate by acrosin could stimulate the prostate epithelial cells to secrete seminal fluid. Another trypsin-like serine protease named prostasin has been found localised in the human prostate and is capable of hydrolysing substrates with a P1 arginine residue (Yu et al., 1994). Prostasin has a unique 19-amino acid hydrophobic portion which makes it suitable to anchor in the cell membrane. Using RT-PCR analysis, its expression has been detected in other tissues including liver, salivary gland, kidney, lung, pancreas, colon, bronchus and renal proximal

tubular cells (Yu et al., 1995). PAR-2 is highly expressed in all these tissues except the salivary gland, and prostatin is another candidate for PAR-2 activation.

Tryptase is a tetrameric trypsin-like enzyme present in mast cells, which is released in an active form upon mast cell degranulation (Schwartz, 1994; Schwartz et al., 1981). In its active tetrameric form it is stabilised by heparin, to which it is ionically bound under physiological conditions (Alter et al., 1987). In the absence of heparin, tryptase subunits dissociate to inactive monomers (Schwartz and Bradford, 1986). Mast cell-derived tryptase can be isolated from either skin or lung due to the abundant distribution of mast cells in these tissues (Schechter et al., 1993; Smith et al., 1984). Although the physiological function *in vivo* is still unknown, tryptase catalyses a number of reactions *in vitro* including cleavage of fibrinogen (Schwartz et al., 1985) and high-molecular-mass kininogen (Maier et al., 1983). Tryptase also activates the anaphylatoxin C3a and collagenase (Gruber et al., 1988) as well as the inactivation of neuropeptides such as vasopressin and neurotensin (Braganza and Simmons, 1991). These observations suggest that tryptase may be involved in a wide variety of processes including the inhibition of coagulation and promotion of bronchoconstriction *in vivo*. Human lung mast cell tryptase is not only a specific and potent growth factor for fibroblasts (Ruoss et al., 1991) and epithelial cells (Cairns and Walls, 1996), but is also able to inhibit the mitogenic effects of thrombin (Hartmann et al., 1994). The subsite specificities of both human skin and lung tryptase have been investigated. The substrate KGR-*p*-nitroanilide was among the best for both forms of tryptase, suggesting that it is a possible physiological activator of PAR-2 (Tanaka et al., 1983).

Only catalytically active tryptase induces the proliferation of primary culture keratinocytes, as this mitogenic effect is blocked by active site inhibitors (Eder et al., 1995). This suggests it may cleave a specific substrate on the surface of the keratinocytes to effect this response. Human primary keratinocytes have been demonstrated to express high levels of PAR-2 (Santulli et al., 1995) suggesting that the effects of tryptase could be mediated by PAR-2 activation. Indeed, tryptase was rapidly inactivated by biotin-SKGR-CH_2Cl with a k_{on} value of $(1.48 \pm 0.02) \times 10^5\ M^{-1}\ s^{-1}$ which is in accord with a putative role for tryptase as an activator of PAR-2. Other results, however, indicate that PAR-2 is

not involved in the mitogenic response to tryptase; the PAR-2 agonist peptide inhibits growth of primary culture keratinocytes (Derian et al., 1996). These results argue strongly against the hypothesis that mast cell tryptase cleaves PAR-2 and suggest that there may be another specific receptor for mast-cell tryptase to exert this mitogenic response. The treatment of human primary keratinocytes with TRAP exerts a strong mitogenic response analogous to thrombin (Algermissen et al., 1995). It is possible that tryptase is cleaving and activating the thrombin receptor to yield a similar response.

Factor Xa, a protease common to both the intrinsic and extrinsic blood coagulation pathways, hydrolyses two bonds in the conversion of prothrombin to thrombin, the hydrolysis sites in bovine prothrombin are IEGR/I and IEGR/S. The P2 Gly residue in these sequences is the same as that observed in the PAR-2 sequence. Factor Xa was able to accommodate a lysine in the P3 position in biotin-SKGR-CH_2Cl. The observed k_{on} value was among the best that have been observed with factor Xa (Kettner and Shaw, 1981). The ability of factor Xa to accommodate the negatively charged Glu and positively charged Lys in the P3 position appears contradictory, but examination of the crystal structure of factor Xa suggests the Lys side chain may be protruding out of the active site into the surrounding solvent (Padmanabhan et al., 1993b).

Thrombin, the last protease in the blood coagulation cascade, catalyses the conversion of fibrinogen to fibrin by the hydrolysis of two peptide bonds, liberating fibrinopeptides A and B. It also activates the transglutaminase factor XIII which cross-links the blood clot. The amino acid sequences preceding the hydrolysed bonds are GVR and VPR for the α-chain of fibrinogen and factor XIII, respectively. Chloromethylketone inhibitors corresponding to each of these sequences are effective inhibitors of thrombin with VPR being the most reactive (Kettner and Shaw, 1981). The preparation of a chloromethylketone with the substitution of D-Phe in the P3 position (D-Phe-Pro-Arg-CH_2Cl) is a potent selective inhibitor of thrombin, attributed to the aryl-binding pocket in thrombin (Kettner and Shaw, 1979; Bode et al., 1989). Biotin-SKGR-CH_2Cl was a poor inhibitor of α-thrombin compared to trypsin. These results are in accord with those of

Nystedt et al. (1994) that showed PAR-2 is cleaved at trypsin concentrations of 0.3 nM, but the receptor is resistant to cleavage with α-thrombin concentrations up to 100 nM.

Plasma kallikrein's physiological function is the hydrolysis of an Arg-Ser bond in kininogen, liberating the C-terminal portion of bradykinin, a potent vasodilator (-FSPFR-OH). The chloromethylketone tripeptide analogue corresponding to the C-terminal of bradykinin, PFR-CH_2Cl, readily inactivates plasma kallikrein (Kettner and Shaw, 1978). The P2 Phe residue of the inhibitor was important for reaction with plasma kallikrein while the amino acid residue at P3 was of minor importance in the enzyme's specificity (Kettner and Shaw, 1981). Thus, the small glycine at P2 of biotin-SKGR-CH_2Cl would explain the slow reaction of the inhibitor with plasma kallikrein.

Plasmin hydrolyses multiple bonds of its physiological substrate, fibrin, during the lysis of blood clots. The specificity of plasmin for fibrin *in vivo* does not appear to be highly dependent on the sequence of its substrate, but rather on the binding of plasmin and plasminogen on the surface of fibrin. The use of affinity labels for this protease has revealed that plasmin displays a two fold greater preference for lysine than arginine as the P1 residue (Kettner and Shaw, 1981). A large bulky Phe residue was preferred in P2, while at P3 Glu, Ala and Phe were tolerated with an alanine residue being the best (Kettner and Shaw, 1981). The presence of both an arginine at P1 and the small glycine residue at P2 in the SKGR sequence provide explanations for the poor reactivity of biotin-SKGR-CH_2Cl with plasmin and suggest PAR-2 is not activated by plasmin *in vivo*.

Apart from the role of tissue plasminogen activator (tPA) in haemostasis, tPA is associated with a number of pathological processes, which include malignant cell transformation, inflammation, and the promotion of carcinogenesis. Tissue plasminogen activator hydrolyses the PGR/V sequence in plasminogen to generate active plasmin. Peptide chloromethylketone inhibitors have pointed to the importance of a Gly at P2, although its substitution by alanine is also tolerated (Kettner and Shaw, 1981). tPA did not display similar kinetic parameters to those observed with pancreatic trypsin, acrosin and mast-cell tryptase. This suggests that tPA does not activate PAR-2.

Activated Protein-C (APC) is a serine protease capable of proteolytically inactivating factors Va and VIIIa in conjunction with protein S. Thus activated protein C shuts off the coagulation cascade by degrading factors which are essential for the amplification reactions that lead to the formation of thrombin. The cleavage specificity of APC has been investigated using peptide chloromethylketone inhibitors (Stone and Hofsteenge, 1985), and the enzyme displayed a marked preference for a bulky non-polar group in the P2 position such as Pro or Phe. This cleavage specificity is very similar to plasma kallikrein (Stone and Hofsteenge, 1985). Compared with all the other trypsin-like serine proteases involved in the processes of blood coagulation and fibrinolysis, APC was most slowly inactivated by biotin-SKGR-CH_2Cl.

In the complement system, the first component C1 consists of three proteins; C1q and the trypsin-like serine proenzymes C1r and C1s, which form a complex in the presence of calcium (Arlaud et al., 1987). This complex binds to antigen-antibody aggregates inducing a conformational change in C1r to form activated C1r (C1r~), which cleaves C1s at a single Arg-Ile bond in the sequence KQR/IIG to yield activated enzyme (C1s~) (Spycher et al., 1986). C1s~ in turn cleaves a single Arg-Ala bond in the sequence LQR/ALE of the complement component C4 (Moon et al., 1981; Press and Gagnon, 1981) and cleaves a single Arg-Lys bond in the sequence LGR/KIQ in complement component C2 (Kerr, 1979). The subsite specificities of C1r and C1s have been investigated using peptide thioester substrates. Human C1r~ and C1s~ prefer a Gly and Ala respectively at P2, combined with a P1 Arg (McRae et al., 1981). From the substrate specificity of C1r~ for the proenzyme C1s *in vivo*, it would appear to accomodate a P3 Lys as well as a P1 Arg, but the biotin-SKGR-CH_2Cl inhibitor proved a poor inhibitor of C1r~. The kinetic parameters were investigated for isolated C1r~, however, the kinetic profile of C1r~ bound to C1q may be different. Although the SKGR sequence had residues at P1 and P2, which from the experiments performed by McRae et al. (1981) would be favourable for C1s~, the presence of a P3 Lys was not tolerated by the C1s~ enzyme.

The physiological substrates of cytotoxic T-lymphocyte granule-associated serine proteases (referred to as "granzymes") remains unclear (Masson and Tschopp, 1987).

Granzyme A has been reported to play a critical role in target cell destruction, including apoptosis and DNA fragmentation in target cells (Smyth and Trapani, 1995). However, whatever the role of granzyme A in apoptosis, it is clear that it does not function as a physiological activator of PAR-2. Although granzyme A is able to activate the thrombin receptor on neuronal cells (Suidan et al., 1994), the results of Sower et al. (1996) suggest that it is capable of activating another receptor and inducing interleukin-6 (IL-6) production by monocytes, whereas trypsin, thrombin and TRAP were unable to induce IL-6 secretion. The poor reaction of granzyme A with biotin-SKGR-CH_2Cl is probably due to the P2 Gly. All previously identified substrates for granzyme A have either a proline or phenylalanine in the P2 position (Simon et al., 1986; Masson et al., 1986; Odake et al., 1991).

5.4 Conclusion

The use of a multiple antigenic peptide clearly demonstrated an advantage over conventional coupling of peptides to carrier proteins in the generation of anti- hPAR-2 rabbit polyclonal antibodies. The resultant affinity purified hPAR-2 antibodies were used successfully for the immunological detection of hPAR-2. Three potential activators of PAR-2 were also detected using a chloromethylketone inhibitor based on the PAR-2 activation site.

Chapter 6

Construction And Expression Of A Recombinant scFv Antibody Containing A Thrombin Loop

6.1 Introduction

Thrombin has a variety of actions on a number of soluble components of the coagulation cascade (Figure 1.2) and on cells (Figure 1.4). Thrombin's substrate specificity is dependent on the anion-binding exosite. It plays a key role in interactions with fibrinogen, fibrin, hirudin, thrombomodulin, Glycoprotein Ib (GPIb) and the thrombin receptor (Stubbs and Bode, 1995). The crystal structure of thrombin bound to hirudin, an inhibitory protein from the medicinal leech, has revealed in great detail the exact structure of the surface loops involved in the anion-binding exosite and the molecular interactions made by the acidic carboxy-terminal region from hirudin and this basic site (Rydel et al., 1991; Stubbs et al., 1992). Overall binding is stabilised by a general complementarity of electrostatic fields, as well as the burial of hydrophobic residues in apolar crevices that line the thrombin exosite (Karshikov et al., 1992).

A sequence similar to that of the exosite-binding region of hirudin is found in the thrombin receptor (Figure 1.7). Interaction of receptor peptides at the anion-binding exosite of thrombin appears to involve the $D^{50}KYEPF^{55}$ motif of the thrombin receptor in a manner analogous to that seen for the hirudin peptide homologues, hirugen and hirulog1 in complexes with thrombin (Mathews et al., 1994; Skrzypczak-Jankun et al., 1991). Studies with mutant thrombin receptors show that thrombin's induced platelet activation of the receptor is dependent on a productive interaction between the anion-binding exosite and the hirudin-like region of the receptor (Vu et al., 1991a; Vu et al., 1991b).

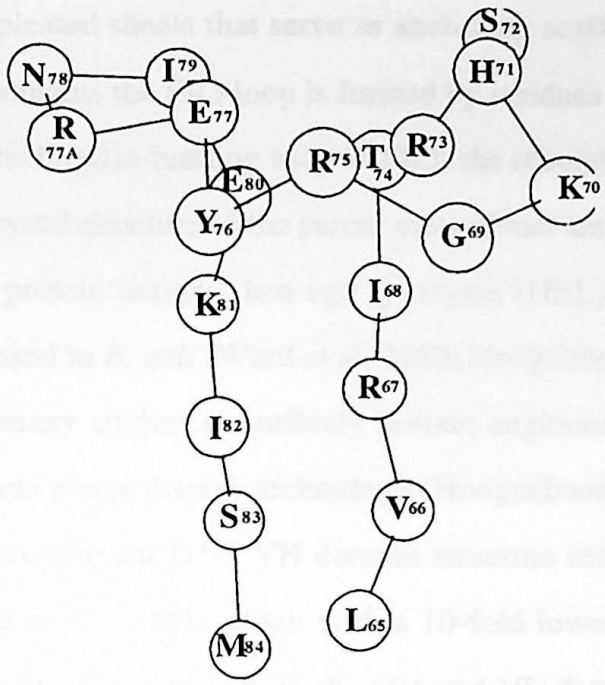
The thrombin receptor extracellular domain interacts with thrombin in a bidentate manner, with the protein C-like sequence LDPR bound to the active site of thrombin and the hirudin-like domain docked in the anion binding exosite of thrombin (Vu et al., 1991a). However, bidentate docking was not observed in crystal structures of thrombin complexes

with synthetic thrombin receptor peptides (Mathews et al., 1994). Instead, an intermolecular binding mode was revealed in which the hirudin-like sequence docked in the expected manner to the exosite of one thrombin enzyme and the protein C-like sequence docked in an unusual non-productive mode with the catalytic centre of a neighbouring thrombin molecule. This manner of binding is probably an artefact produced in the formation of stable thrombin-peptide complex crystals (Mathews et al., 1994).

Maraganore et al. (1992) have shown that a synthetic peptide corresponding to an important surface loop from the anion-binding exosite-1 (L^{65}VRIGKHSRTRYERNIEKIS^{83}) is a thrombin receptor antagonist. This peptide presumably blocks thrombin's binding to and subsequent activation of the thrombin receptor. The IC_{50} for the synthetic linear peptide was 200 μM and the generation of a cyclized peptide resulted in a ten-fold increase in blocking thrombin-induced platelet aggregation (Maraganore et al., 1992). A further increase in potency may be possible if this loop were presented in a conformation resembling the native thrombin loop, with the increase in affinity compared with a simple cyclized peptide, the chimeric protein may provide a novel anti-thrombotic agent.

Sites at the ends of this thrombin hirudin-binding loop (Figure 6.1a), resembling hairpin loops present in highly conserved immunoglobulin structures, were searched for using the available molecular modelling programs. A suitable candidate to graft this thrombin loop was the β-turn loop of the framework region three domain (FR3) of the immunoglobulin variable heavy chain. (Figure 6.1b). This was performed by Dr. A. M. Lesk (Department of Haematology, University of Cambridge, UK). Antibodies have loops at the antigen binding tip of each variable domain, made up by residues from the complementarity determining regions (CDRs) (Figure 4.2). The structural diversity of the CDR loops through length and sequence variation results in diverse binding surfaces for antigens (Chothia and Lesk, 1987) and thus determines the specificity of a particular antibody. In contrast, the loop linking the anti-parallel β-strands in FR3 has a fixed length in either VH or VL domains (being two residues shorter in VL as opposed to VH) and has been considered by Chothia and Lesk (1987) to be part of the conserved framework

(a)



(b)

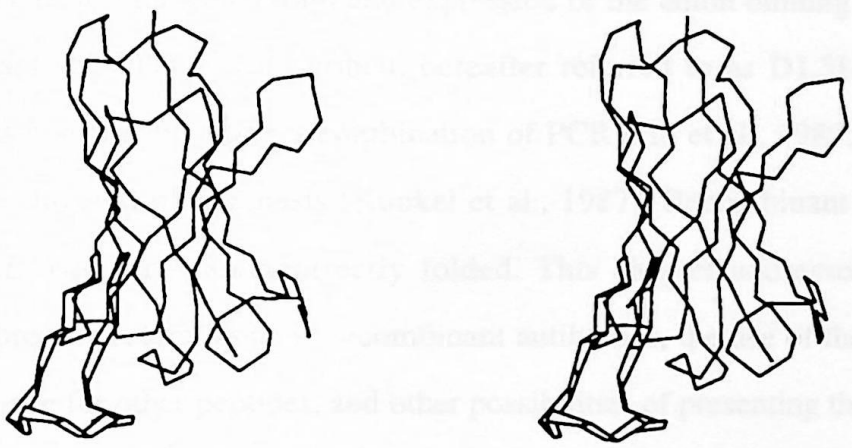


Figure 6.1
(a) *Schematic representation of the overall fold of the hirudin-binding loop from the thrombin anion-binding exosite.* Amino-acid residues are indicated by the single letter code and are numbered using chymotrypsin notation.
(b) *Stereodiagram of a single antibody variable domain with the integrated thrombin loop.* The variable heavy domain is shown as a single black line, with the grafted thrombin loop shown as a ribbon, integrated in the β-turn loop of the framework region three domain (FR3) at the bottom of the antibody. The VH CDR antigen binding loops H1, H2 and H3 are at the top of the diagram.

structure of two β–pleated sheets that serve as anchoring scaffolds for attachment of the CDR loops. In VH domains the FR3 loop is formed by residues 72-77 (Kabat, 1987).

To express the hirudin-binding loop in FR3, the recombinant scFv antibody D1.3 was chosen, as the crystal structure of the parent monoclonal antibody has been determined in complex with its protein antigen, hen egg lysozyme (HEL) (Amit et al., 1986). D1.3 scFv has been expressed in *E. coli* (Ward et al, 1989; Hoogenboom et al., 1991) and it has been the subject of many studies in antibody protein engineering such as CDR grafting (Jones et al., 1986) and phage display technology (Hoogenboom et al., 1991; Mc Cafferty et al., 1990). The recombinant D1.3 VH domain structure retains the ability to bind its target antigen (Ward et al., 1989), albeit with a 10-fold lower affinity compared to the corresponding scFv which contains both the VH and VL domains linked together by a flexible polypeptide linker (Glockshuber et al., 1990, Appendix 4).

In this chapter, the generation and expression of the anion binding exosite loop in the recombinant scFv D1.3 is described, hereafter referred to as D1.3Loop. The scFv D1.3Loop was constructed using a combination of PCR (Ho et al., 1989; Higuchi et al., 1988) and site directed mutagenesis (Kunkel et al., 1987). Recombinant D1.3Loop was expressed in *E. coli* but was incorrectly folded. This chapter addresses the aspect of engineering foreign protein loops in recombinant antibodies, the use of the FR3 loop as a potential graft site for other peptides, and other possibilities of presenting the anion-binding exosite loop in an antibody structure.

6.2 Results

6.2.1 Recombinant construction of D1.3Loop using PCR

The anion binding exosite loop DNA sequence (Appendix 3) was amplified using amplitaq (section 2.2.6) from a cDNA clone of the human prothrombin gene using the oligonucleotide primers T1 forward and T2 reverse (Appendix 2). This generated a 101 bp DNA fragment (Figure 6.2a), incorporating the anion binding exosite loop (60 bp), flanked on each side by D1.3 nucleotide sequence identical to the desired insertion site for the thrombin loop. This identical nucleotide region is shown underlined in the D1.3 DNA sequence, with the thrombin loop insertion site labelled in bold type in Appendix 4. Ten primary PCR amplifications were performed, and the pooled 101 bp product was gel-purified (section 2.2.3) before use in a second round PCR. Approximately 50 ng of this 101 bp product was used as one oligonucleotide primer, in two separate PCRs using 5 ng of the scFv D1.3 DNA sequence obtained in a pUC119 plasmid as the template. The other oligonucleotides used with the common 101 bp fragment in two separate PCR reactions where, MF10 and MF20 (Appendix 2). The priming sites for these two oligonucleotide primers are shown in Appendix 3. Two final products with the common 101 bp DNA sequence were generated. Using primers MF10 and MF20 fragments of 429 bp (Frag1) and 674 bp (Frag2) were generated (Figure 6.2b).

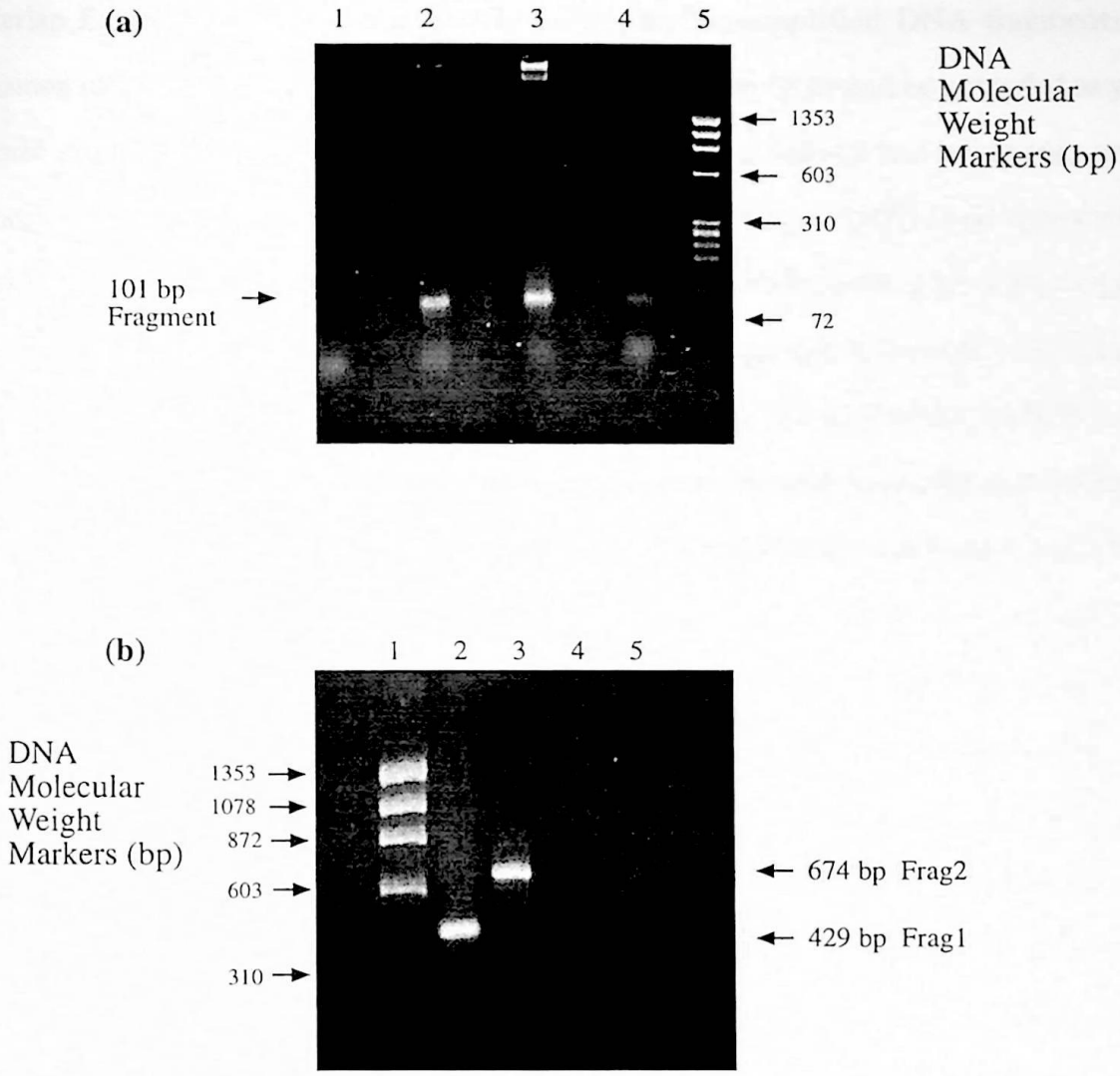


Figure 6.2
(a) *2% agarose gel showing the 101 bp DNA PCR fragment.*
Lane 1, PCR negative control containing no template DNA. Lane 2, PCR using 5 ng of template DNA. Lane 3, PCR using 100 ng of template DNA. Lane 4, PCR using 0.1 ng of template DNA. Lane 5, ΦX174-Hae III digest double stranded DNA molecular weight markers (bp); 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

(b) *1.5% agarose gel showing the PCR fragments Frag1 and Frag2.*
Lane 1, ΦX174-Hae III digest double stranded DNA molecular weight markers (bp); 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. Lane 2, Frag1 429 bp. Lane 3 Frag2 674 bp. Lane 4, Frag1 PCR negative control containing no template DNA. Lane 5, Frag2 PCR negative control containing no template DNA.

The two separate fragments were gel purified and mixed together in a PCR "Splicing by Overlap Extension" (SOE) reaction. In SOE, the two amplified DNA fragments with common identical sequences at one end can prime on each other and be extended to yield a hybrid product. So that if they are purified, mixed and reamplified using two primers annealing at the non-complementary ends, the hybrid will be amplified and can be cloned. This general principle can be used to rejoin two halves of the same gene with a sequence change or an insertion incorporated in the overlap (see Figure 6.3, Ho et al., 1989; Higuchi et al., 1988). The SOE reaction was performed (section 2.2.6). 100 ng of both purified DNA fragments (Frag1 and Frag2) were mixed together and subjected to 10 rounds of amplification, after which the outside primers MF10 and MF20 were added, and a further 20 rounds of amplification were performed.

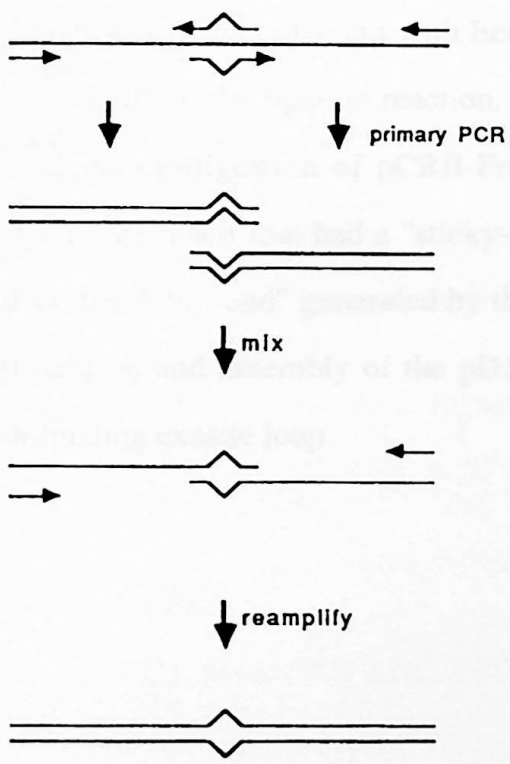


Figure 6.3 *Pictorial description of "Splicing by Overlap Extension" (SOE).*
Two rounds of primary PCR are performed with a common identical sequence at each end. These are then isolated, mixed and reamplified with the two primers annealing at the non-complementary ends, resulting in a final hybrid product.

6.2.2 Assembly of the D1.3Loop construct through step-wise cloning

As the SOE reaction did not generate any full length product, a stepwise cloning approach was used to assemble the two fragments together. The two DNA products Frag1 and Frag2 (section 6.2.1) were cloned using the 3' A-overhangs, generated by amplitaq in a non-template dependent fashion during the PCR process, into plasmid pCRII, a commercial 'T'-vector bearing a single thymine 5'-overhang as described in section (2.2.1 - 2.2.4), (Appendix 5; DNA sequence of pCRII, Holton and Graham, 1991; Marchuk et al., 1991). This generated vectors, pCRII-Frag1 and pCRII-Frag2. To assemble the two fragments, use was made of the common EcoR V restriction site in the introduced thrombin loop, present in both Frag1 and Frag2.

Frag1 was subcloned from pCRII-Frag1 into a new pUC119 vector as a Hind III - EcoR I insert using the Hind III site present within the 5' end of the Frag1 insert and an EcoR I site at the opposing end within the pCRII vector polylinker sequence. This newly created vector pUC119-Frag1 was then double cut with EcoR V and EcoR I. This double cut vector was gel purified and used in a ligation reaction, with a Frag2 insert which had been obtained by a corresponding digestion of pCRII-Frag1 with the same restriction enzymes. The ligation of a Frag2 insert that had a "sticky-end" overlap generated by the EcoR I digestion as well as the "blunt-end" generated by the EcoR V restriction enzyme, permitted the successful ligation and assembly of the pD1.3Loop plasmid encoding the integrated thrombin anion-binding exosite loop.

6.2.3 Mutagenesis of PCR introduced stop codon

After the step-wise cloning assembly, the resulting plasmid D1.3Loop construct was sequenced (section 2.2.5) which revealed a PCR induced error. Codon AGA which encodes for an arginine (nucleotide bp number 295 in Appendix 4) had been mutated to a stop codon TGA (Figure 6.4). Consequently, the plasmid was transformed into the *E. coli* strain CJ236, single stranded uracil template prepared and site directed mutagenesis was performed according to section (2.2.5).

Normal wild-type sequence:

N S A L K S R L S I S K D
5'-AAT TCA GCT CTC AAA TCC AGA CTG AGC ATC AGC AAG GAC-3'

Sequence with PCR induced stop codon:

N S A L K S **Stop** L S I S K D
5'-AAT TCA GCT CTC AAA TCC **T**GA CTG AGC ATC AGC AAG GAC-3'

Sequence with annealed MUTSTOPGO3 mutagenesis primer:

N S A L K S **Stop** L S I S K D
5'-AAT TCA GCT CTC AAA TCC TGA CTG AGC ATC AGC AAG GAC-3'
3'-G TTT AG**A** **T**CT GAC TCG TAG-5'

New mutated sequence with Xba I site

N S A L K S R L S I S K D
5'-AAT TCA GCT CTC AAA **TCT AGA** CTG AGC ATC AGC AAG GAC-3'

Figure 6.4 *Outline of site-directed mutagenesis strategy of the PCR induced stop codon using the MUTSTOPGO3 oligonucleotide primer.* The amino acid residues are indicated by the single letter code. The nucleotide region to which the primer anneals to is shown together with the final corrected pD1.3Loop sequence incorporating a new Xba I restriction site.

The MUTSTOPGO3 primer was designed to both revert the stop codon back to the original arginine and simultaneously introduce a unique Xba I site which would only be present in the corrected plasmid. The presence of an Xba I site was used as an efficient screen for the desired mutants. Figure 6.5 shows the results obtained. Digestion of the plasmid with Xba I and Xho I generated a DNA fragment of 567 bp in the mutated plasmid. The plasmids were sequenced to check that both the newly introduced mutation and the rest of the DNA sequence was correct (Figure 6.6).

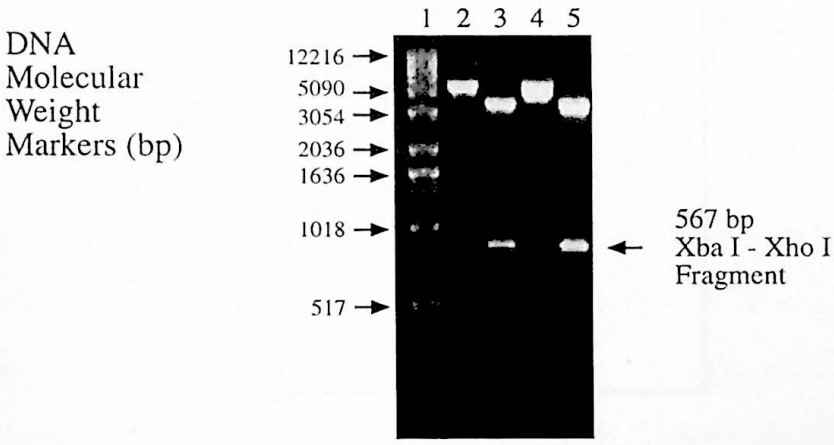


Figure 6.5 *1% agarose gel showing the Xba I -Xho I double restriction digests of selected number of pD1.3Loop plasmid mini-prep clones.*
Lane1, 1 Kb ladder double stranded DNA molecular weight markers (bp); 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517.
Lane 2, miniprep-1 cut with Xba I. Lane 3, miniprep-1 cut with Xba I and Xho I. Lane 4, miniprep-2 cut with Xba I. Lane 5, miniprep-2 cut with Xba I and Xho I.

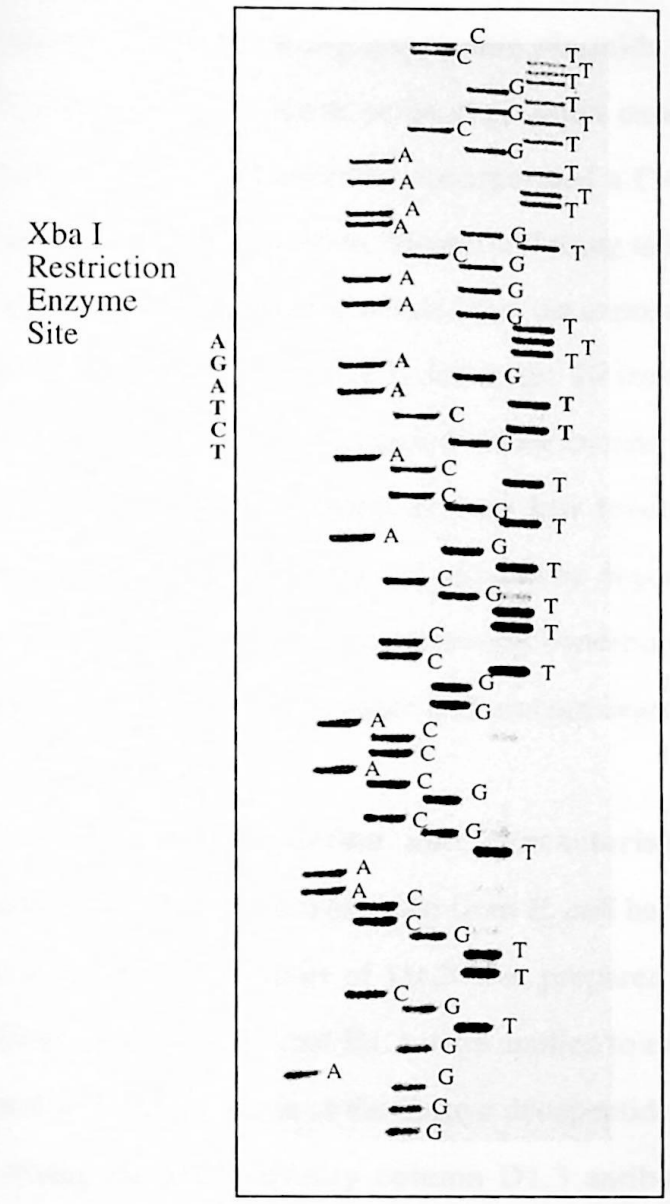


Figure 6.6 *DNA sequence of the pD1.3Loop plasmid with the introduced Xba I restriction site*. The sequence shown, reading from top to bottom is the lower non-coding strand with the Xba I restriction site highlighted in bold type. This can be translated into the corresponding amino acid coding-strand, with part of the integrated anion-binding exosite-1 loop indicated in bold type above the DNA sequence:

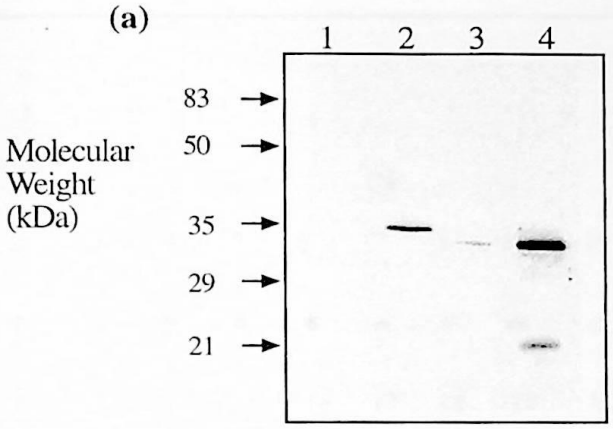
```
    G   N   T   D   Y   N   S   A   L   K   S   R   L   S   I   S
5'-GGA AAC ACA GAC TAT AAT TCA GCT CTC AAA TCT AGA CTG AGC ATC AGC

    K   D   L   V   R   I   G   K   H   S
  AAG GAC CTG GTG CGC ATT GGC AAG CAC TCC-3'
```

6.2.4 Expression of D1.3Loop

Both D1.3 and D1.3Loop expression plasmids were transformed into the *E. coli* strain XL1-Blue F' and a small scale expression experiment was carried out (section 2.3.3). Both recombinant antibodies incorporated a C-terminal C-myc tag that could be used for detection and purification. Western blotting using the 9E10 monoclonal antibody to the C-terminal C-myc tag demonstrated that the expressed recombinant D1.3Loop protein was slightly larger than D1.3. This is due to the 20 residues that have been introduced by the incorporation of the thrombin anion binding exosite loop. The scFv D1.3 antibody was secreted into the periplasm, albeit at very low levels compared to that found in the cytoplasmic preparation. Some sign of proteolytic degradation of the cytoplasmic fraction was also observed. Using identical expression conditions, the scFv D1.3Loop protein was not secreted into the periplasmic space and was not degraded (Figure 6.7a).

6.2.5 Large scale expression and characterisation of D1.3Loop

A large scale (2 l) preparation was made from *E. coli* harbouring the D1.3Loop plasmid. As a control a similar preparation of D1.3 was prepared (section 2.3.3). Clarified *E. coli* lysates (20 ml) of D1.3Loop and D1.3 were applied to either of two affinity matrices: 9E10 monoclonal antibody directed to the C-myc decapeptide tag or a hen egg lysozyme (HEL) column. Using the HEL affinity column D1.3 antibody was affinity purified, but no D1.3Loop protein was detected in the final eluted fraction; but D1.3Loop protein could be however detected in the original *E. coli* lysate using Western blotting (Figure 6.7b). A similar result was obtained using the 9E10 column; dot-blots were performed on the fractions. D1.3 bound to the column as immunoreactive material was eluted (Figure 6.8a). In contrast, D1.3Loop did not bind to the 9E10 column and was found in the flow-through fractions (Figure 6.8b).



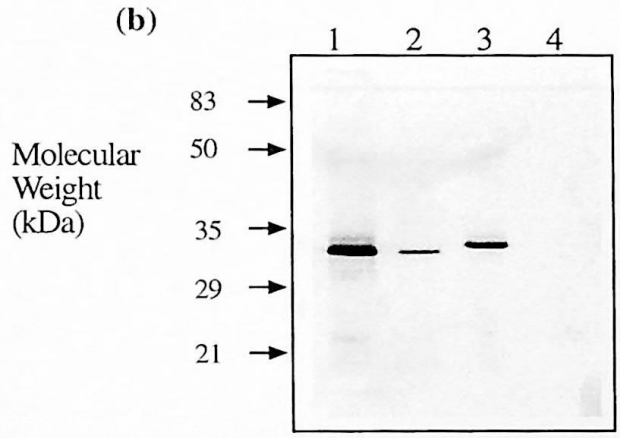
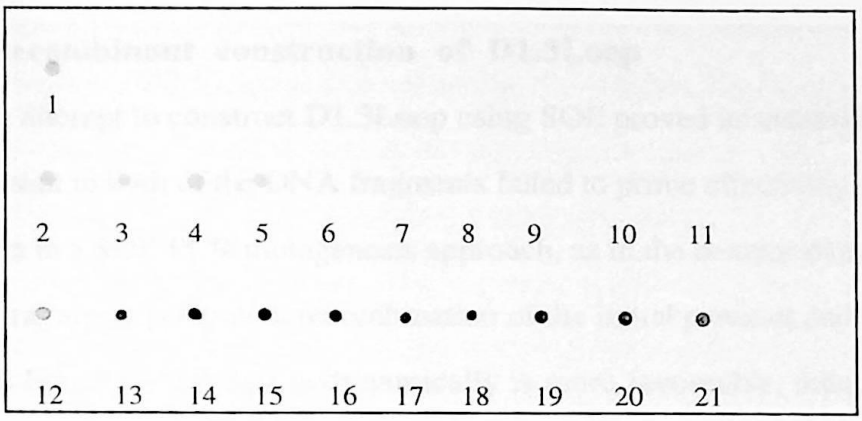


Figure 6.7
(a) *Western blot of the small scale expression of D1.3 and D1.3Loop from 10 ml of E. coli XL1-Blue F'*. 50 µl samples were loaded on a 12% SDS-PAGE gel. Lane 1, D1.3Loop periplasmic fraction. Lane 2, D1.3Loop cytoplasmic fraction. Lane 3, D1.3 periplasmic fraction. Lane 4, D1.3 cytoplasmic fraction. The position of the molecular weight markers are indicated.

(b) *Western blot of the affinity purification on the hen egg lysozyme affinity column from the large scale expression of D1.3 and D1.3Loop*. 50 µl samples have been loaded each on a 12% SDS-PAGE gel. Lane 1, clarified D1.3 *E. coli* lysate before application to the column. Lane 2, eluted D1.3 fraction. Lane 3, clarified D1.3Loop *E. coli* lysate before applied to the column. Lane 4, eluted D1.3Loop fraction. The position of the molecular weight markers are indicated.

(a)



(b)

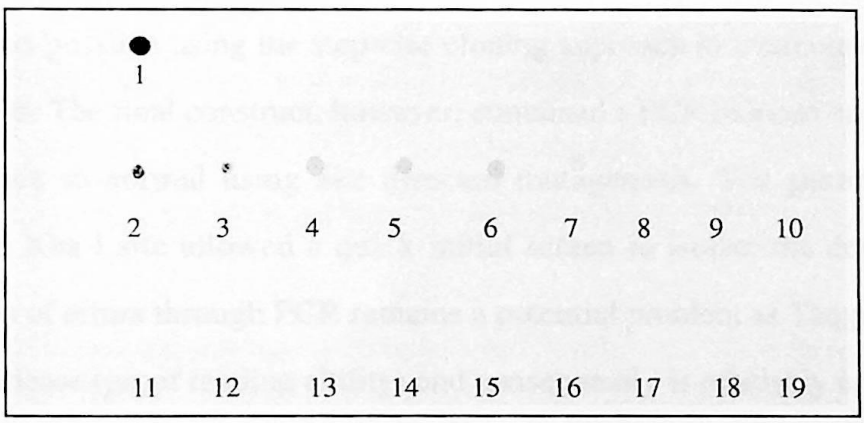


Figure 6.8
(**a**) *D1.3 dot-blot of fractions from the 9E10 affinity column.*
(**b**) *D1.3Loop dot-blot of fractions from the 9E10 affinity column.*

In each case (Figure 6.8a and 6.8b), 5 µl of each 5 ml fraction was applied to the membrane and are numbered underneath the position of the dots which correspond to the following; 1= clarified lysate before applied to the column; 2 - 8= column pass through and initial (PBS plus 0.5 M NaCl) wash fractions; 9 - 11= final washes with PBS before subsequent elution; 12 - 21= column elution fractions with 0.2 M glycine-HCl pH 2.5.

6.3 Discussion

6.3.1 Recombinant construction of D1.3Loop

The attempt to construct D1.3Loop using SOE proved unsuccessful, as the common overlaps present in both of the DNA fragments failed to prime effectively on each other. This is a problem in a SOE PCR mutagenesis approach, as in the denaturation and mixing of the two DNA fragments generated, recombination of the initial parental duplexes of both Frag1 429 bp and Frag2 674 bp thermodynamically is more favourable, than the formation of a single 101 bp duplex overlap between Frag 1 and Frag2. The generation of fragments 1 and 2 as single stranded DNA would enable only the common 101 bp sequence to anneal. Single stranded DNA can be produced by asymmetric PCR (Gyllensten and Erlich, 1988). Other methods for generating single-stranded DNA have also been described. These include strand specific lambda exonuclease digestion (Higuchi and Ochman, 1989) and fractionation based upon biotinylated primers (Mitchell and Merril, 1989).

It was possible using the stepwise cloning approach to overcome the failure of the SOE reaction. The final construct, however, contained a PCR induced error. This error was reverted back to normal using site directed mutagenesis. The generation of a newly constructed Xba I site allowed a quick initial screen to isolate the desired mutant. The introduction of errors through PCR remains a potential problem as Taq polymerase lacks a 3'-5' exonuclease (proof reading ability) and consequently is relatively error-prone (Tindall and Kunkel, 1988). Repeated rounds of amplification, which are necessary in a SOE procedure, can result in a high error rate, 1 misincorporation per 1800 bp amplified (Horton et al., 1989). These problems can be minimised by using thermostable proof-reading enzymes such as Vent polymerase (New England Biolabs) and Pfu polymerase (Stratagene, Cambridge, UK). These enzymes reportedly have a six-fold increase in cumulative fidelity in PCR over Taq polymerase, which is attributed to the presence of 3'-5' exonuclease activity. But this proof-reading ability can sometimes hinder the amplification of DNA templates with oligonucleotide primers that bear nucleotide mismatches, or extra 5' overhangs.

6.3.2 Expression and characterisation of D1.3Loop

As the thrombin anion-binding exosite loop was engineered within the highly conserved antibody FR3 region and not within the antigen binding site, it was envisaged that the recombinant scFv D1.3Loop would still retain its ability to bind to its target antigen hen egg lysozyme (HEL). This was not the case and the D1.3Loop could not be readily affinity purified on a HEL column. D1.3Loop also did not bind to a 9E10 monoclonal antibody affinity column. This demonstrated that D1.3Loop was expressed as a full length protein, but folded in a different conformation, with the C-terminal decapeptide tag not freely exposed in solution and unable to bind to the 9E10 affinity matrix. Yet under the denaturing conditions of Western blotting the C-myc tag was clearly present. Correctly folded recombinant scFv fragments have been solubly expressed in *E. coli* (Pluckthun, 1991a, 1991b). Expression of scFv protein resulting as bacterial inclusion bodies, requiring to be refolded to regain their functional activity has also been described (Gibbs et al., 1991; Power et al., 1992). D1.3Loop, although inactive, was not formed as inclusion bodies, but expressed in the soluble *E. coli* fraction as an incorrectly folded protein. It is clear that the presence of the 20 residue insertion disrupts the normal folding pathway of the D1.3Loop scFv , leading to a thermodynamically stable, but differently folded soluble protein.

Experiments performed by Knappik and Pluckthun (1995) demonstrated that point mutations at the β-turns within a recombinantly expressed *E. coli* scFv either improved or reduced its *in vivo* folding and final yield of functional antibody. The presence of the high density of positive charges in the thrombin loop at the β-turn of FR3 could interfere with folding by making ionic interactions with negatively charged residues present in the D1.3Loop scFv. These electrostatic interactions could disrupt the normal charge interactions that play a role in the scFv folding process leading to a stable, trapped, scrambled intermediate which is functionally inactive.

6.4 Conclusion

The expression of the thrombin-binding exosite loop within the FR3 region of D1.3 proved unsuccessful. This was attributed to the final mutated antibody protein being improperly folded due to the presence of the 20 amino acid insertion loop.

Chapter 7

General Discussion

7.1 Monoclonal antibodies to the human thrombin receptor

The ability of monoclonal antibodies 2/389 and 3/17 to inhibit thrombin-induced platelet aggregation suggests their potential use as anti-platelet agents for the treatment of cardiovascular disease. In the process of cardiovascular disease, damage due to an atherosclerotic plaque results in exposure of adhesive glycoproteins in the sub-endothelial matrix. Platelets can adhere to this matrix, via receptors for these glycoproteins. This adhesion results in platelet activation and induces a conformational change in the glycoprotein II_b/III_a receptor ($GPII_b/III_a$) that allows high affinity binding to fibrinogen and von Willebrand factor with consequent platelet aggregation. A monoclonal antibody directed to $GPII_b/III_a$ (7E3) has been used to block platelet activation (Coller et al., 1991). A recombinant chimeric Fab version of this monoclonal antibody, named c7E3, containing the mouse variable regions grafted onto a human constant region, has proved successful in both Phase II and Phase III clinical trials. Platelet aggregation is profoundly inhibited when increasing concentrations of mouse monoclonal or recombinant humanised c7E3 Fab are added to platelet-rich plasma *in vitro,* or when administered to animals or humans *in vivo* (Tcheng et al., 1994; Simoons et al., 1994).

In addition, prevention of platelet activation would decrease thrombin generation by reducing the available surface area for the generation of active thrombin from prothrombin, so acting as useful clinical anticoagulants. An anti-thrombin receptor polyclonal antibody proved effective in the prevention of intravascular thrombus formation, evaluated with an *in vivo* model of platelet- dependent cyclic flow reduction in the carotid artery of the African green monkey (Cook et al., 1995), without affecting coagulation or bleeding times. Thus, in a primate model of arterial thrombosis, blockage of the platelet thrombin receptor prevents arterial thrombus formation, without altering other haemostatic parameters (Cook et al., 1995). This can be achieved without inhibiting the enzymatic activity of thrombin for

fibrinogen, therefore reducing bleeding complications which are frequently observed with existing anti-thrombotic therapies, such as heparin treatment.

Epitope mapping of the three mouse monoclonal antibodies revealed that the IgA antibody 3/138 binds to the receptor on the N-terminal side of the thrombin cleavage site. Although 3/138 did not prevent thrombin-induced platelet aggregation it could be used to distinguish between cleaved and uncleaved thrombin receptors; after cleavage by thrombin or other proteases this epitope would be lost. This antibody could be useful in the assay of cleaved versus intact receptors, with various applications including the quality control of platelet preparations.

An affinity column prepared using monoclonal antibody 2/389 was successfully used for the immunoaffinity purification of native platelet thrombin receptor. To date, only recombinantly engineered antibody epitope tags have been used for the immunoprecipitation of recombinantly expressed receptor (Hein et al., 1994; Vouret-Craviari et al., 1995b). The resulting purified platelet thrombin receptor could be subjected to N-terminal amino acid sequencing to determine if the sequence corresponds to ARRPESK as deduced from the cDNA clone (Vu et al., 1991a). To date, this N-terminal amino acid sequence has not been confirmed with native protein. The immunoaffinity purified platelet thrombin receptor could be used as an antigen for the generation of either improved polyclonal antibodies or monoclonal antibodies to the whole receptor. When purified receptor is used, every part of the immunogen may induce a response and antibodies may be directed to epitopes located in the extracellular, transmembrane, or intracellular portions. The resulting hybridoma supernatants could be screened either by Western blotting to the purified native receptor. Alternatively, since recombinant thrombin receptor can be functionally expressed in Sf9 insect cells, which normally do not express the native receptor (Chen et al., 1996), a rapid screen to identify monoclonal antibodies that bind to Sf9 thrombin receptor expressing cells and not to wild-type Sf9 cells could be employed. Monoclonal antibodies could be further screened with a functional assay to identify antibodies that directly block agonist peptide binding, and might lead to the identification of a specific thrombin receptor blocking agent.

The opposite is possible, where antibodies directed to the binding site may mimic the agonist peptide binding and result in receptor activation.

7.2 DNA sequencing of the monoclonal antibodies

The cloning and cDNA sequencing of the antibody VH and VL domains allows the further opportunity for the construction, expression and characterisation of corresponding single chain Fv fragments (scFv). Complete antibodies (150 kDa) are large molecules, but the much smaller scFv fragments (35 kDa) retain their antigen binding ability, and are attractive for use *in vivo* as they penetrate tissue boundaries more effectively (Yokota et al., 1992). The construction of scFvs of 3/17 and 2/389 would permit the mapping by site directed mutagenesis of critical CDR amino acid residues involved in binding TRED. The use of phage display technology, combined with random mutagenesis, could be used to improve the antibody affinity for TRED.

Although scFvs or single VH domains (Ward et al., 1989) may be the smallest antigen binding species, it has been noted that some single CDR peptides ('minimal recognition units') retain antigen binding capabilities (Taub et al., 1989; Williams et al., 1989; Sivolapenko et al., 1995; Levi et al., 1993). With the knowledge of the antibody CDR loops involved in binding to the human thrombin receptor, synthetic peptides, either linear or cyclized, could be synthesised and tested as possible thrombin receptor antagonists. The most likely candidate CDR loop that retains antigen binding affinity is the H3 CDR loop (Sivolapenko et al., 1995; Levi et al., 1993). This could reflect the potential high diversity of this CDR, as its derivation is from D, J and N immunoglobulin segments (Yancopoulos, 1986).

Knowledge of the antibody CDR loops permits the further construction of "humanised antibodies" (Winter and Milstein, 1991) for anti-thrombotic therapy. The direct use of a rodent mAb in clinical therapy elicits an immune response to the rodent framework. Rodent antibodies have been extensively manipulated to resemble more fully human antibodies. This process, known as CDR grafting (Jones et al., 1986), involves the re-design of the variable region domain so that the amino acids constituting the rodent binding

site are integrated into the framework of a human antibody. This humanisation process both decreases the immune response and increases the plasma retention time of the antibody (Begent et al., 1990). This could permit use of a humanised anti- thrombin receptor antibody for long term anti-thrombotic therapy. For short term clinical therapy the construction of a single chain Fv is desirable, as they have a relatively short plasma half-life and are rapidly excreted by the kidneys compared to larger antibodies (Yokota et al., 1992).

7.3 Characterisation of PAR-2

The use of a multiple antigenic peptide (MAP) antigen to generate rabbit anti-hPAR-2 antibodies was successful, as the native hPAR-2 receptor could be recognised with the resulting affinity purified antibodies. Mouse monoclonal antibodies, generated using a MAP peptide antigen to either the human or mouse PAR-2 receptor peptide sequences, would possibly be an important future prospect. To generate improved rabbit polyclonal antibodies, the PAR-2 extracellular domain from either the human or mouse receptor could be expressed as a recombinant GST fusion protein as previously undertaken with the thrombin receptor extracellular domain (Bahou et al., 1993a; Bouton et al., 1995; Parry, 1995).

Polyclonal antibodies to PAR-2 can be used to study of the tissue distribution of the receptor. In contrast to Northern analysis, immunohistochemistry can be used to identify which specific cell-type expresses PAR-2. In addition, antibodies to the activating site of PAR-2 will be useful in elucidating which response elicited by trypsin or other enzymes are due to activation of PAR-2. Studies with biotin-SKGR-CH_2Cl, highlighted two physiologically relevant enzymes, mast cell derived tryptase and acrosin, that may activate PAR-2 *in vivo*. These two serine proteases could be tested in conjunction with the polyclonal antibodies directed to the putative cleavage site of PAR-2, in order to investigate whether they elicit a similar response as that seen with with trypsin.

Antibodies directed to the PAR-2 cleavage site can be utilised in the detection of new protease-activated receptors. Previously, it was assumed there was only one protease activated receptor on the surface of HUVECs, with intracellular responses due to TRAP

stimulation resulting solely from activation of the thrombin receptor. It is apparent with the cloning and characterisation of PAR-2, that TRAP can bind and activate PAR-2. The combined use of anti-thrombin receptor and anti-PAR-2 antibodies, to simultaneously block both of these protease receptors, should permit the identification of as yet undiscovered protease activated receptors.

7.4 Construction and expression of a recombinant scFv antibody containing a thrombin loop

The introduction of the anion-binding exosite loop into a scFv antibody was unsuccessful, as the final recombinant antibody was produced in an incorrectly folded form. The presence of the thrombin loop interfered with the overall folding of the D1.3Loop scFv antibody, possibly due to the size or charge characteristics of the engineered loop. The effect of loop insertions in FR3 has been investigated by Simon and Rajewsky (1992). The presence of a four residue insertion within the β-turn of the FR3 VH domain of B1-8, a recombinant murine scFv antibody specific for the hapten 4-hydroxy-3-nitro-phenylacetyl, did not disturb folding or secretion of the mutant B1-8 antibody (Simon and Rajewsky, 1992). Thus, although the FR3 β-turn loop can accommodate small peptide insertions, larger insertions such as the 20 amino acid residue thrombin loop cannot be tolerated. Applications, using smaller insertions into the FR3 loop can be envisaged. The FR3 loop could serve as a potential peptide display site. The important cell adhesion motif Arg-Gly-Asp (RGD) has been expressed in an antibody CDR loop, with full activity (Zanetti et al., 1993; Lee et al., 1993). The results of Simon and Rajewsky (1992) suggest that this RGD sequence could be functionally incorporated into a FR3 loop.

The use of antibody CDR loops could be further explored as potential thrombin loop graft sites. The use of CDR loops has been explored in a number of applications, including the expression of the RGD peptide (Zanetti et al., 1993; Lee et al., 1993), display of known and random polypeptide libraries in a constrained conformation for epitope mapping (Sollazzo et al., 1990; Zanetti et al, 1992) and alpha-helical DNA binding domains (McLane et al., 1995). These studies have mainly focused on the use of the CDR3 loops of

either the VH or VL domain. With the possible engineering of the 20 amino acid thrombin loop into a CDR loop, an attractive graft site would be the long CDR1 loop present in the VL domain of the well characterised phosphorylcholine binding antibody McPC603 (Perlmutter et al., 1984). This antibody has been the subject of many protein engineering experiments (Pluckthun et al., 1987; Knappik and Pluckthun, 1995), and its atomic structure has been solved in detail (Steipe et al., 1992; Satow et al., 1986). However, engineering of a thrombin loop within the McPC603 antigen binding site will directly disrupt the mutant scFv binding to phosphorylcholine. The recombinant mutant scFv could be rapidly purified by metal chelate affinity chromatography using a hexa-histidine 'tag' engineered at the C-terminus of the McPC603 scFv (Skerra et al., 1991). With the advent of powerful selection systems based on the display of scFv fragments on the surface of filamentous phage (Clackson et al., 1991a), the scFv McPC603 loop mutant could be displayed and assayed for direct binding to the thrombin receptor. By a combination of random mutagenesis with rapid selection using phage display technology, the binding affinity of the thrombin loop could be improved.

Appendix 1

Description And Growth Requirements Of Haematopoetic Cell Lines

Murine cell lines were cultured in DMEM supplemented with 5-10% foetal calf serum (FCS); 200 mM L-glutamine, 100U/ml penicillin and 100 mg/ml streptomycin. Interleukin-3 dependent cell lines (Baf-3; A4) were cultured in RPMI supplemented with 10% FCS; 200 mM L-glutamine, 100U/ml penicillin, 100 mg/ml streptomycin, 50 μM 2 β-mercaptoethanol (2ME) and 10% (v/v) WeHi 3BD+ conditioned medium as a source of IL-3.

Cell Lines	Growth Media	Source/Reference
Murine Erythroid		
F4N	DMEM 5% FCS	(Dube et al., 1975)
J2E	DMEM 5% FCS	(Rapp et al., 1985; Klinken et al., 1988)
Murine Myeloid/Basophil		
Mst	DMEM 10% FCS	Dr. A. R. Green (Cambridge, UK)
P815	DMEM 10% FCS	(Dunn and Potter, 1957)
Murine Myeloid/Macrophage		
WeHi 274	DMEM 10% FCS	American Tissue Culture Collection (ATCC)
J774	DMEM 10% FCS	ATCC

Cell Lines	Growth Media	Source/Reference
Murine Myeloid		
416 B	DMEM 10% FCS	Dr. A. R. Green (Cambridge, UK)
416 BMEG	DMEM 10% FCS	Dr. A. R. Green (Cambridge, UK)
M1	DMEM 10% FCS	Dr. A. R. Green (Cambridge, UK)
WeHi 3BD+	DMEM 10% FCS	(Warner et al., 1969)
Murine T-cell		
BW 5174	DMEM 10% FCS	Dr. A. R. Green (Cambridge, UK)
BWN	DMEM 10% FCS	Dr. A. R. Green (Cambridge, UK)
Murine B-cell		
W231	DMEM 10% FCS	Dr. A. R. Green (Cambridge, UK)
NSO	DMEM 5% FCS	European Collection of Cell Cultures (ECACC)
Il-3 Dependent Cell Lines		
Baf-3	RPMI 10%FCS, 50 μM 2ME, 10% IL3	(Palacios and Steinmetz, 1985)
A4	RPMI 10%FCS, 50 μM 2ME, 10% IL3	Dr. A. R. Green (Cambridge, UK)

Appendix 2

Oligonucleotides

(In order of appearance)

Oligos used in the preparation of antibody cDNA by reverse transcription:

Heavy Chain

MCG12FOR	5'-CTC AAT TTT CTT GTC CAC CTT GGT GC
MCG3FOR	5'-CTC GAT TCT CTT GAT CAA CTC AGT CT
MCMFOR	5'-TGG ATT GGG CAC ATG CAG ATC TCT

Light Chain

MCKFOR	5'-CTC ATT CCT GTT GAA GCT CTT GAC
Oligo dT18	5'-TTT TTT TTT TTT TTT TTT

Oligos used in the generation of antibody primary PCR products:

Heavy Chains

MVH1FOR	5'-TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC
MVH1BACK	5'-AGG T(C/G) (A/C) A(A/G)C TGC AG(C/G) AGT C(A/T)G G

Light Chains

MJK1FOR	5'-CCG TTT GAT TTC CAG CTT GGT GCC
MJK2FOR	5'-CCG TTT TAT TTC CAG CTT GGT CCC
MJK4FOR	5'-CCG TTT TAT TTC CAA CTT TGT CCC
MJK5FOR	5'-CCG TTT CAG CTC CAG CTT GGT CCC

MVKABACK	5'-GAT GTT TTG ATG ACC CAA ACT CCA
MVKCBACK	5'-GAC ATT GTG CT(A/G) ACC CA(A/G) TCT CCA
MVKDBACK	5'-GAC ATC CAG ATG AC(T/C/G/A) CAG TCT CCA
MVKEBACK	5'-CAA ATT GTT CTC ACC CAG TCT CCA
MVKFBACK	5'-GAA AAT GTG CTC ACC CAG TCT CCA

Oligos used for PCR screening and DNA sequencing of inserts cloned in either pUC /M13 or pBluescript based vectors:

MF10 Forward	5'-TTG TAA AAC GAC GGC CAG TG
MF20 Reverse	5'-GGA AAC AGC TAT GAC CAT GAT

Oligos used in the construction of the recombinant thrombin loop swop antibody D1.3Loop:

T1 Forward	5'-GAC TGA GCA TCA GCA AGG ACC TGG TGC GCA TTG GCA AGC AC
T2 Reverse	5'-CAT TTT TAA GAA AAC TTG GCT G**GA TAT C**TT TTC AAT GTT TCG C
MUTSTOPGO3	5'-GAT GCT CAG **TCT AGA** TTT G

D1.3Loop sequencing primers:

MO-LINK-BACK	5'-GGG ACC ACG GTC ACC GTC TCC TCA
MO-LINK-FOR	5'-TGG AGA CTG GGT GAG CTC AAT GTC
T3 Forward Seq	5'-AGA CTG AGC ATC AGC AAG
T4 Reverse Seq	5'-TT TAA GAA AAC TTG GCT

Relevant Restriction sites are highlighted in bold type;

GATATC = EcoRV

TCTAGA = Xba I

Appendix 3

DNA sequence and corresponding amino acid sequence of the anion-binding exosite loop from human prothrombin

5'- CTG GTG CGC ATT GGC AAG CAC TCC CGC ACC
Leu Val Arg Ile Gly Lys His Ser Arg Thr

AGG TAC GAG CGA AAC ATT GAA AAG ATA TCC -3'
Arg Tyr Glu Arg Asn Ile Glu Lys Ile Ser

DNA sequence and restriction enzyme sites present in the multicloning site present in the pUC119/ M13mp 19 based plasmid vectors

MF10 Reverse
5'-G GAA ACA GCT ATG ACC ATG AT-3

[--HindIII ----][-
ACA CAG GAA ACA GCT ATG ACC ATG ATT ACG CCA AGC TTG
Met Thr Met Ile Thr Pro Ser Leu

--SpHI ----][----PstI------][-----SalI -------][----XbaI -----] [---BamHI SmaI KpnI --------] [-
CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG
His Ala Cys Arg Ser Thr Leu Glu Asp Pro Arg Val Pro

-----SacI --][----EcoRI ---]
AGC TCG AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT
Ser Ser Asn Ser Leu Ala Val Val Leu Gln Arg Arg -Lac Z β–
Galactosidase

3' -GT GAC CGG CAG CAA AAT GTT-5'
MF20 Reverse

Appendix 4

Sequence of scFv D1.3myc cloned in pUC119 as a SphI-BamHI Fragment

SphI **{-RBS-}** **[------PELB signal peptide----**

gca tgc aaa ttc tat ttc **aag gag** aca gtc ata atg aaa tac cta ttg cct acg gca gcc **60bp**

A C K F Y F K E T V I M K Y L L P T A A

----------] **PstI**

gct gga ttg tta tta ctc gct gcc caa caa gcg atg gcc cag gtg cag **ctg cag** gag tca **120bp**

A G L L L L A A Q P A M A Q V Q L Q W S

gga cct ggc ctg gtg gcg ccc tca cag agc ctg tcc atc aca tgc acc gtc tca ggg ttc **180bp**

G P G L V A P S Q S L S I T C T V S G F

tca tta acc ggc tat ggt gta aac tgg gtt cgc cag cct cca gga aag ggt ctg gag tgg **240bp**

S L T G Y G V N W V R Q P P G K G L E W

VHD1.3

ctg gga atg att tgg ggt gat gga aac aca gac tat aat tca gct ctc aaa tcc aga ctg **300bp**

L G M I W G D G N T D Y N S A L K S R L

Thrombin loop graft site

agc atc agc aag gac **aac tcc aag** agc caa gtt ttc tta aaa atg aac agt ctg cac act **360bp**

S I S K D **N S K** S Q V F L K M N S L H T

gat gac aca gcc agg tac tac tgt gcc aga gag aga gat tat agg ctt gac tac tgg ggc **420bp**

D D T A R Y Y C A R E R D Y R L D Y W G

BstEII **[----Gly^{4}Ser Linker Peptide------**

caa ggc acc acg **gtc acc** gtc tcc tca ggt gga ggc ggt tca ggc gga ggt ggc tct ggc **480bp**

Q G T T V T V S S G G G G S G G G G S G

----------] **SacI**

ggt ggc gga tcg gac atc **gag ctc** act cag tct cca gcc tcc ctt tct gcg tct gtg gga **540bp**
G G G S D I E L T Q S P A S L S A S V G

gaa act gtc acc atc aca tgt cga gca agt ggg aat att cac aat tat tta gca tgg tat **600bp**
E T V T I T C R A S G N I H N Y L A W Y

cag cag aaa cag gga aaa tct cct cag ctc ctg gtc tat tat aca aca acc tta gca gat **660bp**
Q Q K Q G K S P Q L L V Y Y T T T L A D

VKD1.3

ggt gtg cca tca agg ttc agt ggc agt gga tca gga aca caa tat tct ctc aag atc aac **720bp**
G V P S R F S G S G S G T Q Y S L K I N

agc ctg caa cct gaa gat ttt ggg agt tat tac tgt caa cat ttt tgg agt act cct cgg **780bp**
S L Q P E D F G S Y Y C Q H F W S T P R

XhoI **[----------cMyc Tag------**

acg ttc ggt gga ggc acc aag **ctc gag** atc aaa cgg gaa caa aaa ctc atc tca gaa gag **840bp**
T F G G G T K L E I K R E Q K L I S E E

----------] **Stop codons** **BamHI**

gat ctg aat **taa taa tga** tca aac ggt aat aa**g gat cc**a gct cga att **900bp**
D L N OCH OCH OPA S N G N K D P A R I

Index:

RBS= Ribosome binding site.

VHD1.3= variable heavy chain of the single chain antibody D1.3.

VKD1.3= variable light chain of the single chain antibody D1.3.

N S K = amino acid residues that were substituted

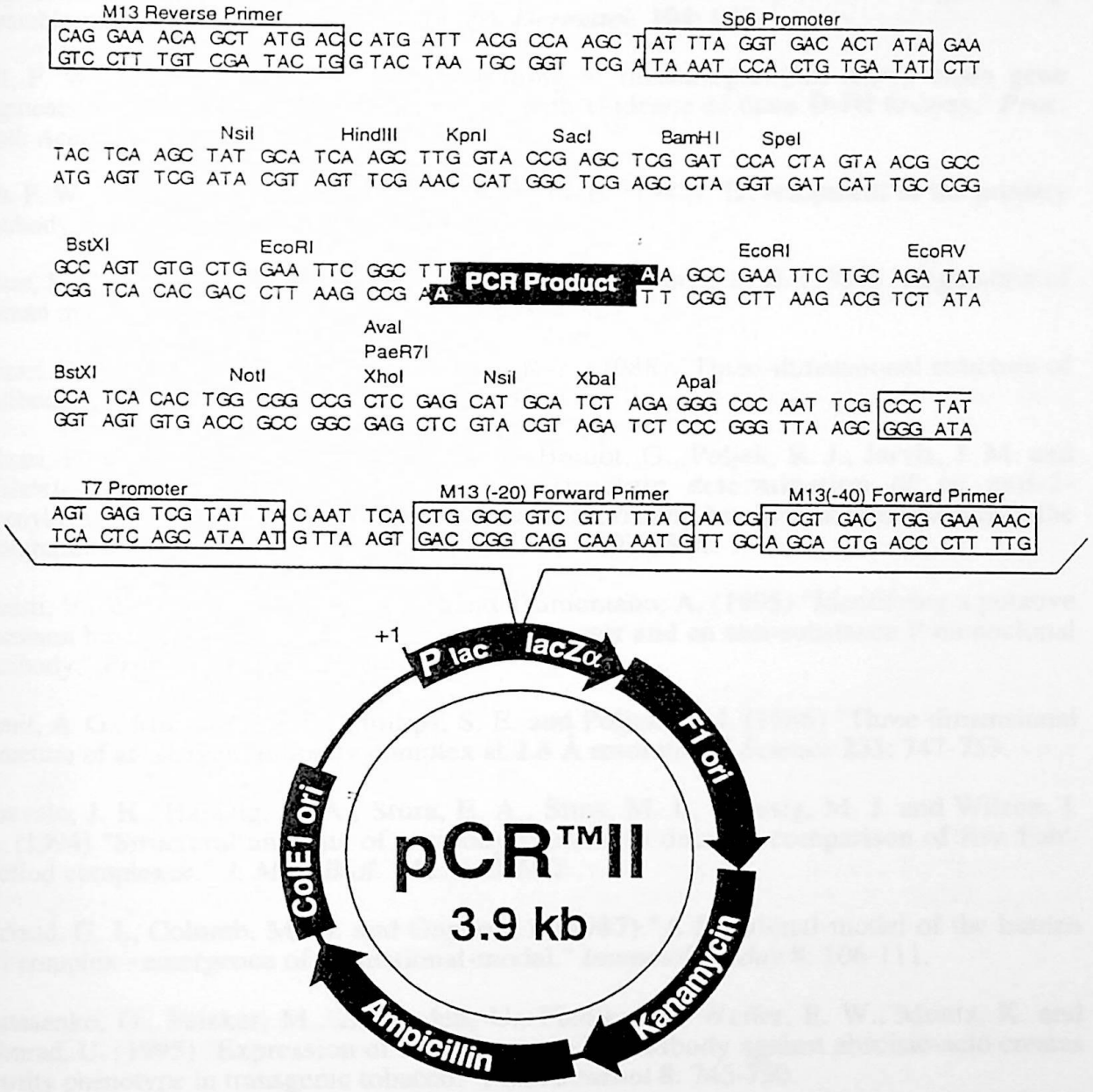
when the thrombin loop was grafted on to the scFV D1.3 framework.

Restriction enzyme sites are labelled above the relevant palindromic sequence

which is shown in bold type.

Relative size of DNA is indicated in basepairs (**bp**) at the end of each line

Appendix 5


M13 Reverse Primer
CAG GAA ACA GCT ATG AC
GTC CTT TGT CGA TAC TG
C ATG ATT ACG CCA AGC T
G TAC TAA TGC GGT TCG A
Sp6 Promoter
AT TTA GGT GAC ACT ATA GAA
TA AAT CCA CTG TGA TAT CTT
NsiI HindIII KpnI SacI BamHI SpeI
TAC TCA AGC TAT GCA TCA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTA ACG GCC
ATG AGT TCG ATA CGT AGT TCG AAC CAT GGC TCG AGC CTA GGT GAT CAT TGC CGG
BstXI EcoRI
GCC AGT GTG CTG GAA TTC GGC TT
CGG TCA CAC GAC CTT AAG CCG AA
PCR Product
A GCC GAA TTC TGC AGA TAT
T T CGG CTT AAG ACG TCT ATA
EcoRI EcoRV
AvaI
PaeR7I
BstXI NotI XhoI NsiI XbaI ApaI
CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA
T7 Promoter
AGT GAG TCG TAT TA
TCA CTC AGC ATA AT
C AAT TCA
G TTA AGT
M13 (-20) Forward Primer
CTG GCC GTC GTT TTA C
GAC CGG CAG CAA AAT G
AA CG
TT GC
M13(-40) Forward Primer
T CGT GAC TGG GAA AAC
A GCA CTG ACC CTT TTG
+1
P lac
lacZα
F1 ori
pCR™II
3.9 kb
ColE1 ori
Ampicillin
Kanamycin

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