J. Mol. Biol. (1994) **244**, 361–369

Isolation of a Peptide Antagonist to the Thrombin Receptor using Phage Display

John Doorbar and Greg Winter

MRC Centre for Protein Engineering
Hills Road, Cambridge, CB2 2QH, U.K.

The thrombin receptor on platelets is an integral membrane protein and is cleaved by thrombin to expose a "tethered ligand" that binds to and triggers the receptor. Here we have explored the power of phage selection technology to make a peptide antagonist of this receptor using platelets directly for the selection. To focus the selection to the thrombin receptor, we eluted the phage with a peptide agonist of the thrombin receptor. A repertoire (1×10^7 phage clones) displaying peptide sequences based on the sequence of the tethered ligand, was constructed and selected by binding to the platelets. After several rounds of selection, we identified phage clones that were able to immunoprecipitate the thrombin receptor from platelets and the encoded peptides were sequenced. This revealed some features in common with the tethered ligand, in particular an arginine residue followed by a proline. Several of the peptides were synthesized chemically and one of the peptides was shown to antagonise platelet aggregation triggered by the agonist peptide, and to inhibit serotonin release and tyrosine phosphorylation triggered by either thrombin or the agonist peptide. Anti-aggregatory activity was about ten-fold higher than that of previously reported peptide antagonists of the thrombin receptor.

Keywords: thrombin receptor; peptide antagonists; peptide fusion phage

1. Introduction

The display of peptides on filamentous bacteriophage by fusion to the N-terminus of the gIII or gVIII coat proteins (Smith, 1985; Greenwood *et al.*, 1991; Felici *et al.*, 1991), has provided the means for selecting peptides with many binding specificities from large repertoires (for a review see Hoess, 1993). For example, peptides have been selected that bind to antibodies (Scott & Smith, 1990) streptavidin (Devlin *et al.*, 1990), immunoglobulin binding protein (BIP†) (BlondElguindi *et al.*, 1993), ribonuclease S (Smith *et al.*, 1993) and the carbohydrate-binding protein concanavalin A (Scott *et al.*, 1992; Oldenberg *et al.*, 1992). The sequences of selected peptides have often proved to be related to each other (Stephen & Lane, 1992), and have helped identify a sequence "motif" required for binding. Sometimes the sequences have resembled the natural peptide ligand; those that have not have been termed mimotopes (Geysen *et al.*, 1986; Scott *et al.*, 1992; Smith *et al.*, 1993). It has been suggested that the technology should be capable of providing new peptide leads for agonists and antagonists of receptors (Cwirla *et al.*, 1990). Indeed the purified extracellular domains of the a_5b_1 fibronectin receptor (Koivunen *et al.*, 1993) and the $a_{IIb}b_3$ platelet fibrinogen receptor (O'Neil *et al.*, 1992) were used to select peptides related to the natural ligand fibronectin, with RGD motif. The selected peptides were able to antagonise integrin-mediated cell adhesion.

The receptors of the G-protein coupled receptor (GPCR) superfamily are of particular pharmaceutical interest. The members of this group all share a highly related structure; seven α-helical transmembrane domains form a ligand binding pocket within the plane of the membrane, and it has been estimated that 80% of all known receptors belong to this family (Bockaert, 1991), including the opiate/muscarinic, tachykinin, 5-hydroxytryptamine and thrombin receptors. Thrombin plays a critical role in haemostasis and thrombosis by activating the thrombin receptor on platelets, and is involved in the mediation of inflammatory responses to vascular injury (Walz *et al.*, 1986; Coughlin, 1993). Thrombin receptor antagonists might therefore help block inflammation and thrombosis.

Here we have tried to make an antagonist of the thrombin receptor. Thrombin cleaves the extracellular N-terminal domain of the thrombin receptor to create a new N-terminus which behaves as a tethered ligand in receptor activation (Vu *et al.*, 1991). The N-terminal six residues of the cleaved receptor are responsible for the agonist activity, and can be mimicked by a free hexapeptide of the same sequence (Vassallo *et al.*, 1992; Chao *et al.*, 1992). As the

Correspondence: John Doorbar. Present address: John Doorbar, Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, U.K.

† Abbreviations used; BIP, immunoglobulin binding protein; GPCR, G-protein coupled receptor; ELIZA, enzyme-linked immunosorbent assay.

0022-2836/94/490361-09 $08.00/0

receptor is an integral membrane protein and cannot be purified as with the integrin receptors, we used platelets for the selection of phages. To focus the selection to the thrombin receptor, we eluted the phages with the agonist peptide.

2. Materials and Methods

(a) *Construction of libraries and phage controls*

Control phage expressing either the SFLLRNP-NDKYE (thrombin receptor activating peptide), RPKPQQFFGLME (substance P), HKTDS-FVGLME (neurokinin A) or DMHDFFVGLME (neurokinin B) ligands were prepared by cloning oligonucleotide pairs between the *Apa*LI and *Not*I sites of the display vector fdtetDOG (Hoogenboom *et al.*, 1991), or between the *Sfi*I and *Not*I sites of the vector fdtetSfi/Not. The pairs were 5'CGG CCA TGG CAT GCA TCT TTC CTA CTA AGA AAT CCG AAC GAT AAG TAC GAG CCG TTC CCA CCA CCA CCA GC 3' and 5' GGC CGC TGG TGG TGG GAA CGG CTC GTA CTT ATC GTT CGG ATT TCT TAG TAG GAA AGA TGC CAT GGC CT 3' (encoding SFLLRNPND-KYE); 5' T GCA CGT CCT AAA CCT CAA CAA TTT TTT GGT CTT ATG TAG CCA CCA CCA CCA GC 3' and 5'GGC CGC TGG TGG TGG TGG CTA CAT AAG ACC AAA AAA TTG TTG AGG TTT AGG ACG 3' (encoding RPKPQQF-FGLME); 5' T GCA CAC AAA ACT GAC AGC TTC GTC GGT CTA ATG TAG CCA CCA CCA CCA GC 3' and 5' GGC CGC TGG TGG TGG TGG CTA CAT TAG ACC GAC GAA GCT GTC AGT TTT GTG 3' (encoding HKTDSFVGLME) and 5' T GCA GAT ATG CAT GAT TTC TTC GTC GGT CTT ATG TAG CCA CCA CCA CCA GC 3' and 5' GGC CGC TGG TGG TGG TGG CTA CAT AAG ACC GAC GAA GAA ATC ATG CAT ATC TGC A 3' (encoding DMHDFFVGLME). The tethered ligand library (see Figure 1) was prepared in the vector fdtetSfi/Not following digestion with *Sfi*I and *Not*I. The oligonucleotide used for library preparation, 5' G TTG TTC CTT TCT ATG CGG CCC CAG CCG GCC ATG GCA $(NN(G/T))_6$ CCG AAC GAT AAG TAC GAG CCG TTC CCA CCA CCA CCA GCG GCC GCA GAA ACT GTT CGC GCG-CGC GAA CAG 3', encoded the random hexapeptide from a sixfold repeat of the codon NN(G/T), (where N represents any nucleotide) and had a ten base repeat at the C-terminal end which was able to form a hairpin structure following denaturation at 95°C and rapid reannealing at 4°C. The second strand was synthesised from the 3' end of the hairpin using the Klenow fragment of *Escherichia coli* DNA polymerase (as previously described (Sambrook *et al.*, 1989)), and the resulting double-stranded oligo was digested with *Sfi*I and *Not*I before being cloned into fdtetSfi/Not. Annealing, synthesis and enzyme digestion were monitored by polyacrylamide gel electrophoresis. A second hexapeptide library was provided by G.P. Smith, University of Missouri (Scott & Smith, 1990). The vector fdkanDOG was a gift from A. Griffiths, and had been constructed by removal of the tetracycline resistance gene from fdtetDOG (as a *Nsi*I fragment) followed by its replacement with a *Pst*I fragment derived from the vector pUC-4K (Pharmacia) which encodes kanamycin resistance.

(b) *Selection of competing peptides*

Platelets were obtained from the East Anglia Blood Transfusion service (Long Road, Cambridge, U.K.) on the day of bleeding, and were washed once at room temperature in PBS (135 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2), 10 mM EDTA, 0.25% (w/v) bovine serum albumin, before resuspension in 2% (w/v) dried milk (in PBS) at a concentration of 1×10^8 platelets/ml. A total of 2.5×10^{11} phage particles were mixed with 5×10^7 platelets and the volume was made up to 1 ml with PBS, 2% milk. Binding was allowed to proceed for one hour at room temperature with end over end rotation. The unbound phage were removed by five cycles of low speed centrifugation (5000 *g*, Eppendorf centrifuge) and washing with PBS, 2% milk. The resulting platelet/phage pellet was resuspended in 200 µl of PBS, 2% milk containing 1mM SFLLRNPNDKYE peptide and incubated at 37°C for 30 minutes. The eluted phage were recovered using *E. coli* TG1 as previously described (Hoogenboom *et al.*, 1991; Marks *et al.*, 1991) before being used in subsequent rounds of selection.

Each round of selection was monitored by the ratio of eluted to input phage, and by platelet ELISA. Washed platelets were suspended in PBS, 10 mM EDTA at a concentration of 1×10^8/ml before being added to ELISA plates (100 µl/well) which had been previously coated with poly L lysine hydrobromide. The ELISA plates were prepared by incubation in 0.1 mg/ml poly L lysine hydrobromide for 30 minutes at room temperature followed by three washes with PBS. Platelets were left to bind for 30 minutes at room temperature, after which unbound platelets were washed off, and those remaining were lightly fixed with PBS, 0.05% (v/w) glutaraldehyde for 15 minutes. Following five washes in PBS, the plates were blocked by incubation at 37°C with PBS, 2% milk before phage ELISA (McCafferty *et al.*, 1990). For competition ELISA, the fixed platelets were incubated with either thrombin or the SFLLRNP-NDKYE peptide (Sigma, Poole, U.K.) for 15 minutes at room temperature before the phage were added. (With thrombin, the plates were washed three times before phage was added.) Phage DNA templates were prepared for sequencing (Sanger *et al.*, 1977) as previously reported (Marks *et al.*, 1991).

(c) *Biological activity of selected peptides*

Peptides were synthesised by Neosystem (Strasbourg, France) or were obtained from Sigma (SFLLRNPNDKYE, RGDS, TKPR, VGSE: Poole, U.K.) or Bachem (MS, MM, SFLLRN; Bubendorf,

Switzerland). Platelet aggregation was performed on a PAP four channel platelet aggregometer using platelet rich plasma as described by Vassallo *et al.* (1992). Potential antagonists were added one minute before the addition of agonist, and the response was monitored for four minutes after this. Serotonin release was according to Chao *et al.* (1989, 1992), and tyrosine phosphorylation according to Vassallo *et al.* (1992) except that phosphotyrosine was detected using a rabbit polyclonal antibody (ICN Immunobiologicals, Cosa Mesa, CA).

Lactoperoxidase was used to radioactively iodate platelet surface proteins according to Harlow & Lane (1988) and platelet membranes prepared by differential centrifugation (Harman & Jamieson, 1985) were fragmented by sonication on ice (6 × 10 second bursts, miniprobe). For immunoprecipitation, phages were prepared as follows: clones were grown overnight in TG1 cells (100 ml); the bacteria were pelleted at 4000 *g* for ten minutes; 1/5th volume of 20% polyethylene glycol 6000, 2.5 MNa Cl was added to the supernatant; and after one hour at 4°C phage were pelleted (8000 *g*, 15 minutes) resuspended in 1 ml of PBS, filtered through an 0.45 mM sterile filter and titred (10^{12} TU/ml). Platelet membranes (50 μg) were incubated with 1×10^9 phage particles in PBS, 2% milk for one hour, and 100 μl (50 μl packed volume) of antiphage Dynabeads were added. (The anti-phage Dynabeads were prepared by coating anti-mouse Dynabeads (Dynal, Wirral, U.K.) with excess mouse anti-sheep antibody (250 μl of 1 mg/ml solution), followed by excess sheep anti-M13 antibody (a gift from Cambridge Antibody Technology) and then washed three times with PBS.) The trapped receptor on the Dynabeads was solubilised in gel sample buffer (1% SDS, 15 mM β-mercaptoethanol, 5% glycerol, 10 mM Tris-HCl, pH 8.8) and the proteins were analysed by Western blotting according to Harlow & Lane (1988), using the monoclonal antibody ATAP 138 which binds to the thrombin receptor (Brass *et al.*, 1992).

3. Results

(a) *Selection against the thrombin receptor on platelets*

A recombinant phage was constructed in which the 12 residue sequence (SFLLRNPNDKYE) exposed following cleavage of the thrombin receptor by thrombin, was fused to the gene III coat protein of the phage fd-tet-Sfi/Not (derived from fd-tet-DOG1) (Hoogenboom *et al.*, 1991). The phage was used to spike a preparation of kanamycin resistant phage (fd-kan-DOG) (see Materials and Methods) and the mixture was panned against either platelets or HEL cells, both of which express the thrombin receptor at high levels (Brass *et al.*, 1991, 1992), or against COS-1 cells, which display low levels of receptor on their surface (Hung *et al.*, 1992). The phage were eluted with the peptide agonist, at a concentration of 1 mM (100-fold higher than required for platelet activation) and enrichment was detected by determining the ratio of tet^+ to kan^+ colonies after infection of *E. coli*. The phage bearing the natural peptide ligand could be enriched on platelets or HEL cells, but not on COS-1 cells (Table 1). We therefore used platelets for the selection of peptide repertoires.

(b) *Construction of a tethered ligand library and selection for novel binders*

As the N-terminal six residues of the tethered ligand appear to have binding (indeed agonist) activity, we randomised this region, but retained eight residues of the tether (Figure 1). After three rounds of selection on fresh platelets, 59/96 of the eluted phage clones bound to fixed platelets as shown by ELISA. For most of these clones, the binding to platelets was consistently reduced in the presence of the agonist peptide, or after treatment of the platelets with thrombin (Figure 2). Clones were also screened for binding in the presence of other peptides not

Table 1

Enrichment for peptide fusion phages

Displayed peptide	Selection target	Enrichment 1:1,000,000	1:1000	1:200	1:50
Thrombin receptor ligand	Platelets	57x	77x	108x	87x
SFLLRNPNDKYE	HEL cells	13x		9x	
	COS cells	1.3x		1.5x	
NK-3 ligand	Platelets	1.3x	1.0x	1.1x	1.0x
DMHDFFVGLM	HEL cells	1.4x		1.4x	
	COS cells	1.7x		1.4x	
fd DOG gene III	Platelets	1.35x	1.2x	1.1x	1.2x
QVQLQELEIKRA	HEL cells	1.1x		1.8x	2.1x
	COS cells		1.5x		1.8x

The enrichment of tet^+ phage displaying 3 different peptides (SFLLRNPNDKYE, DMHDFFVGLM and QVQLQELEIKRA) after selection using 2 different cell types (HEL and COS) or platelets. The tet^+ phage were spiked with kan^+ phage (displaying the peptide QVQLQELEIKRA) in the ratio of $1/10^6$; $1/10^3$, 1/200 and 1/50 and the degree of enrichment calculated after plating of the phage before and after selection, as tet^+ colonies/tet^+ + kan^+ colonies after selection/tet^+ colonies/tet^+ + kan^+ colonies before selection. Results are typical of duplicate experiments.

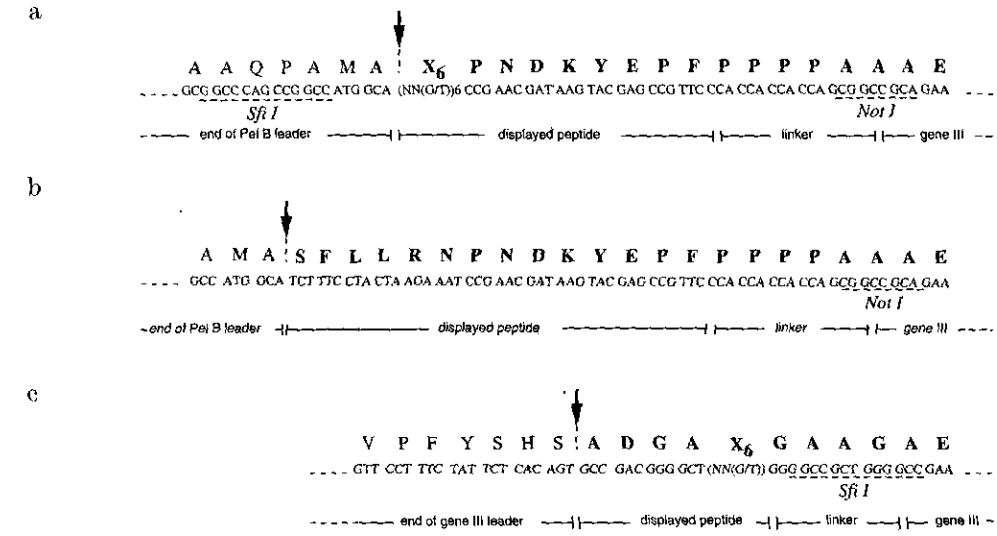

Figure 1. Fusion phage constructs. a, Thrombin ligand repertoire. Following cleavage of the N-terminal pel B signal sequence the random hexapeptide (X_6) is displayed at the N terminus of the gene III protein. Eight amino acids derived from the thrombin receptor tether region are encoded C-terminal to the random peptide. The displayed sequences are linked to the gene III protein by a rigid poly-proline spacer. The cleavage site for removal of the signal sequence is indicated by an arrow. b, Tethered ligand library. c, Hexapeptide library (Scott & Smith, 1990) that incorporates 4 fixed amino acid residues at the N terminus.

expected to compete for the thrombin receptor. Included among these was the tetrapeptide RGDS which interacts with the highly abundant IIb/IIIa receptor present on the platelet surface. With only one exception (T16), the selected phage showed no reduction in platelet binding in the presence of this peptide, even at concentrations as high as 5 mM.

(c) *Peptide sequences*

A selection of phage clones binding to platelets (and competed with agonist peptide) were sequenced. All of the selected hexapeptide sequences included a basic residue (arginine, histidine or lysine), usually an arginine as in the natural sequence, and frequently preceded by either a serine or alanine residue (Figure 3). When the peptides were aligned by the basic residue (Figure 3), we also noted a proline residue to the C-terminal side usually within three residues. Proline is present as the first fixed residue of the tether in the library, but was often also present within the selected hexapeptide sequence. Only three amino acid residues (methionine, serine and glycine) were seen at the N-terminus of the selected peptides, suggesting that these residues are selected and may also form part of the receptor binding motif. Certainly many different amino acid residues were encoded at the N-terminal position in the unselected phages. Thus the sequences of 44 phage clones isolated after three rounds of growth (but without selection on platelets), encoded 17 different amino acid residues, with arginine, alanine, tryptophan and valine occurring most frequently. When we used a second peptide library, but in which the first four N-terminal residues were fixed (Scott & Smith, 1990), we also noted an arginine/proline motif (Figure 3).

(d) *Immunoprecipitation of the thrombin receptor from platelets using phages*

To provide direct evidence that the phages bound to the thrombin receptor, whole platelets were surface labelled with I^{125} using lactoperoxidase, and the phages incubated with a high speed membrane preparation (Harmon & Jamieson, 1985). The phages were then captured on paramagnetic beads coated with anti-M13 antibody, and screened by Western blotting for the thrombin receptor using Mab ATAP 138 (Brass *et al.*, 1992). The presence of the receptor was detected with phages displaying peptides T1, T3 and T8 (and possibly T5, Figure 4). However, we failed to detect the thrombin receptor using phage displaying the natural agonist peptide, perhaps due to poor affinity.

(e) *Biological activity of selected peptides*

Peptides T1 and T8 were synthesized and screened for their activity as antagonists (or agonists) for the thrombin receptor, using platelet aggregation, serotonin release and tyrosine phosphorylation assays on human platelets. The peptides T5, T9, T14 and T16 were also synthesised and screened as controls, as well as shorter variants of the T1 peptide.

(i) *Inhibition of platelet aggregation*

Both thrombin and the peptide agonist 'SFLLRNPNDKYE' stimulate aggregation of platelets at concentrations of around 1 nM and 10 uM, respectively (Chao *et al.*, 1992; Vassallo *et al.*, 1992). Only peptides T1 and T16 were able to inhibit the aggregation (Figure 5). However, peptide T1 was unable to block platelet aggregation triggered by other platelet activators such as ADP, epinephrine

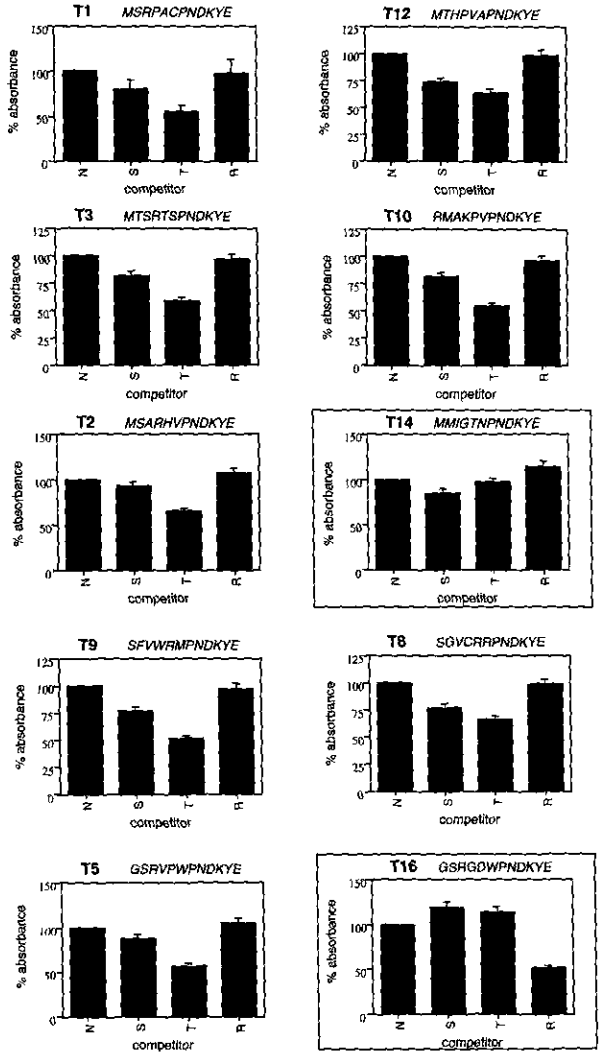

Figure 2. Competition of phage binding. The binding of phage displaying selected peptides to platelets by ELISA in the absence of competitor peptide (track **N**; 100%), in the presence of 1 mM SFLLRNPNDKYE peptide (track **S**), and following pretreatment of platelets with thrombin (track **T**). In addition competition was carried out in the presence of 3 other peptides, RGDS (track **R**), TKPR and VGSE (not shown, but no significant inhibition seen). The results represent the mean signals of 5 ELISA assays. Standard deviations are indicated by the error bars.

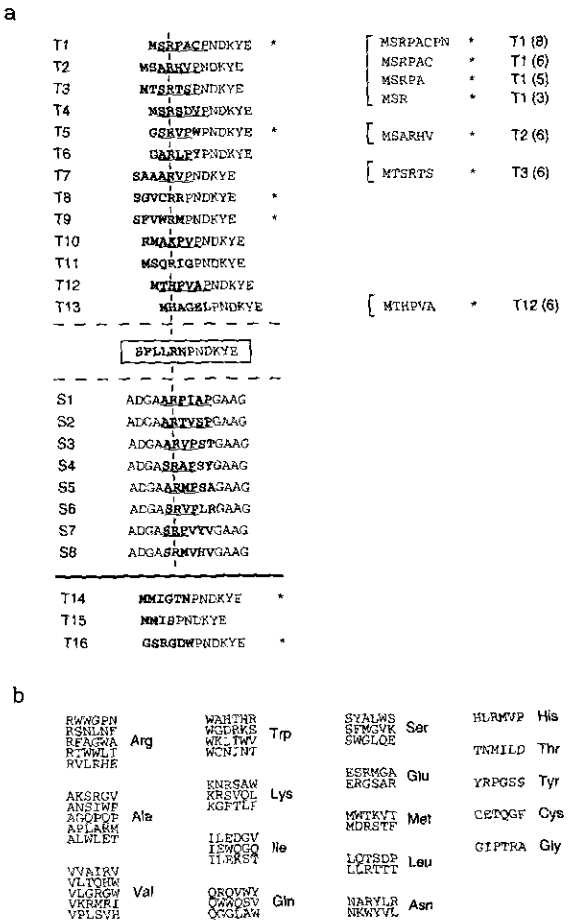

Figure 3. Sequences of displayed peptides. a, Sequences of the displayed peptides in phage showing reduced binding to platelets both in the presence of the SFLLRNPNDKYE peptide and following platelet pre-treatment with thrombin. The peptides are aligned by a basic residue. Peptides T1 to T16 were selected from the thrombin ligand library, while S1 to S8 were derived from the hexapeptide library. Peptides which were synthesised and tested for biological activity are indicated by an asterisk. The motif common to many of the selected sequences is underlined. Peptides T14 to T16 bound to platelets but apparently not to the thrombin receptor. b, The sequences of peptides displayed on 44 phage picked at random following 3 rounds of "mock" selection.

and the thromboxane A2 analog U46619, suggesting that it was specific to the thrombin receptor. Peptide T1 was found to lose activity on prolonged storage in solution, presumably by oxidation of the cysteine residue to cystine as purification on a solid phase reducing column (Pierce, U.K.) restored the activity. By contrast peptide T16 (which contains an RGD motif) blocked platelet aggregation triggered by other activators (ADP, epinephrine, U46619; data not shown), presumably by blocking the activated IIb/IIIa integrin (which mediates platelet aggregation through the binding of fibrogen and von Willebrand factor). The peptides were also screened for agonist activity, and peptide T9 was found to stimulate the aggregation of platelets (EC50 about 100 mM, Figure 6).

(ii) *Inhibition of serotonin release*

Only peptide T1 proved to be effective inhibitor of serotonin release by thrombin (Figure 7). Such inhibition is a characteristic of thrombin receptor antagonists (Brass *et al.*, 1992), as peptide antagonists of the IIb/IIIa receptor, although potent inhibitors of platelet aggregation do not effect serotonin release by platelets (Shattil *et al.*, 1985; Eldor *et al.*, 1985; Huang *et al.*, 1987). Peptide T9 proved to be an agonist (Figure 6).

(iii) *Suppression of tyrosine phosphorylation*

Both thrombin and the agonist peptide induce tyrosine phosphorylation of multiple platelet proteins

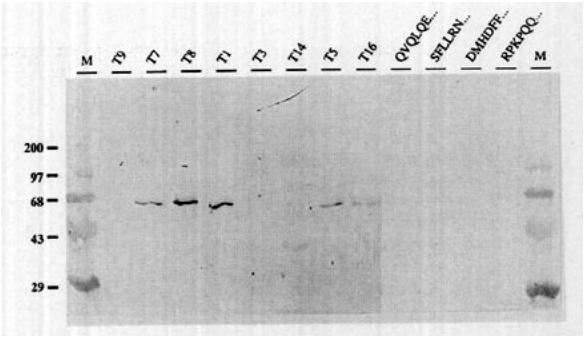

Figure 4. Immunoprecipitation of thrombin receptor by phages. Fragmented platelet membranes were incubated with phage, and then the phage captured on paramagnetic beads. The receptor was detected on Western blots using a monoclonal antibody ATAP 138 after incubations with phage displaying the peptides T7 (SAAARVPNDKYE), T8 (SGVCRRPNDKYE), T1 (MSRPACPNDKYE) and T5 (GSRVPWPNDKYE), but not the other phages. Molecular weight markers are shown in tracks M.

(Vassallo *et al.*, 1992; Golden & Brugge, 1989). This induction can be blocked by an antibody which binds to the tethered peptide of the thrombin receptor (Brass *et al.*, 1992). The peptides were assayed for their ability to prevent tyrosine phosphorylation by either thrombin or SFLLRNPNDKYE. Although we could detect only a limited range of phosphorylated proteins using a rabbit anti-phosphotyrosine polyclonal antibody, the intensity of the signal was consistently reduced when activation was carried out in the presence of T1 as compared to other peptides (Figure 8). However, this was not the case when U46619 was used as the activator.

4. Discussion

The thrombin receptor is cleaved by thrombin to expose a new N terminus that acts as a tethered ligand to activate the receptor. A peptide with the same sequence as the N-terminal portion of the cleaved receptor (SFLLRNPNDKYE) is able to act as agonist, but only at high concentrations (EC 50 = 10 mM), indicating that the peptide sequence has a poor affinity for the receptor. This emphasises the role of the tether in binding the natural ligand, and suggests that the ligand has evolved towards high turnover with the receptor binding site. Perhaps not

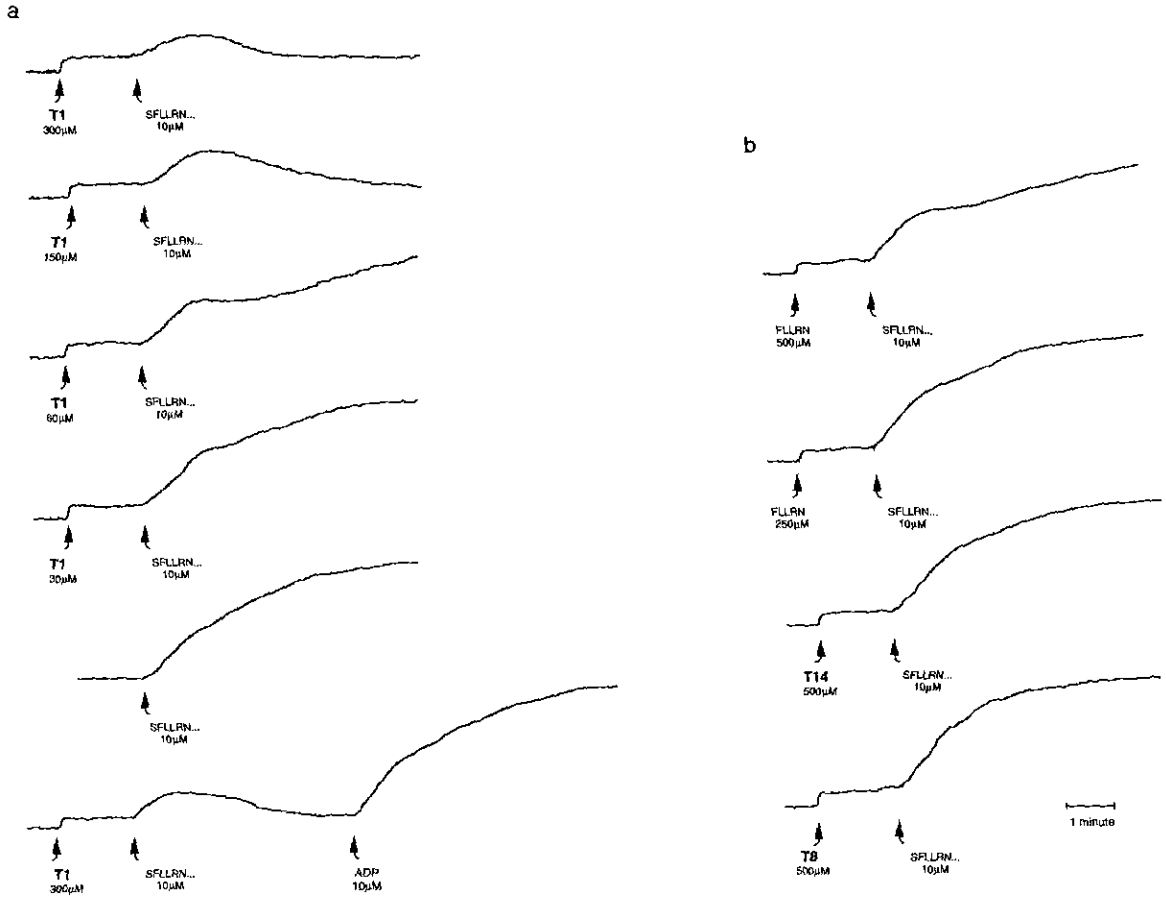

Figure 5. a, Inhibition of platelet aggregation by peptide T1 (MSRPACPNDKYE). Platelet rich plasma was incubated with peptide 1 minute before addition of the agonist peptide SFLLRNPNDKYE and aggregation detected using a 4 channel aggregometer. The response to other agonists such as ADP (10 mM) is also shown. The results are typical of triplicate experiments. b, Inhibition of platelet aggregation by other peptides.

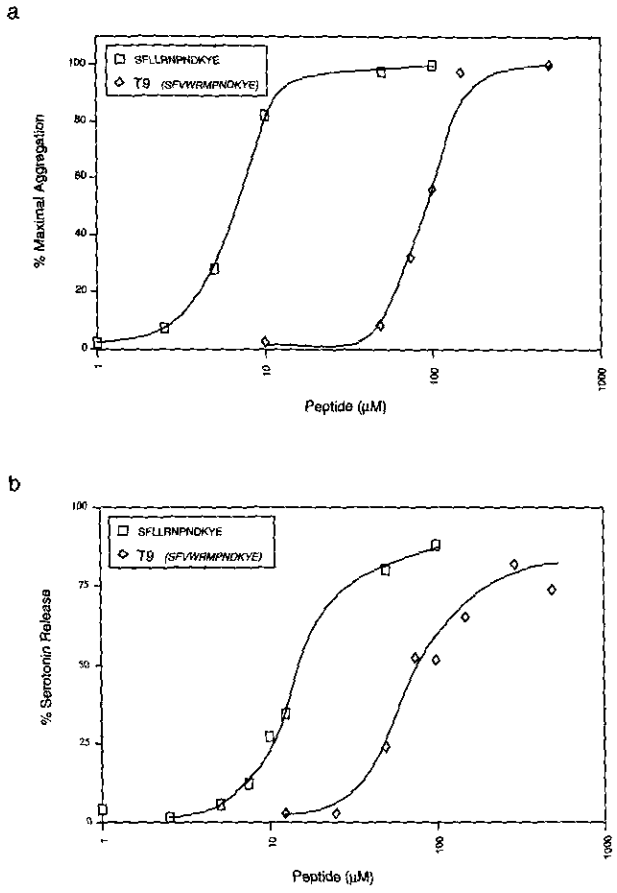

Figure 6. Agonist activity of peptide T9 (SFVWRMPNDKYE). a, Aggregation of platelets in platelet rich plasma by peptide T9. 100% aggregation was taken as that induced by 500 mM agonist peptide (SFLLRNPNDKYE). b, Serotonin release from platelets by peptide T9. 100% release was taken as that induced by 500 mM agonist peptide SFLLRNPNDKYE. The values represent the mean of duplicate experiments.

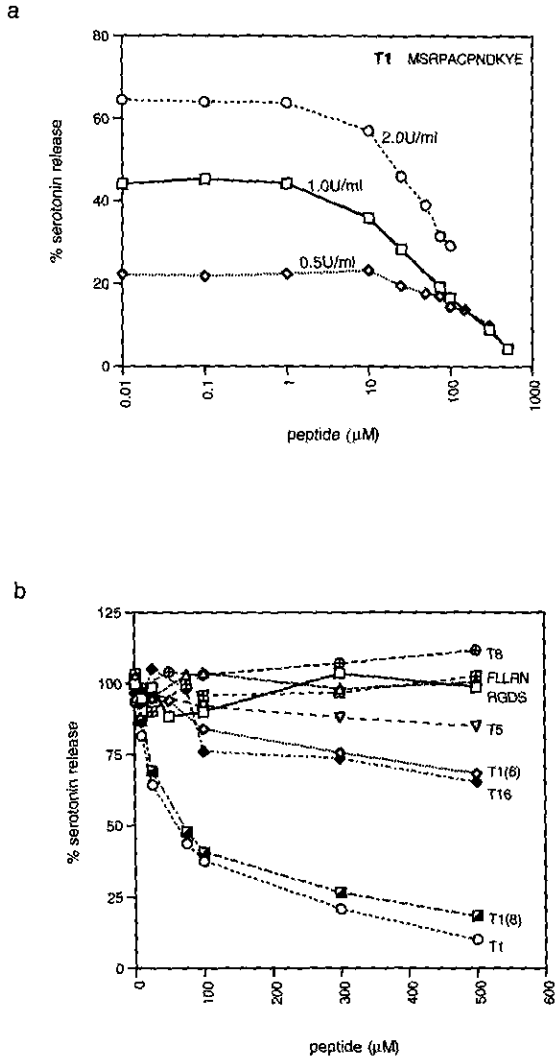

Figure 7. a, Inhibition of serotonin release by peptide T1 (MSRPACPNDKYE). Platelet rich plasma (serotonin loaded) was incubated in the presence of increasing concentrations of peptide for 1 minute before the addition of thrombin (0.5 U/ml to 2.0 U/ml). Serotonin release was measured as described in Materials and Methods. 100% release corresponds to the total amount of [^{3}H] serotonin taken up by the platelets during the labelling period and that could in principle be released (on addition of thrombin). However, it was not possible to achieve 100% release with the highest level of thrombin (2 U/ml) used here. b, Inhibition of serotonin release by other peptides.

surprisingly it has proved difficult to make potent antagonists based on this sequence. With the exception of FFLLRNP and YFLLRNP (Vassallo *et al.*, 1992) modifications of the natural ligand such as SALLRNP, SLLRNP, SFLRNP and propFLLRNP do not act as either agonists or antagonists (Rasmussen *et al.*, 1993).

Here we have tried to harness selection technology to build a peptide antagonist of the thrombin receptor using the agonist peptide. Thus we built a repertoire of sequences based on portions of the agonist peptide, bound the phage to platelets, and eluted with the agonist peptide. However, we do not know whether displacement of the bound phage was due to simple competition or to receptor activation. After several rounds of selection, we succeeded in selecting phage that bound to platelets, and in which the binding could be inhibited with the agonist. At least one of the phages (encoding the peptide T1) could be used as a reagent to immunoprecipitate the thrombin receptor from membranes, and the free peptide T1 was able to antagonise the agonist (or thrombin) triggered aggregation of platelets, serotonin release and tyrosine phosphorylation. From another phage (encoding peptide T9) we identified a free peptide capable of receptor activation. Thus we succeeded in isolating peptides binding to the receptor with both agonist and antagonist activities.

Presumably peptides with binding activity will only be agonists if they make specific contacts with the receptor. Not surprisingly the sequence of the selected agonist (T9) is rather close to that of the eluting agonist peptide whilst that of the antagonist bears a more distant relationship. Thus the sequence of T1 (and several other peptides selected by binding to the thrombin receptor), includes an arginine

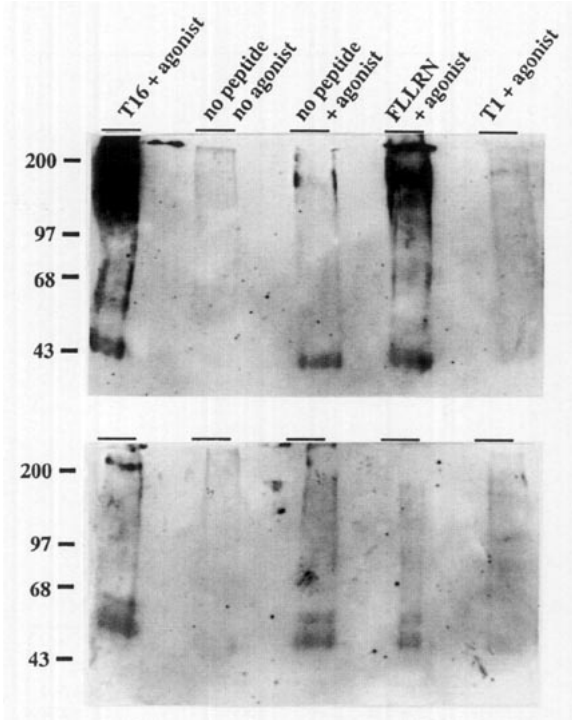

Figure 8. Inhibition of tyrosine phosphorylation. Gel filtered platelets were incubated with either 300 mM peptide (T16, T1 or FLLRN), or with PBS for 2 minutes before the addition of either thrombin (1 U/ml, upper panel) or SFLLRNPNDKYE peptide (10 mM, lower panel). After 2 minutes in the presence of agonist, cell lysates were prepared and tyrosine phosphorylated proteins were detected by Western blotting using a rabbit polyclonal antibody.

residue followed (within three residues) by a proline. As the N-terminal portion of the tethered ligand also includes an arginine residue essential for agonist activity (Chao *et al.*, 1992; Vassallo *et al.*, 1992; Van Obberghen-Schilling *et al.*, 1993), and a proline, we suspect that the antagonist peptide T1 may make some of the same interactions as the agonist. The arginine may well form a salt bridge with an acidic residue of the receptor, and the proline may facilitate a turn as has been suggested (Chao *et al.*, 1992). However, the antagonist is presumably making additional interactions, as it is more potent (at least ten fold) than the antagonist peptides FLLRNP, YFLLRNP (Vassallo *et al.*, 1992; Rasmussen *et al.*, 1993) based on the natural sequence. Furthermore it appears to be more potent than an antagonist Mpr-F-Cha-Cha-RKPND described more recently (Giesberts *et al.*, 1993). The sequence of T1, and other such peptides, may therefore prove to be a useful lead for building yet more potent antagonists.

We thank S. Stone and other colleagues for advice, D. Horwell for advice and continuing support, L. F. Brass for the monoclonal antibody ATAP 138, G. Smith for the hexapeptide phage display library and H. Griffin for help with the phage sequencing. J.D. was supported by Parke Davis Neuroscience Research Centre, U.K.

References

BlondElguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F. & Gething, M. J. H. (1993). Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell*, **75**, 717–728.

Bockaert, J. (1991). G proteins and G-protein-coupled receptors: structure, function and interactions. *Curr. Opin. Neurobiol.* **1**, 32–42.

Brass, L., Manning, D. R., Williams, A. G., Woolkalis, M. J. & Poncz, M. (1991). Receptor and G protein-mediated responses to thrombin in HEL cells. *J. Biol. Chem.* **266**, 958–965.

Brass, L., Vassallo, R. R., Belmonte, E., Ahuja, M., Cichowski, K. & Hoxie, J. A. (1992). Structure and function of the human platelet thrombin receptor. *J. Biol. Chem.* **267**, 13795–13798.

Chao, B. H., Jakubowski, J. A., Savage, B., Chow, E. P., Marzec, U. M., Harker, L. A. & Maraganore, J. M. (1989). Agkistrodon piscivorus platelet aggregation inhibitor: A potent inhibitor of platelet activation. *Proc. Nat. Acad. Sci., U.S.A.* **86**, 8050–8054.

Chao, B. H., Kalkunte, S., Maraganore, J. M. & Stone, S. R. (1992). Essential groups in synthetic agonist peptides for activation of the platelet thrombin receptor. *Biochemistry*, **31**, 6175–6178.

Coughlin, S. R. (1993). Thrombin receptor structure and function. *Thromb. Haemost.* **66**, 184–187.

Cwirla, S. E., Peters, E. A., Barrett, R. W. & Dower, W. J. (1990). Peptides of phage: a vast library of peptides for identifying ligands. *Proc. Nat. Acad. Sci., U.S.A.* **87**, 6378–6382.

Devlin, J. J., Panganiban, L. C. & Devlin, P. E. (1990). Random peptide libraries: a source of specific protein binding molecules. *Science*, **249**, 404–406.

Eldor, A., Vlodavsky, I., Martinowicz, U., Fuks, Z. & Coller, B. S. (1985). Platelet interaction with subendothelial extracellular matrix: platelet-fibrinogen interactions are essential for platelet aggregation but not for the matrix-induced release reactions. *Blood*, **65**, 1477–1483.

Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R. & Cesarini, G. (1991). Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J. Mol. Biol.* **222**, 301–310.

Geysen, H. M., Rodda, S. J. & Mason, T. J. (1986). A priori delineation of a peptide which mimics a discontinuous antigenic determinant. *Mol. Immunol.* **23**, 709–715.

Giesberts, A. N., Willigen, G. & Akkerman, J.-W. N. (1993). Evidence for multiple pathways for signal transduction by the α thrombin receptors in platelets. *Thrombosis and Hemostasis*, **69**, 1342.

Golden, A. & Brugge, J. S. (1989). Thrombin treatment induces rapid changes in tyrosine phosphorylation in platelets. *Proc. Nat. Acad. Sci., U.S.A.* **86**, 901–905.

Greenwood, J., Willis, A. E. & Perham, R. N. (1991). Multiple display of foreign peptides on a filamentous bacteriophage. *J. Mol. Biol.* **220**, 821–827.

Harlow, E. & Lane, D. (1988). *Antibodies, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Harmon, J. T. & Jamieson, G. A. (1985). Thrombin binds a high affinity 900 000 dalton site on human platelets. *Biochemistry*, **24**, 58–64.

Hoess, R. H. (1993). Phage display of peptide and protein domains. *Curr. Opin. Struct. Biol.* **3**, 572–579.

Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P. & Winter, G. (1991). Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucl. Acids Res.* **19**, 4133–4137.

Huang, T. F., Holt, J. C., Lukasiewics, H. & Niewiarowski, S. (1987). Trigamin. A low molecular weight peptide inhibiting fibrinogen interaction with platelet receptors expressed on glycoprotein IIb/IIIa complex. *J. Biol. Chem.* **262**, 16157–16163.

Hung, D. T., Vu, T.-K. H., Nelken, N. A. & Coughlin, S. R. (1992). Thrombin-induced events in non-platelet cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor. *J. Cell Biol.* **116**, 827–832.

Koivunen, E., Gay, D. A. & Ruoslahti, E. (1993). Selection of peptides binding to the a5b1 integrin from phage display library. *J. Biol. Chem.* **268**, 20205–20210.

Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. & Winter, G. (1991). By-passing immunization: human antibodies from V gene libraries displayed on phage. *J. Mol. Biol.* **222**, 581–597.

McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature (London)*, **348**, 552–554.

Oldenburg, K. R., Loganathan, D., Goldstein, I. J., Schultz, P. G. & Gallop, M. A. (1992). Peptide ligands for a sugar-binding protein isolated from a random peptide library. *Proc. Nat. Acad. Sci., U.S.A.*, **89**, 5393–5397.

O'Neil, K. T., Hoess, R. H., Jackson, S. A., Swamy Ramachandran, N., Mousa, S. A. & DeGrado, W. F. (1992). Identification of novel peptide antagonists for GPIIb/IIIa from a conformationally constrained phage peptide library. *Proteins: Struct. Funct. Genet.* **14**, 509–515.

Rasmussen, U. B., Gachet, C., Schlesinger, Y., Hanau, D., Ohlmann, P., Van Obberghen-Schilling, E., Pouyssegur, J., Cazenave, J.-P. & Pavirani, A. (1993). A peptide ligand of the human thrombin receptor antagonises a-thrombin and partially activates platelets. *J. Biol. Chem.* **268**, 14322–14328.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Nat. Acad. Sci., U.S.A.* **74**, 5463–5467.

Scott, J. K. & Smith, G. P. (1990). Searching for peptide ligands with an epitope library. *Science*, **249**, 386–390.

Scott, J. K., Loganathan, D., Easley, R. B., Gong, X. & Goldstein, I. J. (1992). A family of concanavilin A-binding peptides from a hexapeptide epitope library. *Proc. Nat. Acad. Sci., U.S.A.* **89**, 5398–5402.

Shattil, S. J., Hoxie, J. A., Cunningham, M. & Brass, L. F. (1985). Changes in the platelet membrane IIb/IIIa complex during platelet activation. *J. Biol. Chem.* **260**, 11107–11114.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, **228**, 1315–1317.

Smith, G. P., Schultz, D. A. & Ladbury, J. E. (1993). A ribonuclease S peptide antagonist discovered with a bacteriophage display library. *Gene*, **128**, 37–42.

Stephen, C. W. & Lane, D. P. (1992). Mutant conformation of p53. Precise epitope mapping using a filamentous phage epitope library. *J. Mol. Biol.* **225**, 577–583.

Van Obberghen-Schilling, E., Rasmussen, U. B., Vouret-Craviari, V., Lentes, K.-U., Pavirani, A. & Pouyssegur, J. (1993). Structure-activity analysis of synthetic a-thrombin-receptor-activating peptides. *Biochem. J.* **292**, 667–671.

Vassallo, R. R., Kieber-Emmons, T., Cichowski, K. & Brass, L. F. (1992). Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem.* **267**, 6081–6085.

Vu, T.-K. H., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*, **64**, 1057–1068.

Walz, D., Fenton, J. W. & Shuman, M. (1986). Bioregulatory functions of thrombin. *Ann. NY Acad. Sci.* **485**, 1–449.

Edited by R. Huber

(Received 20 June 1994; accepted 16 September 1994)