Antibody mapping of Tissue Factor implicates two different exon-encoded regions in function

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Tissue Factor (TF), a small transmembrane glycoprotein, is the cellular receptor for the zymogen Factor VII and the serine protease Factor VIIa (VIIa). TF provides cofactor function for VIIa in the catalytically active (TF:VIIa) binary complex. To explore the structural loci of TF that are responsible for binding of VII and VIIa, monoclonal antibodies (MAbs) and sequence-specific polyclonal antibodies to the native TF protein were analysed for inhibition of VII binding. Two independent epitopes of MAbs were localized by reciprocal competition and by binding of the MAbs to different proteolytic fragments of TF. The epitopes were also characterized in part by progressive C-terminal deletional mutation of the TF protein. Reactivity of the anti-(locus II) MAb TF9-6G4 is consistent with epitope localization in residues Thr⁴⁰–Val⁸³, encoded by exon 3. In contrast, the anti-(locus I) MAb TF9-5G9 was reactive with fragments encompassing exon 4 (Thr¹⁰⁶–Lys¹⁶⁵). Antibodies to linear sequences encoded by the same two exons also inhibited VII binding. These data suggest a minimum requirement for two of the four exon-encoded regions of TF for the functional integrity of this receptor cofactor with respect to ligand recognition and high-affinity binding.

INTRODUCTION

Cellular initiation of coagulation is efficiently mediated by Tissue Factor (TF) (Maynard *et al.*, 1975), the cell-surface receptor for the coagulation zymogen Factor VII and the receptor and cofactor for the serine protease Factor VIIa (VIIa) (Broze, 1982). Protein–protein interactions between VIIa and the extracellular domain of TF (Ruf *et al.*, 1991) are considered to be essential for both receptor and cofactor functions, the latter being defined as the catalytic enhancement of VIIa-mediated proteolytic activation of the zymogen substrates Factors X and IX (Bach, 1988). It has been inferred that conformational alterations of the VIIa protease domain that are required for proper alignment of the catalytic triad occur upon binding to TF (Nemerson & Gentry, 1986). Positive co-operativity has been observed for the binding of VII to purified TF as well as to TF expressed as an integral cell-membrane protein (Bach *et al.*, 1986; Fair & MacDonald, 1987). These studies indicate that TF may preferably function as a dimer.

Although the primary sequence of this small cell-surface receptor cofactor has been established, functional sites which provide the interactive region for VII binding have not been defined. Certain inhibitory antibodies to TF have been shown to block VII binding, demonstrating that binding is a critical requirement for cofactor function, namely the formation of a catalytically active (TF:VIIa) complex (Carson *et al.*, 1985; Morrissey *et al.*, 1988b). The limited surface area [6.4–7.5 nm² (640–750 Å²)] of antibody ‘footprints’ on proteins (Davies *et al.*, 1988) suggests that monoclonal antibodies (MAbs) may serve as highly specific probes for relatively small solvent-exposed loci on proteins, and thereby may help to identify functional regions. Despite the potential caveat of secondary allosteric effects, antibodies have provided topographical insight into the structure and function of proteins (Dueweke & Gennis, 1990; Brakenhoff *et al.*, 1990; Westmeyer *et al.*, 1990). In the present study we have addressed the localization of functional epitopes on the cell-surface domain of TF which were defined by inhibitory MAbs as well as by sequence-specific polyclonal antibodies. Engagement

of epitopes in the exon 3- and exon 4-encoded regions of the TF extracellular domain resulted in loss of VII binding function. We suggest that at least two regions of the TF extracellular domain, each encoded by a different exon, participate in VII binding and resulting expression of TF cofactor function.

MATERIALS AND METHODS

Materials

PAGE reagents, Protein A and Enzymobeads radioiodination reagent were obtained from Bio-Rad (Richmond, CA, U.S.A.). Carrier-free Na¹²⁵I from Amersham (Arlington Heights, IL, U.S.A.) was used. The plasmid pcDNAII and the *Escherichia coli* strain NM522 were from Invitrogen (San Diego, CA, U.S.A.), the Erase-a-base system was from Promega (Madison, WI, U.S.A.), and restriction enzymes were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). All other reagents used were analytical grade or better.

Proteins

Human Factor VII was purified from plasma as described (Fair, 1983). Twenty-four hybridomas producing MAbs against TF have previously been described in detail (Morrissey *et al.*, 1988b). Hybridomas were propagated in ascites growth and the MAbs were purified from ascites using adsorption of MAb to immobilized Protein A in the presence of 1.5 M-glycine/3 M-NaCl, pH 8.9, followed by elution with 100 mM-citric acid, pH 4.0. Human brain TF was purified as described (Morrissey *et al.*, 1988a). All proteins were of the expected size and were homogeneous by SDS/PAGE (Laemmli, 1970).

Antibodies of predetermined specificity were produced by peptide immunization followed by affinity purification on immobilized TF. The peptides were based on the primary amino acid sequence of human TF and were synthesized on an Applied Biosystems Model 430 synthesizer using standard t-butoxy-carbonyl chemistry and HF cleavage. All peptides had a C-terminal cysteine which was coupled to keyhole-limpet haemocyanin (KLH) via an *m*-maleimidobenzoyl *N*-hydroxy-

Abbreviations used: TF, Tissue Factor; VII/VIIa, Factor VII/VIIa; MAb, monoclonal antibody; KLH, keyhole-limpet haemocyanin; TBS, 20 mM-Tris/150 mM-NaCl, pH 7.4.

succinimide ester linkage (Liu *et al.*, 1979). Rabbits were repeatedly immunized with the conjugate, and immunoglobulin fractions from rabbit serum were obtained by twice precipitating and redissolving at 45% satd. $(\text{NH}_4)_2\text{SO}_4$ at 4 °C followed by chromatography on DEAE-Sephacel. Anti-peptide antibodies reactive with the native TF protein were then isolated by affinity chromatography on TF that was immobilized to Affigel 15 (Morrissey *et al.*, 1987). Bound antibody was eluted with 5 M-guanidine hydrochloride. MAbs to peptide TF-1-30 were produced by standard hybridoma technology (Morrissey *et al.*, 1988b) using spleen cells from mice immunized with KLH-conjugated peptide. The hybridomas were selected for reactivity with affinity-purified human brain TF. The anti-TF-1-30 MAbs which reacted with TF were predominantly reactive with peptides spanning residues 11-28.

Immunochemical analysis of epitope assignment to TF fragments

Samples (60 µg) of purified human brain TF in 125 mM-Tris/1 mM-EDTA/0.1% SDS/20% glycerol, pH 6.8, were placed in a single gel lane sample trough, and were overlaid with 5 µg of V8 protease in the same buffer containing 10% glycerol (Cleveland *et al.*, 1977). Once the proteins had entered the stacking gel, electrophoresis (Laemmli, 1970) was interrupted for 30 min to allow proteolytic digestion at ambient temperature. After completion of electrophoresis, the proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH, U.S.A.), followed by blocking with 5% (w/v) non-fat dry milk in 20 mM-Tris/150 mM-NaCl, pH 7.4 (TBS). Reactivity of antibodies was analysed by incubating the nitrocellulose membranes with 1:10-diluted culture supernatant from individual hybridomas, or with affinity-purified peptide-specific antibody at 10 µg/ml in 5% non-fat dry milk in TBS. This was followed by five washes in TBS and detection of bound antibody by an appropriate alkaline-phosphatase-coupled secondary antibody using NitroBlue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M-Tris/0.1 M-NaCl/5 mM-MgCl₂, pH 9.5, as a colour-developing reagent.

Binding assays

Binding analysis of radiolabelled molecules to cell-surface TF were performed on a J82 cell monolayer (ATCC HTB 1) in the presence of 5 mM-CaCl₂ as previously described (Drake *et al.*, 1989). Binding was corrected for non-specific binding, which was determined in the presence of a 50-fold molar excess of the same unlabelled protein. (i) Epitope identity or overlap between MAbs was analysed by competitive inhibition. This involved binding of representative ¹²⁵I-labelled MAbs at 3 nM to TF-expressing cells for 2 h at 37 °C in the presence or the absence of a 100-fold molar excess of unlabelled competing MAb. Typically, the same MAb or other MAbs to an overlapping epitope consistently produced more than 85% inhibition of binding of the labelled MAb, whereas non-competitive MAbs produced less than 20% inhibition. (ii) For analysis of inhibition of VII binding to TF, TF⁺ J82 cells were incubated with ¹²⁵I-VII (5 nM) in the presence of 5 mM-CaCl₂ and various concentrations of the competing MAb for 2 h, after which binding was determined in comparison with a control reaction using an unrelated MAb (ATCC TIB 115). Values given are means from duplicate determinations, and the dose titration was repeated three times. (iii) Inhibition of VII binding by polyclonal antibodies against linear sequences of TF were not performed under equilibrium conditions, since the affinity of the antibodies for TF was much lower than the affinity of the competing ligand VII. J82 cells were preincubated with the highest available concentration of antibody or buffer control for 2 h followed by addition of 5 nM-VII for 30 min. Unbound

radiolabelled protein was removed by rapid washes as above, and the decrease in VII binding was calculated relative to the buffer control. Means ± S.D. calculated for three repeats are given.

Deletion mutagenesis of TF

A 775 bp *Eco*R1 fragment coding for residues 1-219 of TF (Ruf *et al.*, 1991) was inserted into pcDNA II to give in-frame expression of the TF coding sequence as part of a truncated β -galactosidase fusion protein. After producing a protected 3' protruding end by *Apal* cleavage and an unprotected 5' protruding end by *Xho*I cleavage of the construct, exonuclease III digestion was performed using the Erase-a-base system (Promega). The re-ligated constructs were used to transform *E. coli* NM522. The deletion mutants were selected by the length of a *Pvu*II fragment obtained from cleavage of two sites in the vector which flanked the TF coding sequence. The exact length of the deletion was established for selected mutants by DNA sequencing. Single-stranded DNA was rescued using the helper phage R408 and sequenced by the dideoxy method using Sequenase (United States Biochemical Corp., Cleveland, OH, U.S.A.).

Prokaryotic expression and Western-blot analysis

Cells transformed with the various constructs were grown in LB broth containing 100 µg of ampicillin/ml and induced with 10 mM-isopropyl β -D-thiogalactopyranoside at an A_{600} of 0.2. Cells of a 100 ml confluent culture were sedimented, lysed by repeated freeze-thaw cycles after resuspension in TBS, and the supernatant of the lysate was reduced and separated on SDS/PAGE followed by transfer to nitrocellulose (Schleicher and Schuell). After blocking in 5% non-fat milk in TBS, the blots were incubated with 20 µg of MAb/ml for 1 h followed by detection of the bound MAb by alkaline-phosphatase-conjugated anti-mouse IgG (Tago, Burlingame, CA, U.S.A.). Non-specific binding was assessed using an unrelated MAb at an identical concentration.

RESULTS

Competition analysis of MAb binding to TF

Selected MAbs were purified and ¹²⁵I-labelled for analysis of binding to cell-surface-expressed TF. To assess putative identical or overlapping epitopes between MAbs, TF was saturated by a 100-fold molar excess of each of the MAbs in the presence of the ¹²⁵I-labelled test MAb. Two groups of MAbs were non-competitive, which was interpreted as binding to non-overlapping epitopes (Table 1). Within each group, complete and reciprocal inhibition was observed, consistent with epitope overlap of MAbs to the same locus. In addition, the reciprocal inhibition within one group indicated similar affinities of the MAbs for TF. All MAbs to TF produced in the laboratory had either overlapping epitopes with the two loci described here or an additional non-overlapping locus. We were not able to assign the epitope for this latter group of MAbs by the approaches described in this paper. MAbs to locus I included inhibitory (TF8-5G9) as well as non-inhibitory (TF9-10H10) MAbs (Morrissey *et al.*, 1988b). This demonstrated that binding of a MAb to TF in itself is not sufficient to abolish function, and further substantiates the potential of MAbs to define functional and non-functional regions even on a small receptor molecule.

Inhibition of VII binding to J82 cells by antibodies

The inhibition of VII binding to TF by antibodies was analysed (Fig. 1). MAbs TF8-5G9 (against locus I) and TF9-6G4 (against locus II) inhibited VII binding in a concentration-dependent

Table 1. Competitive inhibition of MAb binding to TF

Inhibition of binding of 3 nM ¹²⁵I-labelled MAb by a 100-fold molar excess of competing non-labelled MAb is shown. Experiments were performed by incubating radiolabelled MAb and competitor with TF⁺ J82 cells in a monolayer for 2 h at 37 °C, followed by washes and determination of cell-bound radioactivity. I and II represent locus I and II respectively.

Competing MAb	Bound MAb ...	Inhibition of binding to TF (%)			
		TF8-5G9 (I)	TF9-10H10 (I)	TF9-6B4 (II)	TF9-5B7 (II)
TF8-5G9		97	89	0	0
TF9-10H10		95	91	12	5
TF9-6B4		15	4	99	96
TF9-5B7		2	0	94	97
TF9-6G4		0	6	95	97
TF9-1F1		5	8	95	97
TF9-2F6		0	9	95	97

Fig. 1. Inhibition of VII binding to J82 cells by MAbs

A fixed concentration (5 nM) of radiolabelled VII was incubated with increasing amounts of unlabelled MAb or buffer for 2 h. The amount of VII specifically bound is shown as a percentage of specific binding determined for the buffer control, and is plotted versus the concentration of inhibiting MAb. Means of duplicate determinations are depicted for one typical experiment. MAbs: Δ , TF9-10H10; $\bullet$, TF8-5G9; $\circ$, TF9-6G4.

Table 2. Inhibition of TF binding of VII by sequence-specific antibodies to TF

Inhibition of VII binding by the antibody was tested by incubating TF⁺ J82 cells with the indicated concentration of antibody for 2 h, followed by addition of radiolabelled VII (5 nM) for 30 min. The decrease in binding is given relative to the buffer control. Means $\pm$ S.D. for three replicate determinations are given.

Antibody	Concentration (μ M)	Inhibition (%)
Anti-TF α -1-30	2.1	15 $\pm$ 7
Anti-TF α -40-71	3.6	60 $\pm$ 3
Anti-TF α -72-104	2.1	6 $\pm$ 7
Anti-TF α -94-121	2.5	35 $\pm$ 5
Anti-TF α -121-155	3.5	17 $\pm$ 6
Anti-TF α -146-167	2.6	18 $\pm$ 6
Anti-TF α -161-189	2.7	4 $\pm$ 1

manner. Similarly, Fab fragments of anti-(locus I) and anti-(locus II) MAbs blocked VII binding (results not shown), suggesting competition with the ligand for the same binding site rather than steric hindrance of the Fc portion as the inhibitory

mechanism. In contrast, the non-neutralizing MAb TF9-10H10 did not inhibit binding of VII over the entire concentration range. This is not attributed to a lower affinity of the MAb, since MAb TF9-10H10 was effective in competitively blocking TF8-5G9 binding at a 100-fold molar excess. Inhibition of VII binding was also observed when cell-surface TF was preincubated with purified polyclonal antibodies of predetermined sequence specificity. These anti-peptide antibodies were reactive with the TF protein and were isolated by affinity purification on immobilized TF. The yield of antibody which reacted with the TF protein was limited to a few milligrams per immunized rabbit. In addition, the affinity of the antibodies for TF appeared to be low, with dissociation constants in the micromolar range. This limited detailed quantitative analysis at equilibrium. Two of the sequence-specific antibodies inhibited VII binding when tested at a fixed concentration of antibody (Table 2). Anti-TF-40-71 blocked binding by $60 \pm 3\%$ and anti-TF-94-121 inhibited binding by $35 \pm 5\%$, whereas comparable sequence-specific antibodies to other regions of the TF extracellular domain were without effect. In addition, MAbs to TF-1-30 which bound to purified TF immobilized on nitrocellulose did not block binding of VII to TF (results not shown), further confirming the data with the polyclonal antibodies. This analysis suggests the participation of these two regions in interactions with VII.

Epitope localization of anti-TF MAbs

The entire panel of previously described anti-TF MAbs (Morrissey *et al.*, 1988b) was reactive with the TF extracellular domain (residues 1-219) (Ruf *et al.*, 1991), which is encoded by exons 2-5 (Mackman *et al.*, 1989). In order to localize the epitopes, we analysed the MAbs for reactivity with proteolytic fragments derived from a *Staphylococcus aureus* V8 protease partial digest of TF. Whereas no reactivity of the anti-(locus I) MAb TF9-10H10 with proteolytic fragments of TF was observed, the partially overlapping and inhibitory anti-(locus I) MAb TF8-5G9 consistently reacted with a 27 kDa V8 fragment (Fig. 2). The N-terminal residue of this fragment has been determined as Thr¹⁰⁶ (Morrissey *et al.*, 1987), the first amino acid in the exon 4-encoded region of TF (Mackman *et al.*, 1989). Immunopurification of a 27 kDa fragment from a V8 digest of TF using immobilized TF8-5G9 and N-terminal sequence analysis of the fragment confirmed that exon 4-encoded sequences were recognized by the MAb. In addition, the pattern of reactivity of TF8-5G9 (Fig. 2) resembled the pattern with sequence-specific antibodies against the C-terminal half of the TF extracellular domain. This included immunoreactivity with intact monomeric TF and with TF dimer, with which antibodies against epitopes in the C-terminal half of

Fig. 2. Reactivity of MAbs TF8-5G9 and TF9-6G4 with proteolytic fragments of TF

Western-blot analysis of MAb reactivity with protease fragments of TF is shown. TF (60 µg/lane) was treated with *S. aureus* V8 protease (5 µg/lane) in the stacking gel according to Cleveland *et al.* (1977) for 30 min at ambient temperature, followed by electrophoretic separation under non-reducing conditions and transfer to nitrocellulose. The reactivities of rabbit polyclonal antibodies specific for linear sequences and of MAbs TF8-5G9 and TF9-6G4 are demonstrated. Antibodies to sequences from the C-terminal half of the extracellular domain and MAb TF8-5G9 (lane 6) reacted with (from the top): TF dimer, intact TF, a 40 kDa fragment and a 27 kDa fragment of TF. Anti-TF-161-189 antibody recognized two additional bands of lower molecular mass. Sequence-specific antibodies against the N-terminus of TF reacted with low-molecular-mass bands not recognized by the above. TF9-6G4 was reactive with these fragments, and a pattern similar to staining with anti-TF-40-71 was obtained. The Western-blot analysis used fragments from one digest separated on the same gel. Antibodies used: lane 1, anti-TFα-1-30; lane 2, anti-TFα-40-71; lane 3, anti-TFα-94-121; lane 4, anti-TFα-146-167; lane 5, anti-TFα-161-189; lane 6, TF8-5G9; lane 7, TF9-6G4.

the TF extracellular domain appeared to be more reactive compared with antibodies reactive with more N-terminal aspects of the protein, if antibody binding to nitrocellulose-bound TF was analysed. Two lower-molecular-mass fragments were detected by the sequence-specific antibody to TF-161-189 which encompass the N-terminal sequence encoded by exon 5. The reactivity of anti-TF-161-189 with a fragment of approx. 18 kDa indicates that the fragment may be identical to a predominant V8 protease fragment, with Phe¹⁷⁵ as the N-terminal residue (Morrissey *et al.*, 1987). TF8-5G9 was not reactive with these fragments, suggesting that the TF8-5G9 epitope was encoded by exon 4 rather than exon 5. TF8-5G9 does not react with reduced TF (Morrissey *et al.*, 1988b). Since the analysis was performed under non-reducing conditions, the lack of reactivity with the 18 kDa fragment encompassing the C-terminal disulphide loop (Cys¹⁸⁶–Cys²⁰⁹) of TF indicates that TF8-5G9 is not reactive with the disulphide loop itself or adjacent sequences alone. We therefore suggest that residues in exon 4 contribute to the TF8-5G9 epitope, which is dependent on participation of the disulphide loop in exon 5 for proper expression. We tested binding of TF8-5G9 to short (10–30 residues) synthetic peptides based on the TF sequence, as well as competition of synthetic peptides with TF binding of TF8-5G9. Both attempts to further localize the epitope of TF8-5G9 failed. These data are consistent with current concepts of conformational epitopes on proteins which are formed by 15–22 amino acid residues in a non-linear sequence display on several surface-oriented loops of proteins (Laver *et al.*, 1990).

The anti-(locus II) MAb TF9-6G4 was reactive with certain V8 protease fragments which were also detected by purified anti-peptide antibodies to an N-terminal proximate aspect of TF (Fig. 2). The patterns of recognition of TF by anti-(locus I) MAb TF8-

Fig. 3. Reactivity of MAb TF9-6G4 with deletion mutants of TF

Immunoreactivity of TF9-6G4 with C-terminal deletion mutants of TF fusion proteins is shown. A 1:500 dilution of a polyvalent goat antiserum to TF or MAb TF9-6G4 at 20 µg/ml was incubated with the transferred fusion protein for 1 h at 37 °C, followed by detection with an appropriate alkaline-phosphatase-conjugated secondary antibody. Based on DNA sequencing, the fusion proteins with TF1-157 (a) and TF1-83 (b) had molecular masses of 36 kDa and 26 kDa respectively. Lane 1, αTF; lane 2, TF9-6G4; lane 3, control MAb.

5G9 and anti-(locus II) MAb TF9-6G4 were different, although identical digests of TF were used. To further address this tentative conclusion, a series of progressive C-terminal deletion mutants of TF were generated and expressed in *E. coli*. Sequence analysis of selected mutants confirmed that the observed molecular mass of the fusion protein was consistent with that predicted from the DNA sequence (Fig. 3). Reactivity of TF9-6G4, but not of an unrelated control MAb, was observed with anti-TF-1-157 and anti-TF-1-83 (Fig. 3). TF-1-64 was consistently not reactive with this MAb, although the polyclonal anti-TF antibody revealed expression of TF epitopes by the fusion protein (results not shown). These data demonstrate localization of the TF9-6G4 epitope within TF-1-83. The loss of reactivity upon deletion of amino acids 64–83 suggest either loss of critical residues contributing to the epitope or loss of the proper conformation of the epitope due to the removal of stabilizing and adjacent residues. TF9-6G4 binding to short synthetic TF peptides could not be demonstrated. However, binding of anti-TF-40-71 to TF in a dot-blot assay was blocked by anti-(locus II) MAbs, but not by anti-(locus I) MAbs in comparison with an irrelevant control MAb (results not shown). This, and the similarity in the pattern of TF9-6G4 reactivity (Fig. 2) compared with that of the antibody specific for the linear sequence TF-40-71, indicates that the locus II epitope is localized within residues 40–83 of exon 3.

DISCUSSION

Both MAbs and sequence-specific polyclonal antibodies represent potential probes for biologically active sites of the protein surface and have been successfully used to delineate functional regions of proteins (Dueweke & Gennis, 1990; Brakenhoff *et al.*, 1990; Westmeyer *et al.*, 1990). Both types of antibody have been used in this study to examine functional binding regions in TF, the cellular receptor and cofactor for VIIa. A panel of sequence-specific antibodies to synthetic peptides based on the TF sequence were characterized for interference with VII binding to TF, a prerequisite for cofactor function. Antibodies against TF-40-71 as well as against TF-94-121 intervened in VII binding to TF. These data support a conclusion that more than a single linear sequence in the TF extracellular domain provides the structural basis for the interaction of TF with its ligand, VII. This was

further supported by characterization of the epitopes for two representative neutralizing MABs.

MABs TF8-5G9 and TF9-6G4 were demonstrated to react with non-overlapping epitopes. Additional MABs were reactive with the same loci identified by TF8-5G9 (locus I) and TF9-6G4 (locus II). Among the MABs reactive with locus II was TF9-5B7, a MAB which has been successfully used to prophylactically intervene against the lethality of gram-negative septic shock in baboons (Taylor *et al.*, 1991). Both anti-(locus I) MAB TF8-5G9 and anti-(locus II) MAB TF9-6G4 inhibited VII binding to TF in a concentration-dependent manner. This suggests either that the MAB epitopes overlap the VII binding site or that binding of the MABs conformationally perturbs TF to prevent VII binding. However, this is not a general property of any antibody associated with TF, since certain sequence-specific antibodies as well as one MAB (TF9-10H10) were shown not to interfere in TF receptor function.

We here demonstrate that the neutralizing MAB TF8-5G9 reacted with a proteolytic fragment spanning the C-terminal half of the TF extracellular domain starting at residue Thr¹⁰⁸, the amino acid encoded by the 5' codon of exon 4. This MAB was not reactive with shorter fragments spanning most of the sequence of exon 5, suggesting that exon 4 encodes residues critical to this locus I epitope. Reactivity of anti-(locus II) MAB TF9-6G4 with proteolytic fragments as well as with progressive C-terminal deletion mutants of TF suggested that within the sequence 40–83 are residues necessary for this epitope. This sequence shows a rather low degree of identity to the mouse TF sequence (Hartzell *et al.*, 1989), thus making it a potential epitope. The analysis of MAB epitopes thus confirms the inhibition pattern observed with antibodies specific for linear sequences of TF. Both analyses establish that binding of antibodies to epitopes encoded by exon 3 and exon 4 of TF results in a loss of TF receptor function. Therefore residues in more than one single exon-encoded linear sequence of TF are implicated in receptor function.

MAB-defined epitopes on proteins are most commonly constituted by residues which are not derived from a single linear sequence (Laver *et al.*, 1990), as demonstrated by crystallography of MAB–protein complexes and more recently by hydrogen–deuterium exchange protection analysis (Paterson *et al.*, 1990). MAB binding to a receptor may resemble the interaction of the natural ligand with respect to occupancy of a circumscribed solvent-accessible protein surface. The affinity of some MABs for binding to TF is comparable with and even exceeds that of the natural ligand, VII (Drake *et al.*, 1989). The MAB 'footprint' can reasonably be assumed to consist of spatially proximate amino acid residues rather than a linear sequence. This may explain the advantage of the MAB analysis presented here and the difficulties we have encountered in identifying peptidyl mimetics based on the linear sequence of TF. Mimicry of one site by a linear peptide, even if adopting a favourable conformation, may not result in a sufficient loss of binding function when several additional sites contribute significantly to the binding energy. The functional regions of the TF sequence identified by the antibody analysis are thus likely to represent regions on the TF surface which are part of or in proximity to the VII-interactive sites. These could consist of two remote sequences forming a complex contiguous region of the folded protein or of two spatially separate interactive areas which interact with independent sites on VII. The interaction with independent and separate sites on VII might represent the structural counterpart of the two functional properties of TF, namely to serve as receptor and cofactor for VIIa. Recent studies

have indeed identified two structural sites on VIIa which contribute to the high-affinity binding of the protease to its receptor, namely the VIIa γ -carboxyglutamic acid-domain (Sakai *et al.*, 1990) and residues 195–206 in the catalytic domain (Wildgoose *et al.*, 1990). In addition, a distant homology search suggested that TF is related to the interferon and cytokine receptor superfamily (Bazan, 1990). Accordingly, the TF extracellular domain may consist of two structurally similar domains in tandem, each of which provides an interactive site for the ligand (Bazan, 1990). Our study would be consistent with the proposed model, which will require additional verification by independent approaches.

This is publication 5682B-IMM from the Department of Immunology. This work was supported by National Institutes of Health grant PO1 HL-16411 from the National Heart, Lung and Blood Institute. Fellowship support was obtained by the Deutsche Forschungsgemeinschaft (W.R.) and the California Chapter of the American Heart Association (A.R. and W.R.). The excellent technical assistance of Jennifer Royce, Pablito Tejada and David Revak is acknowledged. We thank Barbara Parker for assistance in the preparation of the manuscript.

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