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Two sites in the tissue factor extracellular domain mediate the recognition of the ligand factor VIIa

(coagulation cascade/photocrosslinking/ligand recognition/receptor–ligand interaction)

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ABSTRACT **Tissue factor (TF) binds the serine protease coagulation factor VIIa and initiates the coagulation protease cascade by forming a catalytic cofactor–enzyme complex. Using a photoactivatable crosslinking reagent coupled to factor VIIa, we have identified interactive sites in the amino-terminal (residues 44–84) and the carboxyl-terminal (residues 129–169) aspect of the extracellular domain of TF. Epitopes of inhibitory antibodies have previously indicated participation of these regions in TF function. The presence of the γ-carboxyglutamic acid domain in factor VIIa appears to facilitate the interaction with the negatively charged, amino-proximate site, whereas crosslinking of TF with VIIa or des-(1–38)-VIIa at the positively charged carboxyl-proximate site was similar. Lack of α-helical secondary structure in the TF extracellular domain is consistent with the proposed structural similarity of TF with the cytokine receptor family. The interactive sites identified for TF are located in sequence spans that demonstrate a low degree of sequence conservation among the members of this receptor family. Regions with highly conserved residues, such as sequences encoded by exon 2 and 5 in TF, were not implicated in ligand recognition, suggesting that conserved residues in the receptor family may maintain the common β-strand architecture, and variable regions provide a pair of nonidentical motifs for oriented ligand recognition.**

The coagulation serine protease cascades can be initiated by tissue factor (TF), a cell-surface receptor and specific cofactor for the serine protease factor VIIa (VIIa) (1, 2). TF exhibits distant sequence and predicted architectural similarity to the interferon receptors, and homology of an ≈200-amino acid unit in the extracellular domains of these receptors with the cytokine receptor family has been proposed (3). The TF extracellular domain provides the sites for high-affinity binding of the protease ligand VIIa (4, 5). This interaction is necessary for conformational alterations in VIIa, resulting in a markedly enhanced catalysis of substrates (4, 6, 7). There are at least two regions in VIIa that appear to participate in the interaction with TF to form a catalytic binary complex (5, 8–10). Because the covalent disulfide bonding of the carboxyl cysteine pair in TF is required for binding of VIIa, structure necessary for binding of VIIa may be localized in the carboxyl aspect of the TF extracellular domain (11). In addition, epitope mapping of anti-TF monoclonal antibodies that inhibit binding of VIIa (12) suggests that more than one exon-encoded linear sequence in TF may participate in the assembly of VIIa. In the present study, we have used chemical crosslinking from the ligand VIIa to search the TF extracellular domain for nearest neighbor residues. This analysis provides evidence for two independent sequence spans of ≈40 residues in the amino- as well as in the carboxyl-terminal aspect of the TF extracellular domain, which are in contiguity with VIIa, and leads to the hypothesis of a pair of nonidentical interactive sites between the receptor and its ligand to achieve oriented assembly.

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EXPERIMENTAL PROCEDURES

Reagents. Recombinant TF was expressed in *Spodoptera frugiperda* (Sf9) insect cells using the baculovirus expression system (Invitrogen, San Diego). Cells were lysed with Triton X-100 (13), and TF was purified on immobilized monoclonal antibody, as described (14). Protein C was obtained as a byproduct during the standard factor X purification (4). The heterobifunctional crosslinking reagent sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate (SASD) and Iodo-Gen were purchased from Pierce. Trypsin (EC 3.4.21.4), *Staphylococcus aureus* V8-protease (endoproteinase Glu-C, EC 3.4.21.19) and the protease for specific cleavage of peptide bonds amino-terminal of aspartic acid residues (endoproteinase Asp-N) were obtained from Boehringer Mannheim.

Photoaffinity Labeling and Proteolytic Digestion of TF. The heterobifunctional crosslinking reagent SASD was coupled to recombinant VIIa (Novo Nordisk, Gentofte, Denmark) at a 1:1 ratio of crosslinker to amino groups in VIIa followed by radioiodination of the crosslinker with ^{125}I using Iodo-Gen. The modified VIIa retained 80% of specific activity, when tested in a coupled amidolytic assay with an excess of purified and phospholipid-reconstituted TF and factor X as a substrate (4). Purified TF (1 μM) solubilized in 250 μM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was incubated with ^{125}I-labeled SASD-conjugated VIIa (0.5 μM) for 10 min at 5 mM $CaCl_2$ to allow TF–VIIa complex formation. The photoaffinity crosslinker was then activated for 4 min by using a Rayonet photochemical reactor (Southern N.E. Ultraviolet, Middletown, CT) equipped with UV lamps with peak output at 254 nm. The TF–VIIa complex was not active after the crosslinking, presumably due to intramolecular crosslinking in VIIa, which may inactivate critical residues in VIIa or may limit the flexibility in the VIIa protease domain. After reduction, TF was separated from VIIa by SDS/PAGE (15). The TF eluted from this first gel was applied to a second gel together with *S. aureus* V8-protease for an initial proteolytic digestion of TF in the stacking gel, according to Cleveland *et al.* (16). After separation, proteolytic fragments were eluted from gel pieces in the presence of 0.1% SDS. More than 95% of the radioactivity was recovered. Fragments were purified by reverse-phase HPLC using a Vydac C_4 column in 0.1% trifluoroacetic acid (buffer A) and linear gradient elution with acetonitrile/0.08% trifluoroacetic acid (buffer B). No radioactivity was retained on the guard column, excluding that reactive and strongly hydrophobic fragments were not recovered. After lyophilization, the HPLC-purified peptides

Abbreviations: TF, tissue factor; VIIa, factor VIIa; SASD, sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate.

were resuspended in appropriate buffers for the enzymes and further digested in solution. Proteolytic fragments were separated by Tricine-PAGE (17) and transferred (18) to ProBlott sequencing membranes (Applied Biosystems) for amino acid composition and amino-terminal sequence analysis (4).

Circular Dichroism Analysis. Circular dichroism spectra were generated on a Jasco J700 instrument with TF_{1-219} diluted in phosphate-buffered saline, pH 7.0, to 8 μM. The scan speed was 10 nm/min. The percentage of α-helix, β-strand, β-turn, and random-coil secondary structure was calculated by using software from Jasco (Easton, MD) based on the procedure described by Yang *et al.* (19).

RESULTS

Specific Photoaffinity Labeling of TF by SASD–VIIa. Ligand recognition sites on TF were identified by chemical crosslinking of contiguous amino acid side chains using an ^{125}I-labeled, heterobifunctional and photoactivatable crosslinking reagent (SASD) coupled to the amino groups of the ligand VIIa. VIIa retained function (>80%) when treated with SASD at a 1:1 molar ratio of crosslinker to amino groups of VIIa. This modified VIIa and purified TF solubilized with CHAPS were incubated at 5 mM Ca^{2+} for 10 min. We have previously shown that VIIa binds to TF in the absence of phospholipid and that the affinities of VIIa binding to TF were similar in solution and on an uncharged phospholipid surface (5). After complex formation, the radiolabeled arylazide groups of the reagent were activated by UV light to permit crosslinking to residues on TF. Specificity was substantiated by the inhibition of crosslinking by EDTA, which prevents the TF–VIIa interaction or an excess of unconjugated VIIa (Fig. 1*A*). Incorporation of the radiolabel on the donor group of the crosslinker into TF was reduced 85% in the presence of EDTA and 73% at a 5-fold molar excess of unconjugated VIIa compared with the uninhibited reaction at 5 mM $CaCl_2$, whereas a 5-fold molar excess of the homologous, but noninteracting protein C relative to the conjugated VIIa did not block the photoaffinity labeling of TF (Fig. 1*A*).

The photoaffinity-labeled TF was separated from VIIa after reduction by SDS/PAGE. TF was then applied to a second gel for digestion with *S. aureus* V8-protease. After digestion and separation, most radioactivity was recovered and found associated to a similar extent with two fragments (Fig. 1*B*) of 14–16 kDa (58 ± 3%) and 8–12 kDa (42 ± 3%; mean ± SD, n = 5), based on densitometry of autoradiographies of prolonged incubations, which demonstrated complete digestion of the 20–27 kDa to the lower-molecular-weight fragments. Amino-terminal sequence analysis demonstrated that the 8- to 12-kDa fragment (2) was derived from the amino terminus (exon 2- and 3-encoded sequence), and the 14- to 16-kDa fragment (1) was derived from the carboxyl-terminal half (exon 4- and 5-encoded residues) of the TF extracellular domain (20). Deletion of the γ-carboxyglutamic acid domain in VIIa [des-(1–38)-VIIa] results in binding to TF with lower affinity, suggesting two distinct interactive sites in VIIa, which act cooperatively to provide high-affinity binding (5). When TF was crosslinked with SASD conjugated to des-(1–38)-VIIa under the same conditions as used for intact VIIa, 82 ± 5% (n = 5) of the total radioactivity was found associated with the carboxyl-proximate crosslinking site, demonstrating a considerable reduction in crosslinking to the amino-terminal site. This suggests that a γ-carboxyglutamic acid-domain-dependent site in VIIa may interact with the amino site in TF and a γ-carboxyglutamic acid-domain-independent site in VIIa may associate with the carboxyl site in TF, resulting in the tight binding of ligand by receptor. To localize the crosslinking sites in the amino and carboxyl aspect of the TF extracellular domain, the initial V8-

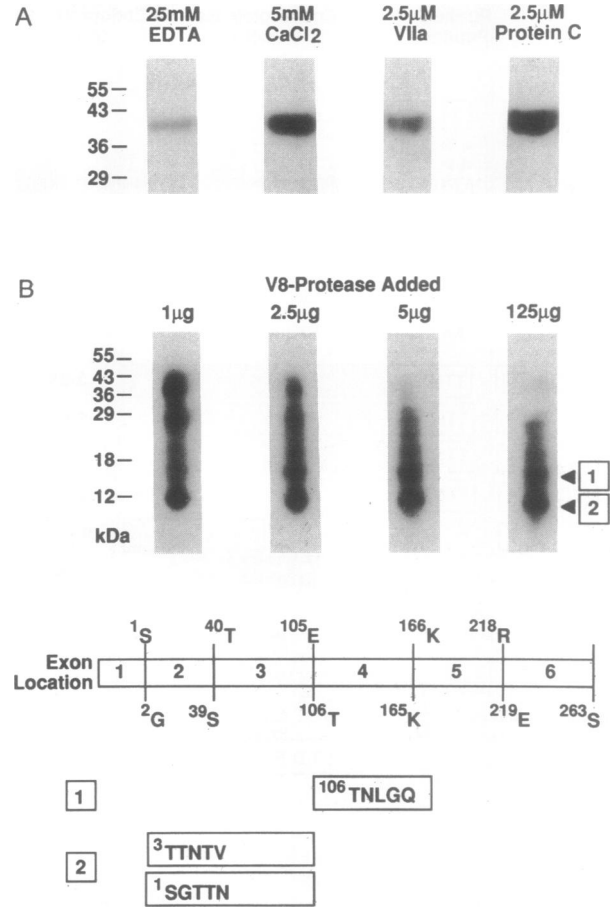

FIG. 1. Photoaffinity labeling of ligand-recognition sites in TF. (*A*) Autoradiography shows photoaffinity-labeled and gel-purified TF. With 25 mM EDTA, complex formation was prevented, and densitometry demonstrated an 85% decrease of radioactivity in comparison with the reaction at 5 mM $CaCl_2$. At 5 mM $CaCl_2$, a 5-fold molar excess of nonmodified VIIa inhibited crosslinking by 73%, whereas an identical concentration of the homologous serine protease zymogen protein C, which does not associate with TF, was noninhibitory (117% of control). (*B*) TF that was photoaffinity-labeled in the presence of 5 mM $CaCl_2$ was digested with the indicated concentrations of V8-protease for 30 min in the stacking gel. The fragments were electrophoretically transferred to sequencing membranes for amino-terminal sequencing. Fragment 1 demonstrated sequence derived from the carboxyl half of the TF extracellular domain (yield, 0.8 pmol of threonine). Fragment 2 had two predominant sequences (yield, 1 pmol of threonine and 0.4 pmol of serine) reflecting the typically staggered amino terminus of TF. Alignment of these sequences with the exon boundaries (20) is indicated in the schematic diagram. Amino acid residues are depicted in one-letter codes.

fragments 1 and 2 were isolated by elution from gel slices and separation by reverse-phase HPLC.

Identification of the Amino-Proximate Crosslinking Site. The 8- to 12-kDa V8 fragments eluted as a single peak from the C_4-reverse-phase column and were resolved by gel electrophoresis into two distinct bands (Fig. 2*A*). Based on amino acid composition and amino-terminal sequence analysis, fragment 3 was identified as residues 3–95, and fragment 4 was identified as residues 3–84. Amino-terminal sequencing of both fragments also demonstrated a minor sequence starting with Ser-1, reflecting the known alternative propeptide cleavage of TF (21). The purified peptide was further digested with endoproteinase Asp-N, and two predominant fragments were obtained. Fragment 5, corresponding to residues 3–60, contained the radioactive crosslinker, and the further degraded fragment 6, corresponding to residues 3–43, lacked

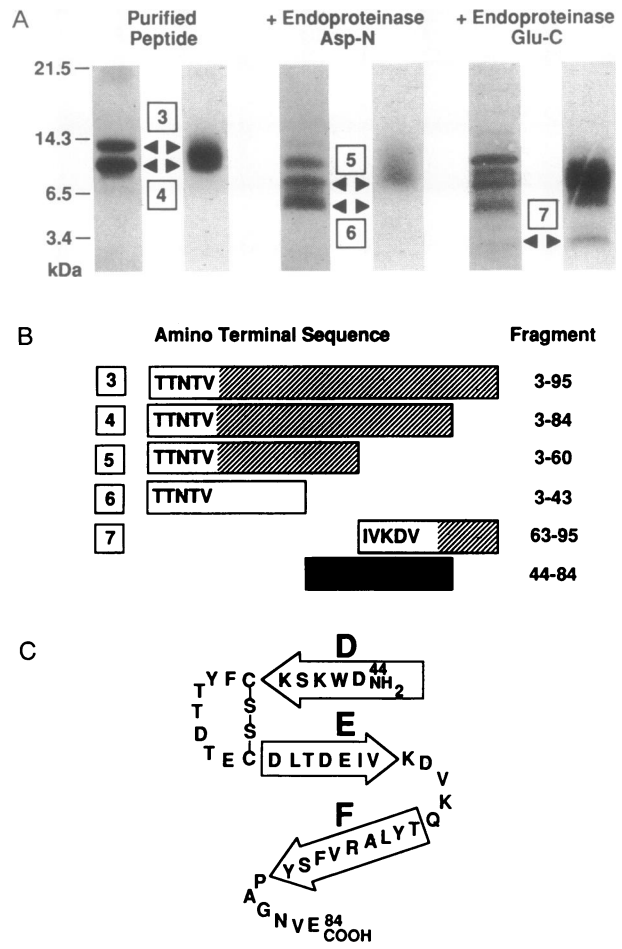

FIG. 2. Localization of the amino-terminal crosslinking site. (*A*) The 8- to 12-kDa peptides from V8-protease digest (Fig. 1*B*) were purified on reverse-phase HPLC. The fraction (fragments 3 and 4) is shown stained with Coomassie brilliant blue after transfer (at left), and crosslinking is visualized by autoradiography (at right). Proteolytic digests of the purified peptide (fragments 5, 6, and 7) with indicated enzymes are similarly displayed. (*B*) Identification of fragments in *A*. Hatched bars were used for radioactive fragments, white bars represent nonradioactive fragments, and the black bar indicates the suggested ligand-recognition site. Sequence range indicated (fragment) is based on amino acid compositions and is consistent with observed electrophoretic mobility. Yields were as follows: fragment 3 (0.3 nmol) and fragment 4 (0.9 nmol) by amino acid composition; fragment 5 (14 pmol of threonine), fragment 6 (36 pmol of threonine), and fragment 7 (14 pmol of isoleucine) by amino-terminal sequencing. (*C*) Schematic alignment of the identified ligand-recognition site in an immunoglobulin-like fold. Orientation of arrows indicates alignment of β-strands in two predicted β-sheets. Sequences are given by the one-letter code for amino acids.

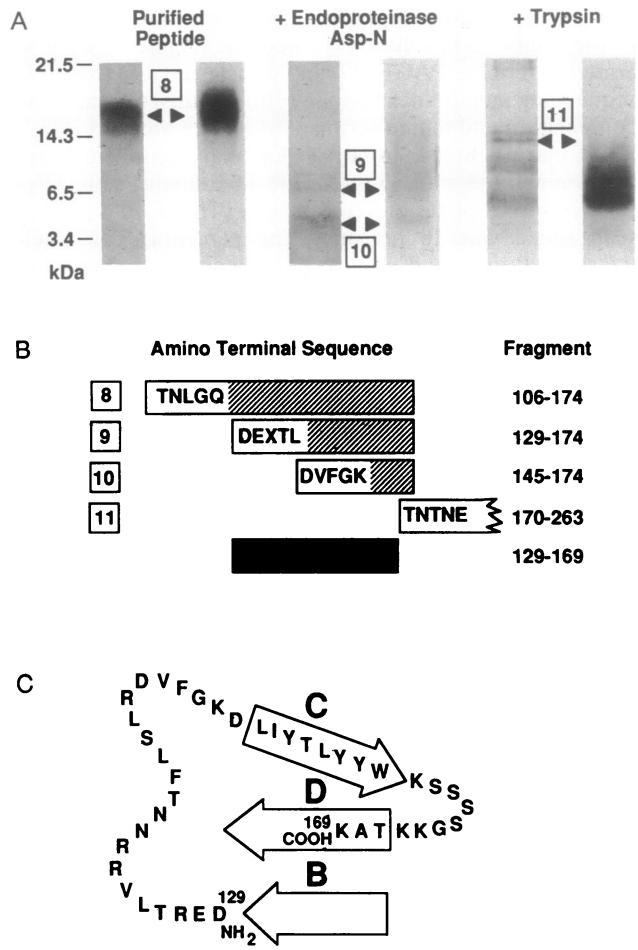

FIG. 3. Localization of carboxyl-terminal crosslinking site. (*A*) The 14- to 20-kDa peptides from V8-protease digest (Fig. 1*B*) were purified by reverse-phase HPLC. The carboxyl-terminal peptide (fragment 8) and its proteolytic fragments (fragments 9 and 10) obtained with endoproteinase Asp-N, as well as a tryptic fragment derived from photoaffinity-labeled, intact TF (fragment 11) is shown by Coomassie staining (at left) and autoradiography (at right). Identification of fragments (*B*) and schematic diagram of the recognition site (*C*) are shown as in Fig. 2. Yields were as follows: fragment 8 (140 pmol) by amino acid composition; fragment 9 (12 pmol of aspartic acid), fragment 10 (32 pmol of aspartic acid), and fragment 11 (10 pmol of threonine) by amino-terminal sequencing.

the radioactive crosslinker (Fig. 2). This excluded exon 2-encoded protein (residues 2–39) and identified sequences starting at residue 44 as part of the ligand-recognition site. This site extended beyond residue 63 because digestion of the amino-terminal fragment with V8-protease (endoproteinase Glu-C) yielded fragment 7 spanning residues 63–95, which had reacted with the radioactive arylazide groups of the crosslinker (Fig. 2). Although not directly excluded, sequences beyond residue 84 are unlikely to contribute to the recognition site because incorporation of radioactivity into fragments 3 (residues 3–95) and 4 (residues 3–84) was quantitatively comparable (Fig. 2*A*).

Identification of the Carboxyl-Proximate Crosslinking Site. The 14- to 18-kDa fragment (8) from the initial V8-protease digest was identified as residues 106-174 (Fig. 3). Digestion of the peptide with endoproteinase Asp-N yielded radioactive peptides containing residues 129–174 (9) and 145–174 (10), whereas repeatedly there was no linkage of the radioactive donor with peptides smaller than 3.5 kDa where peptides (106–128) corresponding to the amino terminus of the exon 4-encoded protein sequence would be present. Although unlikely, we cannot exclude that the 106–128 fragment did not bind to the sequencing membrane and was therefore lost during the electrophoretic transfer. We identified a nonradiolabeled fragment (11) from a partial tryptic digest of photoaffinity-labeled TF that had not been digested with V8-protease. Fragment 11 started at residue 170 and appeared to span the entire carboxyl terminus of TF based on the observed molecular weight (Fig. 3). In addition, we obtained fragments smaller than 8 kDa from the initial V8 digest, which were identified as residues 175–219 by amino acid composition and which also were not labeled by the crosslinker (data not shown). Exon 4-encoded sequences spanning residues 129–169 are, therefore, suggested to provide the carboxyl-terminal VIIa-interactive site.

Alignment of the Recognition Sites in a β-Strand Architecture. Based on sequence-alignment methods and structural

pattern-matching techniques, a similar structural architecture has been proposed for TF and the interferon receptors; this homology was suggested to extend to the extracellular domain of ≈200 amino acid residues, which is common to most members of the cytokine receptor family (3). The basic structural motif of this receptor family is a repeated module of ≈100 amino acids (3, 22), which is encoded by two exons in human TF (20) and growth hormone receptor (23). The modules appear remotely related to the fibronectin type III repeat with a suggested β-strand structure organized into an immunoglobulin-like fold (22, 24). Alignment of the cytokine receptor extracellular-domain sequences to this type of global fold results in two modules, each of 7 (3) or 8 (22) predicted β-strands, which form two sheets of anti-parallel β-strands. Because no crystal structure for any member of the receptor family is known, support for these inferences was taken from a published circular dichroism analysis of an apparently homogeneous protein preparation with TF activity that had only minor α-helical structure (25). However, amino acid composition, carbohydrate content, and the specific activities presented for this protein are not consistent with TF, suggesting that TF represented only a minor fraction in the preparation.

To provide evidence for the proposed β-strand architecture of the TF extracellular domain, purified recombinant TF extracellular domain (4) was used for circular dichroism analysis (Fig. 4). This demonstrated lack of α-helical structure and a high percentage of β-strand and random coil with some β-turn secondary structure (Fig. 4). One can, therefore, reasonably conclude that the extracellular ligand-binding domain of TF is organized in multiple β-strands. Following Bazan's alignment of the TF sequence into a pair of seven β-strand modules of ≈100 amino acid residues (3), the deduced recognition site in the amino-terminal module is formed by D, E, and F β-strands and their connecting surface loops (Fig. 2C). Strand D is located at the edge of the β-sheet and is the least-conserved strand in the receptor family (3). The site in the carboxyl-terminal module (Fig. 3C) also included strand D as well as strand C, and the C–D and C–B connecting loops. The C–B loop in the immunoglobulin V domains typically participates in the antigen-recognition site

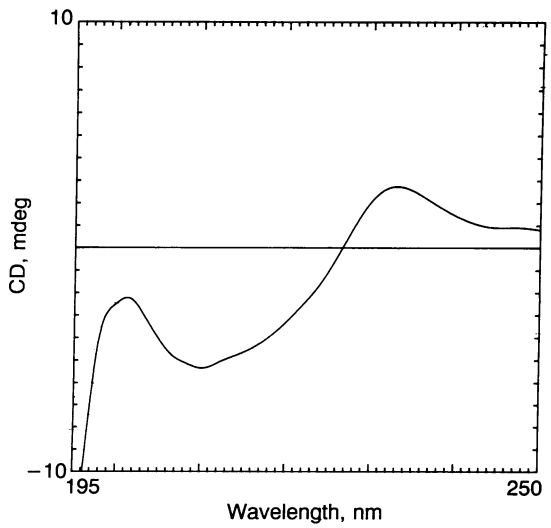

FIG. 4. Circular dichroism spectrum of isolated TF extracellular domain. The spectrum was generated from a sample of purified TF_{1-219} (8 μM), a recombinant TF mutant lacking transmembrane and cytoplasmic domains (4). Repeated analysis indicated lack of α-helix; the data indicating 62–65% β-strand, 23–25% random coil, and 11–13% β-turn secondary structure were obtained from a computer-generated estimate. The circular dichroism (CD) is depicted in millidegrees (mdeg) plotted versus the wavelength.

(26, 27), and this connecting loop is typically extended in the TF/interferon receptor class in comparison with the remaining members of the receptor family (3). Alignment of the ligand-recognition sites in TF with the proposed β-strand architecture, therefore, suggests that nonidentical sets of β-strands form the two ligand sites, one in each of the predicted structural modules of TF.

DISCUSSION

Using functional ligand that has been modified by addition of a photo-activatable crosslinking reagent, we have identified two independent sites for ligand recognition in the extracellular domain of TF. This analysis is consistent with inhibitory sequence-specific polyclonal antibodies and with the epitope assignment of inhibitory monoclonal antibodies, which also has implicated two regions of this receptor as important for function (12). These independent approaches provide convergent evidence for two interactive sites—one in the amino-terminal (N-module) and one in the carboxyl-terminal (C-module) predicted module of the TF extracellular domain.

Sequence-specific polyclonal and monoclonal antibodies to exon 2- and exon 5-encoded regions of TF were noninhibitory, consistent with the lack of crosslinking to these sequence spans. The carboxyl-terminal aspect of the C-module is highly conserved among members of the cytokine receptor family and includes the Trp-Ser-Xaa-Trp-Ser (WSXWS) motif (corresponding to residues 201–205 in TF), which has been suggested to be functionally important by analogy to the Arg-Gly-Asp motif found in approximately the same position in the tenth type-III module of fibronectin (24). In contrast to the lack of crosslinking to the carboxyl aspect of the C-module, the disulfide bonding of the carboxyl cysteine pair appears required for TF function, suggesting a critical importance of the bond for maintaining C-module structure (11). Thus, the highly conserved regions, such as exon 2- and 5-encoded sequences in TF, may be critical for maintaining the characteristic fold of the ligand-binding domain in the receptor family, whereas specificity for cognate ligand is imparted by the variable sequences in nonconserved β-strands and connecting loops. This receptor family may, therefore, follow a general paradigm that nonconserved regions provide specific function within the conserved structural framework of a family of proteins, as shown for the immunoglobulin domains (28).

We have previously shown that des-(1–38)-VIIa, which lacks the γ-carboxyglutamic acid domain, binds to TF only with reduced affinity (5). When des-(1–38)-VIIa was treated with SASD and used to crosslink TF, the radioactive arylazide group was preferentially crosslinked to the C-module. This result suggests that the negatively charged N-module may contribute to tight binding that is γ-carboxyglutamic acid-domain dependent. One can consider that coordinated complex formation of the negatively charged residues in the amino-proximate site could occur with Ca^{2+} bound to the γ-carboxyglutamic acid domain of VIIa, resulting in an interaction that has some similarity to the association of γ-carboxyglutamic acid domains with negatively charged phospholipid (29). The C-module of TF appears to interact with VIIa via a γ-carboxyglutamic acid domain-independent site, which is sufficient for low-affinity binding of VIIa by TF at micromolar Ca^{2+} concentration (5). This γ-carboxyglutamic acid-domain independent site could be located in the epidermal growth factor-like domain of the VIIa light chain (5) or may involve residues in the catalytic domain (8, 10). Because the latter low-affinity interaction of VIIa with TF is sufficient to impart efficient catalytic function to the VIIa protease domain, a role of the C-module in cofactor function of TF is inferred.

Each of the ligand-recognition sites identified in the N-module and C-module contains the infrequent tripeptide sequence Trp-Lys-Ser, which is repeated three times in TF (21). Nevertheless, the sites are nonidentical based on alignment with β-strands and by their net charge, which is negative in the amino-terminal and positive in the carboxyl-terminal module. This fact supports the idea that the repeat modules may form an oriented recognition structure to accommodate specific ligand. Because VII forms a 1:1 stoichiometric complex with TF (30, 31), both ligand-recognition sites in TF are probably occupied by complementary sites on one molecule of VII. Interaction between ligand and receptor involving more than one contact would enhance the affinity and would be characterized by slow dissociation of ligand from the receptor, as demonstrated for VIIa binding to TF (32, 33). Further, two nonidentical sites can more effectively impart both orientation and conformational alteration of the ligand for recognition of additional proteins on the cell surface. This relationship is exemplified by preferential recognition of cell-surface-associated substrate by the TF–VIIa complex (4). The assembly of multisubunit complexes on the cell surface is similarly important to mediate cytokine effects on their respective target cells. Interaction of a cytokine with two or more receptor proteins appears essential for transduction of signals to the responsive cell (34–36). The primary association of ligand with receptor may not only impart significant allosteric effects on the bound ligand and thus facilitate interaction of the ligand with an effector cell-surface protein (4, 34) but may influence ordered alignment between cell-surface receptors in their intra- and/or extracellular domains, resulting in the generation of biological signals.

Note Added in Proof. The recent analysis (37) of the hormone-binding determinants in the growth hormone receptor, a member of the cytokine receptor family and suggested related molecule to TF, provided circular dichroism analysis that, similar to our analysis, is consistent with the proposed structural model for the cytokine/interferon receptor family. In addition, residues with the strongest contribution to the binding of growth hormone by its receptor (W-104, P-106) align with the E–F loop in the amino-terminal recognition site in TF. This may indicate that certain sequence spans serve as ligand recognition sites in several members of the receptor family.

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