Identification of the Factor VIIa Binding Site on Tissue Factor by Homologous Loop Swap and Alanine Scanning Mutagenesis

Craig S. Gibbs,* Sarah N. McCurdy, Lawrence L. K. Leung, and Lisa R. Paborsky*

Gilead Sciences, Inc., Foster City, California 94404

Received May 24, 1994; Revised Manuscript Received September 14, 1994[⊗]

ABSTRACT: Tissue factor (TF) is a membrane-bound glycoprotein that functions as a cofactor for coagulation factor VIIa (VIIa) and initiates blood coagulation at sites of vascular injury. On the basis of sequence alignments, TF was predicted to be a member of the cytokine receptor superfamily. Utilizing the structural information available for the cytokine receptor superfamily, we have used site-directed mutagenesis to identify the binding site on TF for VIIa. The predicted loop regions in TF were systematically replaced with the homologous loops from the γ-interferon receptor (γ-IFN-R), the protein most related to TF in the superfamily of cytokine receptors. Six discontinuous regions (residues 16−20, 40−46, 60−69, 101−111, 129−151, 193−207) were identified that are required for interaction with VIIa and enhancement of activity. Individual substitution of 68 residues within these loops with alanine revealed eight residues (K20, D44, W45, K46, Q110, R135, F140, V207) that are required for cofactor activity. These residues fall into two groups, those that are required only for interactions with VIIa (K46, Q110, R135, F140, V207) and those that are also required to induce the conformational change in VIIa required for enhanced activity (K20, D44, W45). The discontinuous regions of TF required for interactions with VIIa form a single binding surface for VIIa that is analogous to the interface defined by the crystal structure of the complex between growth hormone and its receptor. This suggests that in addition to the conservation of structure between the members of the cytokine receptor superfamily there is also conservation of the mode of ligand binding despite the structural and functional diversity among the ligands.

Tissue factor (TF) is a membrane-bound glycoprotein that acts as a receptor for coagulation factor VII (VII) and factor VIIa (VIIa). TF participates in the proteolytic activation of VII to VIIa (Nakagaki et al., 1991; Yamamoto et al., 1992) and functions as a cofactor for VIIa, enhancing the proteolytic activity of VIIa toward its physiological substrates, coagulation factor IX (IX) (Østerud & Rapaport, 1977) and factor X (X) (Nemerson, 1966). The proteolytic activation of IX to IXa and X to Xa by the TF/VIIa complex leads to the activation of prothrombin and the formation of a fibrin clot (reviewed in Bach, 1988; Nemerson, 1988; Edgington et al., 1991). TF is expressed on the surface of a wide variety of cells in the extravascular space but is not normally expressed on cells in contact with the circulation. Vascular damage or induction of TF expression on monocytes and endothelial cells by various inflammatory signals such as bacterial lipopolysaccharide (Crossman et al., 1990), TNF-α (Scarpati & Sadler, 1989) and IL-1 (Bevilacqua et al., 1984) can lead to the exposure of TF to circulating VII and VIIa. The ability of thrombin itself to stimulate the intrinsic pathway of blood coagulation by feedback activation of factor XI has led to the proposal that VIIa activation by TF is the major mechanism for the initiation of blood coagulation *in vivo* at sites of inflammation and vascular injury (Gailani & Broze, 1991). Because the interaction of TF with VII/VIIa is believed to be such a critical event in the initiation of blood coagulation, we attempted to identify the structural elements in TF that are involved in this interaction.

Human TF is a glycoprotein composed of 263 amino acids and consists of three domains. The extracellular domain (residues 1−219) contains three sites for N-linked glycosylation and four cysteine residues that form two disulfide bonds (C45−C57 and C186−C209) (Bach et al., 1988). However, the functional expression of both full-length recombinant human TF and a mutant TF (C245S) in *Eschericia coli (E. coli)* demonstrated that glycosylation was not required for TF activity (Paborsky et al., 1989). A truncated soluble form of TF (sTF) consisting of only the extracellular domain has been expressed in *E. coli,* cultured Chinese hamster ovary cells and *Saccharomyces cerevisiae* (Waxman et al., 1992; Ruf et al., 1991a,b; Shigematsu et al., 1992). The TF extracellular domain alone appears to be capable of forming high affinity interactions with VIIa and acting as a cofactor for VIIa, enhancing the hydrolysis of small peptidyl substrates and the proteolytic activation of X to Xa. The broad range of affinities reported for this interaction (0.5−100 nM) is generally reduced compared to the values reported for full length TF (7.0 pM−9.0 nM). We chose to analyze the interaction between VIIa and sTF because the interaction can be studied in the absence of potential complications due to the presence of detergents and phospholipids.

On the basis of sequence alignments it was predicted that TF is a member of the superfamily of cytokine receptors that includes receptors for a diverse group of growth factors, hematopoietic factors, interleukins, and interferons (Bazan, 1990a,b). The extracellular domains of the members of this cytokine receptor superfamily have a conserved structure composed of two tandemly repeated folding modules of approximately 100 residues. Each module consists of seven conserved β-strands connected by more variable loop regions

* To whom correspondence should be addressed: Gilead Sciences Inc., 353 Lakeside Drive, Foster City, CA 94404 (telephone 415-574-3000; Fax 415-573-4890).

[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1994.

0006-2960/94/0433-14003$04.50/0

assembled into two antiparallel β-sheets in a manner analogous to the constant domains of immunoglobulin molecules. These predictions were found to be generally correct upon the solution of the crystal structure of the extracellular domain of human growth hormone receptor (hGH-R) in a 2:1 complex with human growth hormone (hGH) (de Vos et al., 1992).

Utilizing the structural information available for the cytokine receptor superfamily to guide our mutagenesis strategy, we attempted to identify the regions of TF important for interactions with VIIa. The predicted loop regions in TF were targeted for mutation because the loop regions in proteins are often exposed on the protein surface and make important contributions to interactions with ligands (Rose et al., 1985; Kuntz, 1972). They are tolerant of considerable sequence variation (Bajaj & Blundell, 1984), and substitution of the residues in these regions has less potential for causing nonspecific structural disruptions. We systematically replaced the loop regions in TF with the homologous regions from the human γ-interferon receptor (γ-IFN-R), the closest relative to TF in the cytokine receptor superfamily. The homologous loop swap mutants were screened for TF cofactor activity. To identify the specific amino acids involved, individual residues in the TF loop regions identified as being important for interactions with VIIa were then substituted with alanine. The alanine replacement mutants were screened for cofactor activity, and the contribution of each residue to the binding energy of the TF/VIIa complex was determined by estimating the change in K_D attributable to the mutation.

EXPERIMENTAL PROCEDURES

Materials. VIIa was from Haematologic Technologies, Inc., and BiosPacific (Emeryville, CA); the chromogenic substrate, H-D-Isoleucyl-L-prolyl-L-arginine-*p*-nitroaniline dihydrochloride (S-2288) was from Chromogenix. The goat anti-TF antibody and the monoclonal antibody against TF (catalogue number 4503) were from American Diagnostica, Inc.; anti-FLAG M1 monoclonal antibody and anti-FLAG M1 affinity gel were from IBI; BCA (bicinchoninic acid) protein assay reagent was from Pierce.

Construction of Vectors for TF Expression. An expression vector, pT7-hTF(T)FLAG was constructed for the expression of sTF in *E. coli.* The 657 nucleotide fragment encoding the extracellular domain of human TF (amino acids 1−219) was isolated by PCR (GeneAmp kit, Perkin Elmer Cetus) from a cDNA clone of human TF, pHTF8 (ATCC 61351) (Scarpati et al., 1987). A stop codon (TAA) was introduced immediately after the codon for residue 219 and *Nsi*I sites were introduced at the 5′ and 3′ ends of the fragment. The fragment was ligated into the *Nsi*I site in pT7-3.0. pT7-3.0 is a pUC-based phagemid expression vector that contains the T7 RNA polymerase promoter upstream of a region encoding the translation initiation site and signal peptide from the ST II enterotoxin gene. This is followed by the T7 transcription termination region and the origin of replication for single-stranded bacteriophage, f1 (Dotto et al., 1981). Synthesis of single-stranded DNA and packaging into filamentous bacteriophage particles can be induced by a helper phage, M13KO7 (Vieira & Messing, 1987). The STII signal sequence in pT7-3.0 has been engineered to cryptically encode unique restriction sites for *Bsm*I, *Nru*I, *Mlu*I, and *Nsi*I that can be used to create fusions between any coding sequence and the ST II signal sequence. This fusion directs secretion across the bacterial inner membrane. High-level expression from this vector can be induced in *E. coli* strain BL21(DE3) containing pLysS (Studier et al., 1990) which supplies the gene for T7 RNA polymerase expressed from the *lac*UV5 promoter. This promoter can be induced by IPTG. Oligonucleotide-directed mutagenesis was used to insert a sequence encoding an octapeptide (DYKDDDDK) known as the FLAG motif (Hopp et al., 1988) at the junction between the carboxyl terminal of the STII signal sequence and the amino terminal of the coding region for sTF to create a fusion protein. The octapeptide motif serves as an epitope for a Ca^{2+}-dependent monoclonal antibody, M1.

Oligonucleotide-Directed Mutagenesis. The procedure used has been previously described in detail (Gibbs & Zoller, 1991). A uracil-containing, single-stranded template of pT7-hTF(T)FLAG was generated by isolating single-stranded DNA from a *dut*$^-$*ung*$^-$ strain of *E. coli*, CJ236, transformed with pT7-hTF(T)FLAG and infected with helper phage M13K07 (Kunkel et al., 1987). Synthetic oligonucleotides were designed that encode various predicted loop regions from the γ-IFN-R or single alanine substitutions flanked by regions of 15 nucleotides complementary to the pT7-hTF(T)-FLAG template. Mutagenic oligonucleotides were synthesized on an Applied Biosystems Inc. solid-phase synthesizer using phosphoamidite chemistry. Phosphorylated oligonucleotides were annealed to the pT7-hTF(T)FLAG template, extended by T7 DNA polymerase, and joined to the newly synthesized strand by DNA ligase. The reaction mixture was used to transform a *dut*$^+$*ung*$^+$ strain of *E. coli*, XL1-Blue, to select against the uracil-containing template strand. Single-stranded DNA from individual colonies was sequenced using dideoxy chain termination and Sequenase 2.0 (United States Biochemicals) to confirm the identity of the mutation. Single-stranded DNA was used to transform *E. coli* strain, BL21(DE3), containing pLysS for expression of mutant constructs.

Expression of sTF Fusion Proteins in E. coli. The sTF mutants were expressed in *E. coli* strain BL21(DE3) containing pLysS. Single colonies were picked from a plate of LB agar containing 200 μg/mL ampicillin and 30 μg/mL chloramphenicol and used to inoculate 2 mL of LB broth containing 200 μg/mL ampicillin and 30 μg/mL chloramphenicol. The 2 mL cultures were grown overnight at 37 °C (250 rpm) and 300 μL was used to inoculate 30 mL of LB broth containing 200 μg/mL ampicillin and 30 μg/mL chloramphenicol. The 30 mL cultures were incubated at 37 °C (250 rpm) for 6 h and induced by the addition of IPTG to a final concentration of 0.4 mM. The incubation was continued at 30 °C (250 rpm) overnight. Cultures were scaled up to 200 and 1000 mL final volumes as required.

Purification of sTF Fusion Proteins. Soluble TF was purified from *E. coli* strain BL21(DE3) containing pLysS culture medium, or combined periplasmic and cytoplasmic extracts, by affinity chromatography using the anti-FLAG M1 monoclonal antibody. The cells were harvested by centrifugation and stored at −20 °C, and the bacterial culture supernatant was concentrated by ultrafiltration. The cell paste was thawed and resuspended in 50 mM Tris-HCl and 150 mM NaCl, pH 7.5, containing 1 mg/mL lysozyme. The extract was sonicated and rotated end over end for 1 h at 4 °C. Poly(ethylenimine) (5% (w/v)) was added to a final concentration of 0.1%, and the extract was clarified by centrifugation. The resulting cell extract or concentrated

culture medium was adjusted to 1 mM $CaCl_2$ and then loaded at 4 °C (20 mL/h) onto the anti-FLAG M1 affinity resin preequilibrated with 50 mM Tris-HCl, 150 mM NaCl, and 1 mM $CaCl_2$, pH 7.5. The column was washed with 10 column volumes of the equilibration buffer and eluted with 50 mM Tris-HCl and 50 mM EGTA, pH 7.5. The fractions were collected and the peak was pooled by optical density at 280 nm. The pool was dialyzed extensively against 50 mM Tris-HCl and 150 mM NaCl, pH 7.5. The protein concentration of wild-type sTF was based on a BCA protein assay using bovine serum albumin as the standard.

ELISA. The amount of sTF antigen expressed was estimated using a sandwich immunoassay employing the anti-FLAG M1 monoclonal antibody and a goat anti-human TF antibody. Purified wild-type sTF was used as standard. The anti-FLAG M1 monoclonal antibody was coated on to 96-well enzyme-linked immunosorbent assay (ELISA) plates (Corning) at 20 μg/mL IgG in 50 mM carbonate buffer, pH 9.2, overnight at 4 °C. The plates were washed with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% BSA, 1 mM $CaCl_2$, 0.02% Tween 20), and the excess sites on the plate were blocked for 1 h with 5% BSA in 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, and 0.02% Tween 20. The plates were washed again, and sTF was added at increasing concentrations and incubated for 1 h at 4 °C. Soluble TF was detected by incubating the plates for 1 h at 4 °C with the goat anti-TF antibody (10 μg/mL) followed by a 1 h incubation at 4 °C with an anti-goat antibody conjugated to horseradish peroxidase (1: 5000). The plates were washed in between each incubation step and developed using the peroxidase substrate, ABTS (Vector Laboratories). The absorbance at 405 nm was determined using a Molecular Devices plate reader. The sensitivity of the assay was 1−10 ng/mL.

sTF Cofactor Activity Assay. To examine the functionality of the sTF mutants, the cofactor activity for VIIa-catalyzed hydrolysis of a peptidyl chromogenic substrate S-2288 was measured. Varying concentrations of sTF were incubated for 5 min at 37 °C in 50 mM Tris-HCl, 100 mM NaCl, and 0.1% BSA, pH 7.5, containing 5 mM $CaCl_2$ and 2 nM VIIa. The chromogenic substrate S-2288 was added, and the reaction was incubated for an additional 10 min at 37 °C. The reaction was terminated by the addition of 50% acetic acid, and the amount of *p*-nitroaniline released was measured by the absorbance at 405 nm using a Molecular Devices plate reader. The K_D for the interaction of purified wild-type sTF with VIIa was calculated by curve fitting using nonlinear regression analysis.

VIIa Binding Assay and Monoclonal Antibody Binding Assay. A 96-well ELISA plate (Corning) was coated overnight at 4 °C with 20 μg/mL anti-FLAG MI monoclonal antibody in 50 mM carbonate buffer, pH 9.2. The excess sites on the plate were blocked with 5% BSA in 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, and 0.02% Tween 20, and the plate was washed with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% BSA, 1 mM $CaCl_2$, 0.02% Tween 20). Soluble TF (3.8 nM) was added and incubated for 1 h at 4 °C. The plate was washed and then incubated with increasing concentrations of biotinylated VIIa (0−1 μM). (VIIa was biotinylated using a Molecular Probes kit according to manufacturer's instructions.) Binding was detected by incubating the plate with avidin−horseradish peroxidase (2.5 μg/mL) for 1 h at 4 °C. The plate was washed and then developed by incubating for 30 min at room temperature with the peroxidase substrate, ABTS. The absorbance at 405 nm was determined using a Molecular Devices plate reader. K_D was determined by curve fitting using nonlinear regression analysis.

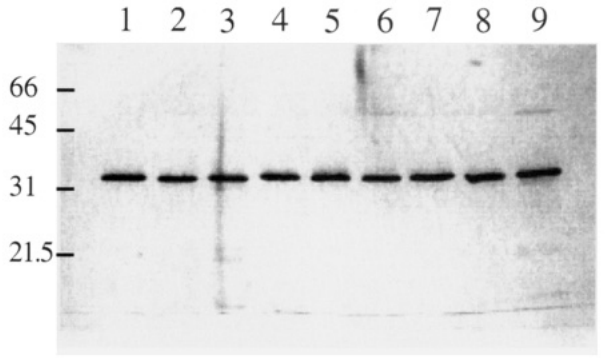

FIGURE 1: SDS−PAGE analysis of purified wild-type sTF and sTF alanine substitution mutants. Wild-type sTF or the TF mutants were purified from *E. coli* cell extracts as described under Experimental Procedures. Two hundred nanograms of sTF was loaded per lane and visualized by silver staining. Lane 1, purified wild-type sTF; lane 2, K20A; lane 3, D44A; lane 4, W45A; lane 5, K46A; lane 6, Q110A; lane 7, R135A; lane 8, F140A; lane 9, V207A. Molecular weight standards are indicated on the left.

The assay described above was modified to test for the conformational integrity of the sTF mutants. The ELISA plate was coated with the M1 antibody, blocked, and incubated with sTF as described. The plate was washed and incubated with increasing concentrations (0−50 nM) of biotinylated monoclonal antibody (American Diagnostica 4503). Binding was detected and K_D was determined as indicated for the VIIa binding assay. The conformational dependence of the monoclonal antibody was established using denatured sTF that had been reduced and carboxymethylated using iodoacetic acid.

RESULTS

Purification and Characterization of sTF. Bacterial culture medium concentrated by ultrafiltration or an *E. coli* cell extract was adjusted to 1 mM $CaCl_2$ and loaded onto the anti-FLAG M1 affinity column. The column was washed with equilibration buffer and eluted with EGTA. The pooled fractions were concentrated and then dialyzed against 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl. The purified sTF appeared as a single band of ~35 kDa when analyzed by nonreducing SDS−PAGE and silver staining (Figure 1, lane 1). This material was assessed to be greater than 90% pure and was subsequently used as the standard in the TF ELISA.

The purified sTF was assayed for cofactor function by determining its ability to enhance the catalytic activity of VIIa toward a peptidyl chromogenic substrate, S-2288 (Figure 2). The concentration required to generate half-maximal enhancement of VIIa activity was 5.3 nM, which is consistent with published values for the interaction between sTF and VIIa (0.5−100 nM) (Waxman et al., 1992; Ruf et al., 1991a,b; Shigematsu et al., 1992). This assay represents a simple method for analyzing the interaction between sTF and VIIa and provides an easy screen to characterize TF mutants.

Identification of Loop Regions in TF Important for Interactions with VIIa by Homologous Loop−Swap Mutagenesis. The amino acid sequence of the extracellular domain of human TF was aligned with the sequences of both the γ-IFN-R, the closest relative to TF in the cytokine

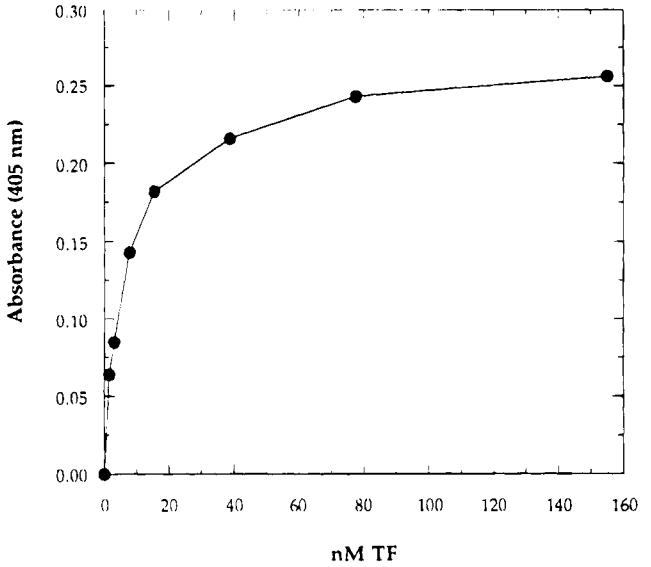

FIGURE 2: Concentration dependence of sTF cofactor activity. Increasing concentrations of purified wild-type sTF were incubated with VIIa (2 nM) and $CaCl_2$ (5 mM) for 5 min at 37 °C. The peptidyl chromogenic substrate, S-2288, was added and allowed to incubate for an additional 10 min at 37 °C. The absorbance at 405 nm was determined using a Molecular Devices plate reader.

receptor superfamily, and the hGH-R, the cytokine receptor for which the most structural information exists (Bazan, 1990b). The 14 regions of β-secondary structure from the hGH-R crystal structure were superimposed on the TF alignment (Figure 3). In order to identify the loop regions in TF which interact with VIIa, structurally nondisruptive homologous loop swap mutations were constructed. The loops connecting β-strands in each module, those connecting β-strands with the N- and C-terminal regions, and the intermodule linker were systematically replaced with the corresponding loop regions from the γ-IFN-R (Table 1) by oligonucleotide-directed mutagenesis. The loop connecting β-strands C-B and C-C was too large to be swapped using a single oligonucleotide primer; thus this loop was swapped in two separate portions, loop regions 10 and 11. Homologous loop swap mutants in which TF loop regions 3 and 11 were replaced could not be stably expressed. Therefore, loop regions 3 and 11 were swapped in two discrete portions, 3a, 3b, 11a, and 11b.

Table 1: Cofactor Activity of Tissue Factor Homologous Loop Swap Mutants[a]

tissue factor loop swap mutant	loop location (connecting β-strands)	tissue factor residues replaced	TF cofactor activity (% wild type)
1	NH_2-N-A	1-9	65
2	N-A-N-B	16-20	8
3a	N-B-N-C	27-28	126
3b	N-B-N-C	29-32	154
4	N-C-N-C′	40-46	4
5	N-C′-N-E	50-54	37
6	N-E-N-F	60-69	2
7	N-F-N-G	77-95	19
8	N-G-C-A	101-111	9
9	C-A-C-B	118-121	124
10	C-B-C-C	129-140	0
11a	C-B-C-C	141-145	5
11b	C-B-C-C	147-151	5
12	C-C-C-C′	161-169	143
13	C-C′-C-E	172-174	49
14	C-E-C-F	181-183	110
15	C-F-C-G	193-207	2
16	C-G-COOH	211-219	150
wild type			100

[a] Predicted loop regions in TF (1-16) were replaced with the corresponding residues from γ-IFN-R according to the alignment in Figure 3. TF mutants were screened for TF cofactor activity at a concentration of 38 nM. Activity is expressed as a percentage of the cofactor activity of wild-type sTF.

A total of 18 sTF loop swap mutants and wild-type sTF were stably expressed in *E. coli*. Culture medium containing the mutant proteins was concentrated by ultrafiltration and the amount of sTF was quantitated by ELISA. The sTF mutants were screened at a concentration of 38 nM for their ability to stimulate VIIa activity toward the chromogenic peptide substrate S-2288. This concentration exceeds the EC_{50} for this reaction by approximately 7-fold. Eight mutants, encompassing six distinct loop regions, had less than 10% of the wild-type cofactor activity, indicating that these loops were important for interactions with VIIa (Table 1). Using the α-carbon trace of hGH-R structure as a model for the topology of sTF (Figure 4A), three of these loop regions were located on one face of the N-terminal module (N-A-N-B, N-C-N-C′, N-E-N-F), two loop regions comprised one face of the C-terminal module (C-B-C-C, C-F-

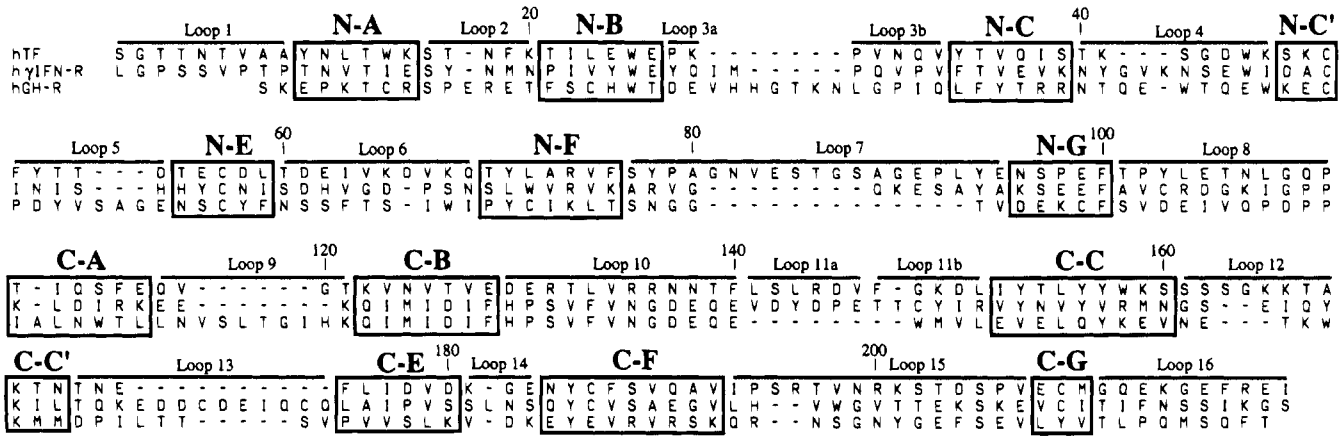

FIGURE 3: Sequence alignment of the extracellular domains of tissue factor, γ-interferon receptor, and growth hormone receptor. Sequence alignments are according to Bazan (1990b). Predicted β-strand regions are enclosed in boxes. The termini of β-strands are defined from the crystal structure of the hGH/hGH-R complex. β-Strands are described by folding module (N- or C-), where each module is comprised of seven antiparallel strands (A, B, C, C′, E, F, G) assembled into two sheets; A, B, and E belong to one sheet and C, C′, F, and G to the other sheet (de Vos et al., 1992). Predicted loop regions (1-16) mutated in this study are overlined and assigned loop numbers. Single letter code for amino acids is used; a dash indicates a gap in the sequence alignment.

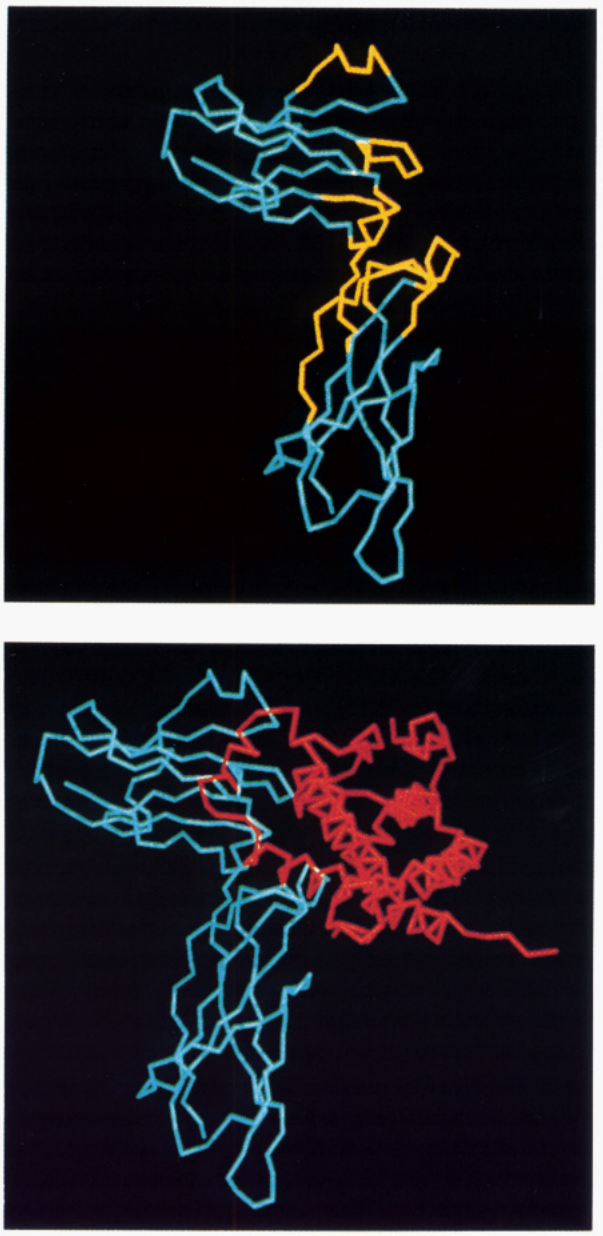

FIGURE 4: Loop regions in tissue factor predicted to be involved in interactions with factor VIIa: comparison with the interface between growth hormone and its receptor. (A, top) The α-carbon trace of the extracellular domain of the hGH-R from the crystal structure of the 2:1 complex of hGH-R with hGH (de Vos et al., 1992) was used as a model for the structure of the extracellular domain of TF. The coordinates of receptor 1 in the complex were used for the model of TF. Using the alignment illustrated in Figure 3, the regions in the hGH-R equivalent to the six loop regions in TF (loop regions N-A−N-B, N-C−N-C′, N-E−N-F, N-G−C-A, C-B−C-C, C-F−C-G) required for interactions with VIIa are highlighted in yellow. (B, bottom) The crystal structure of the 2:1 complex of hGH-R with hGH contains a single hGH molecule bound to two receptors. Although the two binding sites on the hGH molecule are different, the same binding region was utilized on both of the receptor molecules. The interaction between hGH (red) and receptor 1 (blue) illustrates that the same general surface was utilized by hGH-R for the recognition of hGH as was used by TF for interactions with VIIa. The regions utilized by the hGH-R include two loop regions from one face of each of the two subdomains, part of β-strand N-G, and the linker region that connects the two subdomains.

C-G), and the remaining loop region consisted of the intermodule linker (N-G−C-A). These six loop regions form a single continuous binding surface for VIIa on the surface of TF (Figure 4A) that is analogous to the interface identified in the crystal structure of the complex between hGH and hGH-R (Figure 4B).

Identification of Specific Amino Acid Residues in TF Important for Interactions with VIIa by Alanine Scanning Mutagenesis. To identify the individual amino acids involved in specific interactions with VIIa, the 68 residues (excluding glycine) within the six important loop regions of TF were substituted with alanine (Table 2). Four of the 68 alanine substitution mutants could not be expressed in soluble form in *E. coli* (F19A, T60A, F147A, L151A). Three of these mutants involve large hydrophobic residues (phenylalanine and leucine) that are often buried in proteins, and the substitution of such residues may disrupt the overall conformation of the protein (Bowie et al., 1990).

The remaining 64 sTF mutants were isolated in the combined periplasmic and cytosolic extracts from 200 mL of *E. coli* culture and the concentration of each mutant protein was determined by ELISA. Extracts containing the mutant proteins were screened for cofactor activity at a concentration of 13 nM, approximately 2.5-fold in excess of the EC_{50} for wild-type sTF (Table 2). Eight sTF mutants (K20A, D44A, W45A, K46A, Q110A, R135A, F140A, V207A) involving residues from five of the six TF loop regions displayed less than 40% of the cofactor activity of wild-type sTF. These eight sTF mutants were selected for further analysis to determine the energetic contribution of each residue toward interactions with VIIa.

Quantitative Analysis of the Contribution of Specific TF Residues to Interactions with VIIa. The eight sTF alanine replacement mutants were purified from combined periplasmic and cytoplasmic extracts by M1 affinity chromatography. The mutant proteins were at least 90% pure as estimated by silver-stained SDS−PAGE (Figure 1), and the concentration of each sTF mutant was determined by ELISA.

The conformational integrity of each sTF mutant was assessed using a noninhibitory conformationally sensitive monoclonal antibody in a modified ELISA format (Table 3). The K_D for native sTF was 0.13 nM while the K_D for denatured sTF was 1.0 nM. The affinity of each of the sTF mutants for the monoclonal antibody was identical to native sTF (K_D = 0.12−0.16 nM), indicating that the conformational integrity of all mutants was preserved.

The interaction of each mutant with VIIa was quantitatively assessed by the sTF cofactor activity assay (Table 3). Each of the mutants displayed decreased affinity for VIIa. The K_D for each mutant was increased by 4.5 to 61-fold, corresponding to a loss of 0.9−2.5 kcal/mol of free energy of binding for each residue substituted.

When the ability of each mutant to enhance the activity of VIIa was considered, the mutants fell into two classes. Despite the decrease in affinity for VIIa, the ability to enhance VIIa activity at saturating concentrations of TF was undiminished for some mutants (K46A, Q110A, R135A, F140A, V207A). The residues substituted in these mutants are candidates for those that may solely participate in binding interactions with VIIa. For other mutants (K20A, W45A, and to a lesser degree mutant D44A) the level of enhancement of VIIa activity at saturation was diminished by 2−4-fold. In addition to contributing to binding interactions with VIIa, the residues replaced in these mutants are potentially required to induce the conformational change in VIIa postulated to be responsible for the increased activity of VIIa in the TF/VIIa complex.

Table 2: Cofactor Activity of Tissue Factor Alanine Substitution Mutants[a]

tissue factor loop region	alanine substitution mutant	TF cofactor activity (% wild type)	standard error	tissue factor loop region	alanine substitution mutant	TF cofactor activity (% wild type)	standard error
loop region 2 (N-A−N-B)	S16A	115	16.0		L133A	72	3.8
	T17A	114	16.0		V134A	78	5.2
	N18A	103	15.0		R135A	28	1.3
	F19A	not expressed			R136A	81	3.5
	K20A	2.9	1.7		N137A	79	6.8
loop region 4 (N-C−N-C′)	T40A	73	5.5		N138A	85	3.8
	K41A	105	20.0		T139A	74	5.0
	S42A	72	6.7		F140A	13	3.3
	D44A	5	2.5		L141A	116	8.7
	W45A	2.3	2.3		S142A	82	8.4
	K46A	33	10.0		L143A	123	6.7
loop region 6 (N-E−N-F)	T60A	not expressed			R144A	100	3.1
	D61A	76	2.2		D145A	117	5.8
	F62A	74	7.2		V146A	66	2.3
	I63A	86	7.0		F147A	not expressed	
	V64A	97	3.3		K149A	111	9.0
	K65A	91	2.6		D150A	100	4.7
	D66A	96	2.6		L151A	not expressed	
	V67A	109	1.1	loop region 15 (C-F−C-G)	I193A	54	3.4
	K68A	94	2.3		P194A	99	11
	Q69A	96	3.4		S195A	98	12
loop region 8 (N-G−C-A)	T101A	68	6.6		R196A	52	4.3
	P102A	79	6.6		T197A	98	13
	Y103A	72	10.0		V198A	101	7.6
	L104A	91	4.4		N199A	78	7.9
	E105A	72	6.5		R200A	100	2.8
	T106A	78	2.0		K201A	84	5.5
	N107A	86	11.0		S202A	72	1.9
	L108A	80	18.0		T203A	117	3.7
	Q110A	37	13.0		D204A	125	4.4
	P111A	83	4.3		S205A	85	6.5
loop region 10 + 11 (C-B−C-C)	D129A	66	7.3		P206A	105	14
	E130A	56	6.7		V207A	29	10
	R131A	72	2.6	wild type		100	
	T132A	99	17.0				

[a] Sixty-eight residues in loop regions of TF were individually replaced with alanine. TF mutants were screened for TF cofactor activity at a concentration of 13 nM. Activity is the mean of three separate determinations expressed as a percentage of the cofactor activity of wild-type sTF. Single letter code for amino acids is used. Nomenclature for mutants is as follows: substituted residue type, residue number, replacement residue type.

Table 3: Quantitation of Interactions of Purified Soluble Tissue Factor Mutants with Factor VIIa and a Conformationally Dependent Monoclonal Antibody[a]

sTF mutant	conformationally sensitive Mab affinity (K_D, nM)	maximal VIIa activity at saturating [sTF] (% wild type)	VIIa affinity (K_D, nM)	binding energy loss ($\Delta\Delta G$) (kcal/mol)
Wild-type	0.13	100	5.3	
K20A	0.15	25	325	2.5
D44A	0.13	49	198	2.2
W45A	0.15	29	290	2.5
K46A	0.15	110	24	0.9
Q110A	0.16	102	48	1.4
R135A	0.12	111	28	1.0
F140A	0.14	79	223	2.3
V207A	0.15	99	75	1.6

[a] Monoclonal antibody affinity was determined from the concentration dependence of biotinylated antibody binding. Maximal VIIa activity at saturating concentrations and affinity of TF mutants for VIIa were determined from the concentration dependence of VIIa activity. Maximal VIIa activity is expressed as the percentage of VIIa activity induced by wild-type sTF at saturation. Mean standard error for K_D determinations is less than 30%. Loss of free energy of binding ($\Delta\Delta G$) was calculated from the equation $\Delta\Delta G = -RT \ln(K_{D\ mutant}/K_{D\ wild\ type})$.

The interaction of sTF mutants for VIIa was also examined utilizing biotinylated VIIa in a modified ELISA format. The K_D for wild-type sTF under these conditions was estimated to be 38 nM. Despite the nonequilibrium nature of this assay, the rank order of affinity for each sTF mutant was preserved (data not shown), with the exception of V207A which displayed only a slight (24%) decrease in affinity. The interaction of this mutant may be affected by the biotinylation of VIIa.

DISCUSSION

Using a site-directed mutagenesis strategy guided by structural information, we have identified the regions of TF that are important for VIIa binding and cofactor activity. The systematic replacement of predicted loop regions in the extracellular domain of TF with the homologous loops from a protein of related structure (γ-IFN-R) revealed six discontinuous regions that are required for enhancement of VIIa activity (16−20, 40−46, 60−69, 101−111, 129−151, 193−207). The individual substitution of each residue within the six putative loop regions with alanine revealed eight residues (K20, D44, W45, K46, Q110, R135, F140, V207) that are important for binding interactions with VIIa. The possibility exists that the loss of function observed may be due to the nonspecific disruption of protein conformation rather than the replacement of a genuinely functional residue. Our mutagenesis approach was designed to reduce this. Alanine was used for all substitutions as its small size and neutral character impose the least constraints on secondary structure

and tertiary conformation (Klapper, 1977; Rose et al., 1985). Therefore, the effects of the amino acid substitution can be studied with minimal interference from nonspecific structural disruption. Previous studies utilizing alanine scanning mutagenesis or incorporation of homologous residues from related proteins have indicated that gross structural perturbations are an uncommon event (Cunningham et al., 1989; Cunningham & Wells, 1989; Gibbs & Zoller, 1991). The conformational integrity of each of our sTF mutants was established by demonstrating that affinity for a conformationally sensitive monoclonal antibody was preserved.

In addition, several lines of evidence indirectly indicate that structural disruption was not a problem in our study. All of the mutants analyzed were stably expressed in soluble form, suggesting that global conformation was preserved. Among the 64 alanine substitution mutants analyzed only 8 were functionally defective, suggesting that alanine substitutions do not commonly disrupt protein folding. In addition, residues whose replacement with alanine caused large functional defects could be found adjacent to residues that could be substituted without loss of function, suggesting that any structural perturbations were small, localized, and not perpetuated to affect flanking residues. Finally, the energetic contribution of each of the eight TF residues (K20, D44, W45, K46, Q110, R135, F140, V207) to the binding of VIIa, calculated from the change in K_D attributable to the substitution of each residue, varied between 0.9 and 2.5 kcal/mol. These residues have polar, charged and hydrophobic side chains that could contribute hydrogen bonds, salt bridges, and hydrophobic interactions to stabilize the TF/VIIa complex (Janin & Chothia, 1990). The magnitude of the contribution of individual residues to the free energy of binding was comparable to previous determinations demonstrating that hydrogen bonds between polar residues contribute 0.5−1.5 kcal/mol, hydrogen bonds involving a charged residue contribute 3.5−4.5 kcal/mol (Fersht et al., 1985), and salt bridges contribute up to 2.0 kcal/mol (Wells et al., 1987). Thus the magnitude of the functional disruption caused by the alanine substitutions was consistent with the loss of individual hydrogen bonds and salt bridges rather than the gross disruption of the protein structure.

The six regions and the individual residues within these regions characterized as being important for cofactor activity are widely dispersed in the primary sequence of TF, implying that TF must fold to bring these regions into proximity for interactions with VIIa. When the hGH-R structure was used as a model for TF (de Vos et al., 1992), the six dispersed loop regions in TF were found to be in close proximity to each other (Figure 4A) and defined a binding surface for VIIa that was homologous to the interface for hGH on hGH-R (Figure 4B). This suggests that in addition to the conservation of structure between the members of the cytokine receptor superfamily there is also conservation of the mode of ligand binding despite the structural diversity among the ligands. This is remarkable because the bundle of four α-helices that comprise hGH would not be expected to resemble the γ-carboxyglutamic acid, epidermal growth factor-like and protease domains of VIIa which have been proposed to be involved in interactions with TF (Wildgoose et al., 1990, 1992, 1993; Toomey et al., 1991; Kazama et al., 1993).

Six of the eight amino acid residues of TF that are important for interactions with VIIa (K20, W45, K46, Q110, R135, F140) are conserved in the human, bovine, rabbit, and mouse TF sequences. D44 is conservatively substituted with asparagine in bovine, rabbit, and mouse and V207 is nonconserved. The high degree of conservation of these residues among mammalian TF molecules is consistent with the proposed functional importance of these residues (Creighton & Darby, 1989). None of these residues are conserved among the other members of the cytokine receptor superfamily (Bazan, 1991b). Mapping of the residues in the crystal structure of hGH-R reveals 19 residues that are buried in the interface with hGH or make electrostatic interactions with hGH and may contribute to ligand binding (de Vos et al., 1992). The importance of eight of these residues has been confirmed by site-directed mutagenesis (Bass et al., 1991). When the TF and hGH-R sequences are aligned, the residues in each protein predicted to be important for ligand binding are located in similar regions (Figure 3). However, it is difficult to predict whether these residues are in spatially equivalent locations in the respective three-dimensional structures because most of these residues are located in loop regions which are less homologous and more variable in length than the β-strand regions.

The eight residues identified could be divided into two groups based on the effects of their replacement on affinity for VIIa and their ability to mediate maximal enhancement of VIIa activity. The substitution of K46, Q110, R135, F140, and V207 decreased the affinity for VIIa only, suggesting that these residues were involved in simple binding interactions with VIIa. The replacement of K20, W45, or D44 resulted in a decrease in the maximal level of enhancement of VIIa activity at saturation as well as decreased affinity for VIIa. These results suggest that these residues may participate in the conformational change postulated to be responsible for the enhancement of VIIa activity when bound to TF (Nemerson & Gentry, 1986). These residues may make additional or more energetically favorable interactions when bound to VIIa in the active conformation.

Site-directed mutagenesis of cysteine residues in TF suggested that the integrity of the disulfide bond between C186 and C209 in the carboxyl-terminal region of the TF extracellular domain was required for interactions with VIIa (Rehemtulla et al., 1992). Competition studies using monoclonal antibodies directed against TF identified two extended regions (40−83 and 106−165) that are recognized by antibodies that block VII binding (Ruf et al., 1991c). Two similar regions (44−84 and 129−169) were identified by peptide mapping of TF photoaffinity labeled with VIIa (Ruf & Edgington, 1991). The two extended regions identified in those studies encompass four of the six critical loop regions identified in our study. Random substitution of residues in TF and screening crude membrane preparations for TF function in an assay measuring factor Xa activity revealed five mutants defective in VIIa recognition (Ruf et al., 1994). Three of these residues (K20, R135, and F140) were also identified in our study; the other residues implicated (I22 and D58) are located in predicted β-strand regions and were not tested in our analysis.

The grouping of the cytokine receptors into a superfamily that shares a common structure requires assumptions of structural relatedness because the sequence homology at the level of amino acid identity is weak. The identification of a ligand binding site on TF that is spatially homologous to the hormone binding site on the hGH-R validates the relationship between these two diverse members of the superfamily. This also indicates that the mode of ligand

binding and structure−function relationships are likely to be conserved among superfamily members despite the considerable evolutionary distance between sequences and the diversity of receptor function. Our results reveal a common region on all the cytokine receptors that may be exploited as a target for competitive inhibitors of ligand binding and may provide opportunities for the protein engineering of receptors with altered specificities.

ACKNOWLEDGMENT

We are grateful to Regan Shea, Terry Terhorst, Kim Sweetnam, Lisa Crow, and Melissa Klute for the synthesis of the oligonucleotides used in this study, Cheri Mao and Veronica Law for technical assistance, Kyla Dunn, Julie Cherrington, and Jay Toole for critically reviewing the manuscript, and Bart de Vos (Genentech Inc.) for providing the α-carbon coordinates of the crystal structure of the hGH/hGH-R complex.

REFERENCES

Bach, R. R. (1988) *CRC Crit. Rev. Biochem. 23*, 339−368.
Bach, R., Konigsberg, W. H., & Nemerson, Y. (1988) *Biochemistry 27*, 4227−4231.
Bajaj, M., & Blundell, T. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 453.
Bass, S. H., Mulkerrin, M. G., & Wells, J. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4498−4502.
Bazan, J. F. (1990a) *Cell 61*, 753−754.
Bazan, J. F. (1990b) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6934−6938.
Bevilacqua, M. P., Pober, J. S., Majeau, G. R., Cotran, R. S., & Gimbrone, M. A., Jr. (1984) *J. Exp. Med. 160*, 618−623.
Bowie, J. U., Reidhaar-Olson, J. F., Lim, W. A., & Sauer, R. T. (1990) *Science 247*, 1306−1310.
Creighton, T. E., & Darby, N. J. (1989) *Trends Biochem. Sci. 14*, 319−324.
Crossman, D. C., Carr, D. P., Tuddenham, E. G. D., Pearson, J. D., & McVey, J. H. (1990) *J. Biol. Chem. 265*, 9782−9787.
Cunningham, B. C., & Wells, J. A. (1989) *Science 244*, 1081−1085.
Cunningham, B. C., Jhurani, P., Ng, P., & Wells, J. A. (1989) *Science 243*, 1330−1336.
de Vos, A. M., Ultsch, M., & Kossiakoff, A. A. (1992) *Science 255*, 306−312.
Dotto, G. P., Enea, V., & Zinder, N. D., (1981) *Virology 114*, 463.
Edgington, T. S., Mackman, N., Brand, K., & Ruf, W. (1991) *Thromb. Haemostasis 66*, 67−79.
Fersht, A. R., Shi, J., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature 314*, 235−238.
Gailani, D., & Broze, G. J., Jr. (1991) *Science 253*, 909−923.
Gibbs, C., & Zoller, M. J. (1991) *Methods: A Companion to Methods in Enzymology*, Vol. 3, pp 165−173, Academic Press, New York.
Hopp, T. P., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L., & Conlon, P. J. (1988) *Bio/Technology 6*, 1204−1210.
Janin, J., & Chothia, C. (1990) *J. Biol. Chem. 265*, 16027−16030.
Kazama, Y., Pastuszyn, A., Wildgoose, P., Hamamoto, T., & Kisiel, W. (1993) *J. Biol. Chem. 268*, 16231−16240.
Klapper, M. H. (1977) *Biochem. Biophys. Res. Commun. 78*, 1018.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
Kuntz, I. K. (1972) *J. Am. Chem. Soc. 94*, 4009.
Nakagaki, T., Foster, D. C., Berkner, K. L., & Kisiel, W. (1991) *Biochemistry 30*, 10819−10824.
Nemerson, Y. (1966) *Biochemistry 5*, 601−608.
Nemerson, Y. (1988) *Blood 71*, 1−8.
Nemerson, Y., & Gentry, R. (1986) *Biochemistry 25*, 4020−4033.
Neuenschwander, P. F., & Morrissey, J. H. (1992) *J. Biol. Chem. 267*, 14477−14482.
Østerud, B., & Rapaport, S. I. (1977) *Proc. Natl. Acad Sci. U.S.A. 74*, 5260−5264.
Paborsky, L. R., Tate, K. M., Harris, Reed J., Yansura, D. G., Band, L., McCray, G., Gorman, C. M., O'Brien, D. P., Chang, J. Y., Swartz, J. R., Fung, V. P., Thomas, J. N., & Vehar, G. A. (1989) *Biochemistry 28*, 8072−8077.
Rehemtulla, A., Ruf, W., Miles, D. J., & Edgington, T. S. (1992) *Biochem. J. 282*, 737−740.
Rose, G. D., Geselowitz, A. R., Lesser, G. L., Lee, R. H., & Zehfus, M. H. (1985) *Science 229*, 834−838.
Ruf, W., & Edgington, T. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8430−8434.
Ruf, W., Rehemtulla, A., & Edgington, T. S. (1991a) *J. Biol. Chem. 266*, 2158−2166.
Ruf, W., Kalnik, M. W., Lund-Hansen, T., & Edgington, T. S. (1991b) *J. Biol. Chem. 266*, 15719−15725.
Ruf, W., Rehemtulla, A., & Edgington, T. S. (1991c) *Biochem. J. 278*, 729−733.
Ruf, W., Schullek, J. R., Stone, M. J., & Edgington, T. S. (1994) *Biochemistry 33*, 1565−1572.
Scarpati, E. M., & Sadler, J. E. (1989) *J. Biol. Chem. 264*, 20705−20713.
Scarpati, E. M., Wen, D., Broze, G. J., Miletich, J. P., Flermeyer, R. R., Siegel, N. R., & Sadler, J. E. (1987) *Biochemistry 26*, 5234−5238.
Shigematsu, Y., Miyata, T., Higashi, S., Miki, T., Sadler, J. E., & Iwanaga, S. (1992) *J. Biol. Chem. 267*, 21329−21337.
Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
Toomey, J. R., Smith, K. J., & Stafford, D. W. (1991) *J. Biol. Chem. 266*, 19198−19202.
Vieira, J., & Messing, J. (1987) *Methods Enzymol. 153*, 3−11.
Waxman, E., Ross, J. B. A., Laue, T. M., Guha, A., Thiruvikraman, S. V., Lin, T. C., Konigsberg, W. H., & Nemerson, Y. (1992) *Biochemistry 31*, 3998−4003.
Wells, J. A., Powers, D. B., Bott, R. R., Graycar, T. P., & Estell, D. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1219−1223.
Wildgoose, P., Kazim, A. L., & Kisiel, W. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7290−7294.
Wildgoose, P., Jørgensen, T., Komiyama, Y., Nakagaki, T., Pedersen, A., & Kisiel, W. (1992) *Thromb. Haemostasis 67*, 679−685.
Wildgoose, P., Foster, D., Schiødt, J., Wiberg, F. C., Birktoft, J. J., & Petersen, L. C. (1993) *Biochemistry 32*, 114−119.
Yamamoto, M., Nakagaki, T., & Kisiel, W. (1992) *J. Biol. Chem. 267*, 19089−19094.