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Isolation of cDNA clones coding for human tissue factor: Primary structure of the protein and cDNA

(blood coagulation/protein chemistry/DNA sequence/membrane protein)

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ABSTRACT **Tissue factor is a membrane-bound procoagulant protein that activates the extrinsic pathway of blood coagulation in the presence of factor VII and calcium. λ Phage containing the tissue factor gene were isolated from a human placental cDNA library. The amino acid sequence deduced from the nucleotide sequence of the cDNAs indicates that tissue factor is synthesized as a higher molecular weight precursor with a leader sequence of 32 amino acids, while the mature protein is a single polypeptide chain composed of 263 residues. The derived primary structure of tissue factor has been confirmed by comparison to protein and peptide sequence data. The sequence of the mature protein suggests that there are three distinct domains: extracellular, residues 1–219; hydrophobic, residues 220–242; and cytoplasmic, residues 243–263. Three potential N-linked carbohydrate attachment sites occur in the extracellular domain. The amino acid sequence of tissue factor shows no significant homology with the vitamin K-dependent serine proteases, coagulation cofactors, or any other protein in the National Biomedical Research Foundation sequence data bank (Washington, DC).**

Blood coagulation can be initiated by a complex of tissue factor (TF), a membrane-bound glycoprotein, and factor VII, a plasma coagulation factor (for reviews, see refs. 1 and 2). The physiological significance of this extrinsic pathway can be judged by the severe bleeding frequently observed in individuals who are markedly deficient in factor VII (3, 4). In contrast, individuals who have deficiencies or abnormalities in proteins that are involved in the early steps of the intrinsic pathway of coagulation—i.e., high molecular weight kininogen, prekallikrein, and factor XII—are asymptomatic (5). The TF–factor VII complex activates factor IX, a component of the intrinsic pathway, as well as factor X (6). Thus, it is reasonable that the association of TF and factor VII may be the crucial event triggering the initiation of clotting *in vivo*.

The cDNAs for all of the proteins involved in TF-initiated coagulation, with the exception of TF itself, have already been cloned and sequenced (7–13). The TF apoprotein has been purified from both bovine and human sources (14–16). Approximately 50–70% of the amino acid sequence of both species has now been determined, and this has permitted us to select suitable amino acid sequences to serve as a basis for constructing oligonucleotide probes. This in turn has enabled the isolation and characterization of two human placental TF cDNA clones that contain the entire coding region of the mature protein. The nucleotide sequence of these clones, together with amino acid sequence data, has allowed us to formulate a primary structure for the human TF apoprotein[§].

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MATERIALS AND METHODS

TF Purification and Sequencing. A monoclonal antibody (17) prepared against human TF that had been purified by using factor VII affinity columns (15) was used for immunoaffinity isolation of TF. Briefly, TF was extracted from human brain or placental tissue acetone powders with Triton X-100 and then adsorbed onto the immunoaffinity column. The adsorbed TF was washed with Triton X-100-containing buffers at pH 7.5 and was eluted from the column at pH 2.5. Gel filtration on an Ultrogel AcA 34 column in Triton X-100 separated TF from minor contaminants, yielding a homogeneous protein. The purity of each preparation was assessed by PAGE in $NaDodSO_4$ (18). The purification was monitored by a two-stage coagulation assay after reconstitution of the detergent-solubilized protein into phospholipid vesicles (16). Amino acid analysis of each TF preparation was performed on a Beckman 121M analyzer.

Amino acid sequence analysis of intact TF (100–200 μg) and peptide fragments was performed with an Applied Biosystems (Foster City, CA) gas-phase sequenator (19). The C-terminal CNBr peptide was prepared from 60 μg of the placental apoprotein. After precipitation with 10% trichloroacetic acid and acetone extraction, TF was dissolved in 0.8 ml of 6 M guanidine·HCl/50 mM $NaHCO_3$, pH 8.0, and treated with three successive additions of 1 mg of succinic anhydride (Pierce). Triton X-100 was then added to 0.1%, and the succinylated TF was dialyzed versus 50 mM $NaHCO_3$ containing 0.1% Triton X-100. TF was again precipitated and cleaved with 50 μl of CNBr (15 mg/ml in 70% formic acid). After incubation for 24 hr at 25°C, 450 μl of H_2O was added, and the digest was dried twice in a vacuum centrifuge. The protein pellet was sequenced as described above.

Tryptic peptides were prepared from placental TF (120 μg). After precipitation as described above, the protein was solubilized in 50 μl of 8 M urea and diluted with 150 μl of 50 mM NH_4HCO_3. Trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl ketone (Cooper Biomedicals, Malvern, PA) was then added at a trypsin/TF ratio of 1:25 (wt/wt). After 24 hr at 37°C, peptides were isolated on a Vydac C_{18} column (0.45 × 25 cm) equilibrated in 0.05% trifluoroacetic acid, and run at a flow rate of 1 ml/min. Peptides were eluted with linear gradients of buffer B (0.05% trifluoroacetic acid/80% acetonitrile) as follows: 0–63 min (2–37.5% buffer B), 63–95 min (37.5–75% buffer B), and 95–105 min (75–98% buffer B). The elution profile was monitored by absorbance at 210 and 280 nm. Tryptic peptides were sequenced as described above.

Abbreviation: TF, tissue factor.

[§]The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. JO 2931).

Chemical and Enzymatic Deglycosylation. Placental TF (5 μg) was chemically deglycosylated by the method of Edge *et al.* (20) with trifluoromethanesulfonic acid. After precipitation with 5 volumes of acetone, 20 μl of anisole and 40 μl of trifluoromethanesulfonic acid were added, and the reaction was performed under N_2 for 3 hr on ice. The sample was then extracted twice with 3 ml of ether/hexane, 9:1 (vol/vol), with 5 μl of pyridine added to facilitate protein precipitation. Finally, the resulting pellet was extracted once with acetone.

Asparagine-linked carbohydrate was enzymatically cleaved from placental TF (5 μg) with endoglycosidase F (21). After precipitation with acetone, the protein was dissolved in 40 μl of a buffer containing 0.5% β-octyl-D-glucopyranoside, 20 mM EDTA, 50 mM sodium acetate (pH 6.1), and endoglycosidase F (0.1 unit; Boehringer Mannheim, grade II). After incubation for 16 hr at 37°C, the reaction was terminated by acetone precipitation. The products of chemical and enzymatic deglycosylation were analyzed by PAGE on the $NaDodSO_4$/urea gel system of Swank and Munkres (22).

Screening of cDNA Libraries. A human placental cDNA library cloned into λgt11 (23) purchased from Clontech (Palo Alto, CA) was screened for TF coding sequences by using the three oligonucleotide probes shown in Fig. 1. Oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems 380A DNA synthesizer. The probes were purified by HPLC with a Nucleogen DEAE 60-7 column (Macherey & Nagel) and then 5'-end-labeled with [γ-^{32}P]ATP (Amersham) and phage T4 polynucleotide kinase (Pharmacia) to a specific activity of 1×10^8 cpm/μg.

Approximately 30,000–50,000 phage per 85-mm dish were plated on a lawn of *Escherichia coli* Y1088 cells (23) and screened by hybridization of duplicate filter lifts (Colony/PlaqueScreen, New England Nuclear) to ^{32}P-labeled oligonucleotide probes. Hybridizations with probe 1 were carried out at t_m − 25°C (50°C) in 0.90 M NaCl/0.09 M sodium citrate/0.6% $NaDodSO_4$; the nitrocellulose filters were subsequently washed at t_m − 8°C in 0.30 M NaCl/0.03 M sodium citrate/0.2% $NaDodSO_4$. Autoradiography was performed at −70°C with an intensifying screen (DuPont Lightning Plus) for 16–40 hr. Phage that gave positive signals with probe 1 were subsequently plaque-purified and rescreened by hybridization to probes 2 and 3 under standard conditions (24) to eliminate false positives.

Nucleotide Sequence Analysis. *Eco*RI restriction fragments from two TF cDNA clones in λgt11 were cloned into phage M13mp19, to facilitate nucleotide sequence analysis. All sequencing was performed by the dideoxy chain-terminating method of Sanger *et al.* (25) with deoxyadenosine 5'-[α-(^{35}S)thio]triphosphate (Amersham; 500 mCi/mmol; 1 Ci = 37 GBq). Sequencing reactions were analyzed on 6% polyacrylamide/7 M urea gels, which were dried prior to autoradiography on film overnight. Chain-extension reactions were primed by using phage M13 17-nucleotide primers complementary to positions −20 and −40 relative to the *Eco*RI site (New England Biolabs; primers 1211 and 1212). In addition, six different oligonucleotides (18-mers) complementary to the TF gene were used to initiate sequencing reactions within large inserts in M13, as indicated in Fig. 2.

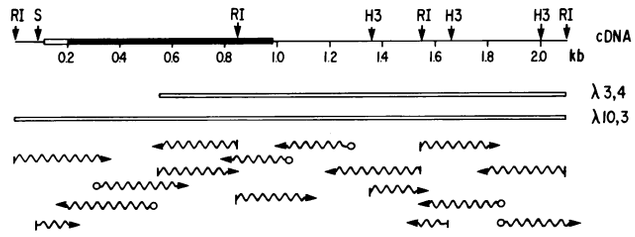

FIG. 2. Schematic representation of the cDNA structure for the TF gene. Restriction sites that were used in generating phage M13 subclones are indicated on the first line. The open and solid boxes indicate the coding region for the signal peptide and mature protein, respectively. The second and third lines indicate the inserts contained in the two λgt11 cDNA recombinants used for the sequence analysis. The wavy lines indicate the length and direction of sequence determined from M13 subclones. Open circles indicate where synthetic primers were used to originate the sequencing reactions. These primers corresponded to the sequence of the TF cDNA at positions 322–339, 539–522, 1075–1058, 1310–1293, 1870–1853, 1853–1870.

RESULTS

Protein Chemistry of Human TF. The N-terminal sequence of human TF was determined for the apoprotein purified from both brain and placenta. The two preparations gave identical sequences; residues 1–22 (Fig. 3) were determined with brain TF and residues 1–38 were determined with the placental material. Each cycle of sequencing from the intact proteins yielded two phenylthiohydantoin-conjugated amino acids giving overlapping sequences that were out of phase by two residues. This interpretation is consistent with the primary structure as derived from the cDNA sequence. Thus, approximately half of the TF apoproteins lack the first two amino acids. No amino acid was found at position 11. This is probably due to glycosylation of the asparagine residue at this consensus N-linked glycosylation site (Asn-Xaa-$\frac{Ser}{Thr}$) (26).

A partial sequence, spanning residues 211–244, of the C-terminal CNBr peptide (residues 211–263), was obtained

TF (145-159) Asp-Val-Phe-Gly-Lys-Asp-Leu-Ile-Tyr-Thr-Leu-Tyr-Tyr-(Arg/Trp)-Lys

Coding Sequence 5'-GA$^{T}_{C}$-GTX-TT$^{T}_{C}$-GGX-AA$^{A}_{G}$-GA$^{T}_{C}$-$^{C}_{T}$TX-AT$^{T}_{C}$-TA$^{T}_{C}$-ACX-CTX-TA$^{T}_{C}$-TA$^{T}_{C}$-$^{A}_{C}$G$^{A}_{G}$-AA$^{A}_{G}$-3'
A

Probe 1 3'-CTA-CAG-AAA-CCG-TTC-CTG-GAC-TAG-ATG-TGG-GAC-ATG-ATG-GCC-CTT-5'

TF Gene 5'-GAT-GTT-TTT-GGC-AAG-GAC-TTA-ATT-TAT-ACA-CTT-TAT-TAT-TGG-AAA-3'
* * * * * * * * * *

TF (24-29) Glu-Trp-Glu-Pro-Lys-Pro

Coding Sequence 5'-GA$^{A}_{G}$-TGG-GA$^{A}_{G}$-CCX-AA$^{A}_{G}$-CCX

Probe 2 3'-CT$^{T}_{C}$-ACC-CT$^{T}_{C}$-GGX-TT$^{T}_{C}$-GG-5'

TF (#210-215) Met-Gly-Gln-Glu-Lys-Gly

Coding Sequence 5'-ATG-GGX-CA$^{A}_{G}$-GA$^{A}_{G}$-AA$^{A}_{G}$-GGX-3'

Probe #3 3'-TAC-CCX-GT$^{T}_{C}$-CT$^{T}_{C}$-TT$^{T}_{C}$-CC -5'

FIG. 1. Oligonucleotide probes used to screen human cDNA libraries for the TF gene. For each of the three probes the protein sequence and the coding sequence are given above the sequence of the complementary synthetic oligonucleotides. For probe 1, the actual sequence of the TF gene determined in these studies is given below the probe sequence, and the asterisks indicate the location of mismatches. Note that residue 158, tentatively identified as arginine, was subsequently identified as tryptophan. The overall homology of probe 1 with the TF gene was 75%.

FIG. 3. Nucleotide sequence of TF cDNAs and deduced amino acid sequence of TF. Nucleotides are numbered from the 5′ end of the λ10,3 cDNA insert, and amino acids are numbered from the N terminus of the mature TF. Underlined residues indicate amino acid sequences determined by gas-phase sequencing. The arrow indicates the boundary of the λ3,4 cDNA and the potential polyadenylylation signal is underlined.

by succinylating the intact protein and cleaving at Met-210 with CNBr, followed by gas-phase sequence analysis. To obtain additional sequence information, tryptic peptides of placental TF were isolated by HPLC and sequenced as described.

The derived amino acid sequence for the mature TF apoprotein (Fig. 3) predicts a M_r of 29,593, which is smaller than the M_r of 44,000–46,000 estimated by PAGE in $NaDodSO_4$ (14, 15). To examine this discrepancy, TF was chemically and enzymatically deglycosylated, and the molecular weight of the resulting protein was redetermined. The untreated protein migrated with an apparent M_r of 42,000 on gels containing $NaDodSO_4$ and 8 M urea (22). This value differs significantly from the apparent M_r of 46,000 observed on a Laemmli gel system (18) and suggests anomalous detergent binding (data not shown). The chemically deglycosylated protein gave an apparent M_r of 34,500 in the Swank and Munkres system (22), whereas the value for the enzymatically deglycosylated material was 33,500. Thus, carbohydrate contributes 7500–8500 to the apparent molecular weight of TF. All of the carbohydrate is probably N-linked, since chemical and enzymatic digestions give essentially the same result.

The derived sequence of human TF was examined for homology with 4668 sequences in the protein sequence data base of the National Biomedical Research Foundation¶. The homology search was performed using the FASTP program (27) and revealed no significant homology between TF and any protein in the data base. In addition, thrombomodulin (28), factor VII (9), and factor V (8) were examined, and no similarities in primary sequence were observed.

Isolation of TF cDNA Clones. Based on the protein sequence data for TF, three oligonucleotide probes were synthesized and used to screen a human placental cDNA library (containing 1.2×10^6 independent recombinants) constructed in λgt11. The probes, shown in Fig. 1, correspond to DNA coding for three regions of the TF protein. Two of the probes (2 and 3) were mixtures of 32 oligonucleotides, each 17 bases in length, which were complementary to all of the possible coding sequences for peptides isolated from the N-terminal region (probe 2) and C-terminal portion (probe 3) of human TF. Probe 1 was a single 45-base deoxyoligonucleotide corresponding to a predicted preferred coding sequence for an internal tryptic peptide. Selection of the optimal coding sequence for the 45-mer probe was based on the codon preference observed in human structural genes as described by Lathe (29).

Approximately 2.5×10^6 recombinant λgt11 phage containing cDNA inserts were screened with the radiolabeled 45-mer. Thirty-six potentially positive phage plaques were isolated from the cDNA library, plaque-purified, and screened further by hybridization to probes 2 and 3. In all cases, the 45-mer probe gave significantly stronger hybridization signals than did the mixed 17-mers. Only two λ phage isolates were reactive to a second probe, one of which hybridized to probe 3 (λ3,4) and the other to probes 2 and 3 (λ10,3). The remaining 34 positive phage plaques were not examined further. Thus, the number of clones containing TF cDNA is estimated to be between 2 and 34 per 2.5×10^6 recombinant phage. This corresponds to a relative abundance of one TF cDNA per 7×10^4 to 1×10^6 cDNAs. Analysis of restriction fragments indicated that the two λ phage recombinants contained approximately 1600- and 2100-base-pair (bp) inserts, respectively.

*Eco*RI, *Sau*3A, and *Hin*dIII restriction fragments of the two recombinant phage DNA inserts were cloned into M13mp19 (30) for sequence determination by the dideoxy method (25). Fig. 2 shows a restriction map of the TF cDNA

¶Protein Identification Resource (1987) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 11.0.

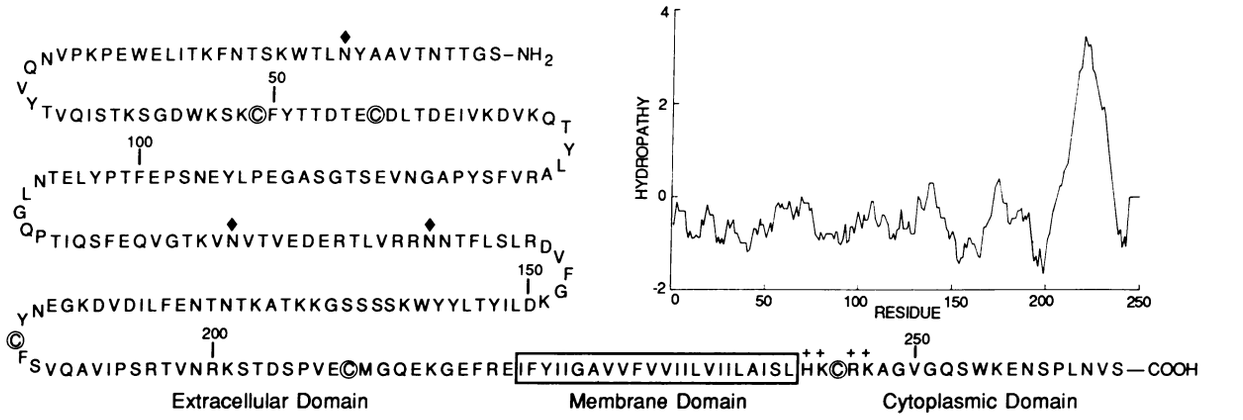

FIG. 4. Amino acid sequence and hydropathy plot of the mature form of human TF. The amino acid sequence is that derived from the cDNA sequence of the human TF gene. The hypothetical domain structure includes an extracellular region (residues 1–219), a membrane-spanning sequence (residues 220–242), and a cytoplasmic tail (residues 243–263). Other features of the structure represented schematically include three potential N-linked glycosylation sites (♦), a cluster of positively charged amino acid side chains at the cytoplasmic interface (+), and the five half-cystine residues in the molecule (circled). Hydropathy was determined by the method of Kyte and Doolittle (32). Each value was calculated as the average hydropathic index of a sequence of 21 amino acids and plotted to the middle residue of each sequence.

and indicates the regions contained in each of the recombinant λgt11 phage. Both of the cDNAs contained two internal *Eco*RI sites, and the smaller cDNA was found to overlap entirely with the larger cDNA insert. The various M13 subclones used in the nucleotide sequence analysis are summarized in Fig. 2. The sequence of large restriction fragments was obtained by using synthetic 18 nucleotide primers complementary to the TF DNA for the chain extension reactions. The continuity of the nucleotide sequence at the *Eco*RI sites was evident from sequencing the *Sau*3A and *Hin*dIII restriction fragments. The nucleotide sequence was determined for both strands of DNA for 100% of the cDNA coding for the mature protein and ≈82% of the overall cDNA sequence.

Phage λ10,3 was found to contain a full-length cDNA insert of 2147 bp, which has a single long open reading frame from nucleotides 112 to 996. The cDNA sequence and the deduced amino acid sequence of TF are given in Fig. 3. Based on the known N-terminal sequence of the mature form of TF, analysis of the cDNA sequence predicts that TF mRNA is translated into a preprotein containing a leader sequence of 32 amino acids, with the mature protein containing 263 residues. The predicted start of the preprotein is based on the location of the sole ATG codon preceding the known start of the mature protein. The 5′ untranslated region of the mRNA is very G+C rich (72% G+C), as are a number of other eukaryotic leader regions. The coding region of the TF mRNA is followed by 1151 nucleotides of 3′ untranslated sequence, which is slightly A+T rich (63% A+T). The sequence AATAAA near the end of the cDNA (position 2119) may serve as a polyadenylylation signal on the mRNA (31), since it is followed 17 bases later by a sequence of 6 adenosine residues.

The accuracy of the DNA sequence was evaluated by comparison with the sequence of a number of TF peptides, indicated by solid underlining in Fig. 3. A total of 71.5% of the coding region for the mature protein was confirmed by amino acid sequence analysis, and all peptide sequences were matched to predicted peptides. With the exception of residue 208, which was predicted to be glutamic acid based on the DNA sequence of both cDNAs and glycine based on gas-phase sequencing of peptide-(202–214), all of the remaining 187 amino acid residues determined by protein or peptide sequencing agreed exactly with the derived sequence shown in Fig. 3.

DISCUSSION

We have determined the sequence of the structural gene coding for human TF from which we have deduced the primary structure of the TF apoprotein. From inspection of this sequence, we propose a domain structure for TF, which is shown schematically in Fig. 4. The salient features of this model are as follows: (*i*) a signal peptide region of 32 residues (not shown); (*ii*) a probable extracellular domain (residues 1–219); (*iii*) a candidate for a membrane-spanning region (residues 220–242); and (*iv*) a potential cytoplasmic domain (residues 243–263). Four potential N-linked carbohydrate attachment sites (Asn-Xaa-$\frac{\text{Ser}}{\text{Thr}}$) are found in the molecule. One of these sites occurs in the presumptive cytoplasmic domain and, therefore, is probably not glycosylated. Of the three sites in the proposed extracellular domain, two have been identified as bearing carbohydrate by amino acid sequence analysis, in that no phenylthiohydantoin-conjugated amino acid could be identified at two positions (11 and 137) predicted to be asparagine. Our model, in which the N-terminal portion of TF is shown protruding from the cell membrane, is supported by numerous examples of membrane-anchored proteins, where carbohydrates have been found attached only on the extracellular domain of the proteins. The molecule contains a total of five half-cystine residues, four of which occur within the extracellular domain and are probably disulfide-linked. The presence of disulfide bridges has previously been inferred by the observation that reduction with 2-mercaptoethanol in the presence of NaDodSO$_4$ results in loss of TF activity, whereas treatment with the detergent alone does not (16). In our model, Cys-245 would not be expected to form an intramolecular disulfide bond because it is segregated on the presumptive cytoplasmic tail. This is consistent with our observation that CNBr cleavage at Met-210 liberates the carboxyl-terminal peptide with or without reduction (data not shown). It is, however, possible that Cys-245 forms intermolecular disulfide linkages, perhaps modulating TF self-association (33) or interaction with other proteins during purification.

The hydrophobic character of the proposed membrane domain is illustrated by the hydropathy plot shown in Fig. 4. This consecutive run of 23 nonpolar amino acids is a good candidate for the membrane-spanning domain. Termination of this hydrophobic region by a cluster of four positively charged amino acids is a common feature of the interface between membrane and cytoplasmic domains of many inte-

gral membrane proteins (34). The 21 amino acids on the C-terminal end of TF constitute the proposed cytoplasmic tail. It resembles the C terminus of at least two other membrane proteins, thrombomodulin and the low density lipoprotein receptor (28, 35), with respect to length, distribution of positively charged amino acids, and the presence of a single cysteine residue.

In addition to carbohydrate residues, other posttranslational modifications may be present. However, after alkaline hydrolysis of bovine TF, no γ-carboxyglutamic acid was detected (16). Thus, although γ-carboxyglutamic acid is required for factor VII activity (36), the Ca^{2+}-dependent association of these species does not require these residues in TF. The derived amino acid sequence, in conjunction with chemical analysis of the mature protein, suggests that human TF is a single polypeptide chain of 263 amino acids. As previously shown with the bovine protein, and in contrast to coagulation cofactors V and VIII, no proteolysis is required for the activator to bind factor VII (33). Likewise, zymogen factor VII is unique among coagulation proteases in that it has demonstrable proteolytic activity when associated with its activator (37). Thus, coagulation may be initiated simply by the complexation of these two factors without a prior proteolytic event. Since there is little or no continuous *in vivo* blood coagulation, it is therefore reasonable to conclude that TF is not normally accessible to blood proteins.

The amino acid sequence of TF bears little relationship to the vitamin K-dependent serine proteases (factors II, VII, IX, and X) or to the other coagulation cofactors (factors V and VIII). This lack of homology is consistent with the unique role of TF as an initiator of a proteolytic cascade without invoking prior proteolysis of either the zymogen or the activator and also implies a separate evolutionary pathway for this protein.

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