Munson, L. M., Stormo, G. D., Niece, R. L., & Reznikoff, W. S. (1984) *J. Mol. Biol. 177*, 663–683.
Murakawa, G. J., Chen, C.-h. B., Kuwabara, M. D., Nierlich, D. P., & Sigman, D. S. (1989) *Nucleic Acids Res. 17*, 5361–5375.
Noll, M., Hapke, B., Schreier, M. H., & Noll, J. (1973) *J. Mol. Biol. 75*, 281–294.
Offengand, J. (1980) in *Ribosomes, structure, function, and genetics* (Chambliss, G., et al., Eds.) pp 497–529, University Park Press, Baltimore, MD.
Reddy, P., Peterkofsky, A., & McKinney, K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 71*, 5656–5660.
Schneider, T. D., Stormo, G. D., Gold, L., & Ehrenfeucht, A. (1986) *J. Mol. Biol. 188*, 415–431.
Schümperli, D., McKinney, K., Sobieski, D. A., & Rosenberg, M. (1982) *Cell (Cambridge, Mass.) 30*, 865–871.
Shine, J., & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1342–1346.
Shinedling, S., Singer, B. S., Gayle, M., Pribnow, D., Javis, E., Edgar, B., & Gold, L. (1987) *J. Mol. Biol. 195*, 471–480.
Sigman, D. S. (1986) *Acc. Chem. Res. 19*, 180–186.
Sigman, D. S., Spassky, A., Rimsky, S., & Buc, H. (1985) *Biopolymers 24*, 183–197.
Stanssens, P., Remaut, E., & Fiers, W. (1985) *Gene 36*, 211–223.
Stefano, J. E., & Gralla, J. D. (1980) *J. Biol. Chem. 255*, 10423–10430.
Steitz, J. A. (1979) in *Biological Regulation and Development* (Goldberger, R. F., Ed.) Vol. 1, pp 349–399, Plenum Press, New York.
Steitz, J. A. (1980) in *Ribosomes, structure, function, and genetics* (Chambliss, G., et al., Eds.) pp 479–495, University Park Press, Baltimore, MD.
Stern, S., Wilson, R. C., & Noller, H. F. (1986) *J. Mol. Biol. 192*, 101–110.

Purification of Recombinant Human Tissue Factor

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ABSTRACT: Tissue factor (TF) is a 263 amino acid membrane-bound procoagulant protein that serves as a cofactor for the serine protease factor VII (fVII). Recombinant human TF (rTF) produced in both human kidney 293 cells and *Escherichia coli* has been immunoaffinity purified by using a TF-specific monoclonal antibody. Recombinant TF produced in 293 cells is glycosylated and migrates on reducing SDS–PAGE with an apparent molecular weight (M_r) of 45K. Some interchain disulfide-bonded rTF dimers are observed under nonreducing conditions. The *E. coli* produced rTF has a molecular weight of 33K and 35K, with the 33K band missing nine amino acids at the carboxy terminus. Although the *E. coli* produced rTF does not contain any carbohydrate, it is fully functional in both a chromogenic assay and a one-stage prothrombin time assay. A variant has been constructed wherein the cytoplasmic cysteine (residue 245) has been mutagenized to a serine residue. The amount of disulfide-linked aggregates is dramatically reduced following immunoaffinity purification of this four-cysteine variant (C2455), which is active in the chromogenic and prothrombin time assays.

Tissue factor (TF)[1] is a membrane-bound glycoprotein cofactor that functions in the initiation of blood coagulation. Following vascular injury, TF forms a complex with both the zymogen and activated forms of the serine protease factor VII (fVII) and modulates the catalytic activity of this enzyme toward its substrates. The importance of this interaction has been further demonstrated by the observation that this TF–fVII(a) complex, which was previously thought to activate only fX, also activates fIX (Osterud & Rapaport, 1977). Because the TF–fVII(a) complex has been shown to activate factors in both the extrinsic and the intrinsic pathways of blood coagulation, this complex is now believed to be the physiological inducer of blood clotting (Nemerson & Bach, 1982). TF apoprotein has been purified from bovine brain (Bach et al., 1981; Carson et al., 1985) as well as from human brain and human placenta (Broze et al., 1985; Guha et al., 1986; Morrissey et al., 1988; Rao & Rapaport, 1987). The apo-

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[1] Abbreviations: TF, tissue factor; fVII, factor VII; TFA, trifluoroacetic acid; DTT, dithiothreitol; TMAC, tetramethylammonium chloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; MTX, methotrexate; dhfr, dihydrofolate reductase; r, recombinant.

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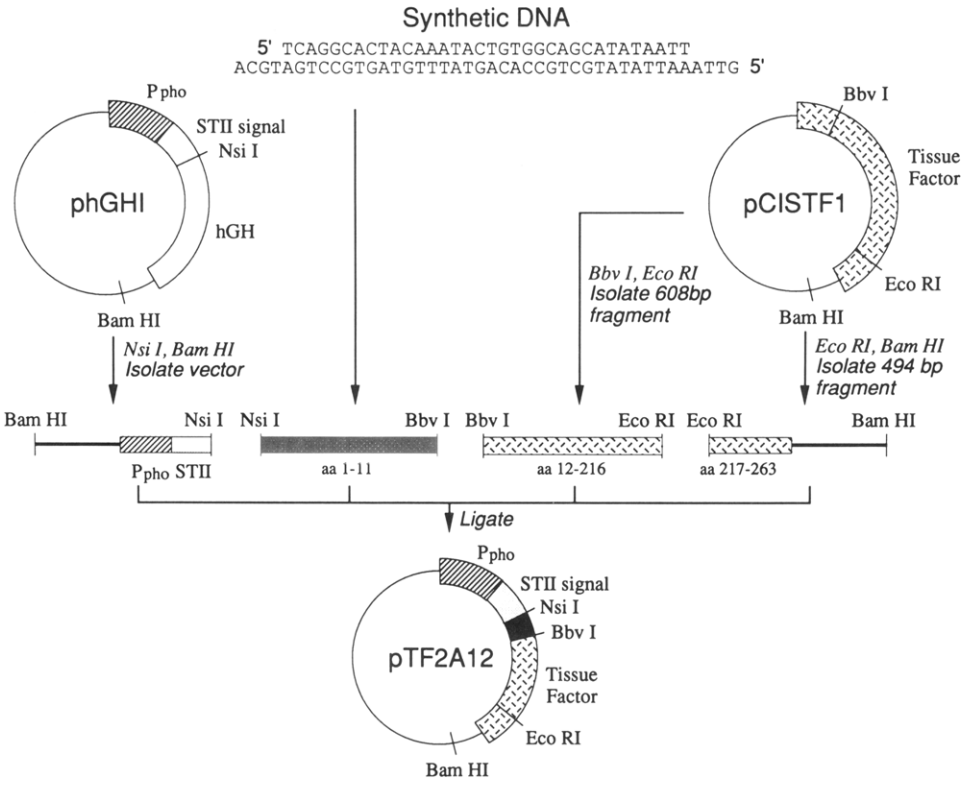

FIGURE 1: Construction of the plasmid pTF2A12 for the expression of TF in *E. coli*. The coding sequence for hGH in the expression plasmid phGH was replaced with the coding sequence for mature TF described under Experimental Procedures. The sequence of synthetic DNA used in the construction was verified by DNA sequencing after cloning.

protein has minimal procoagulant activity and must be reassociated with lipid for full biological function (Pitlick & Nemerson, 1970).

The cDNA cloning of human TF has recently been described (Fisher et al., 1987; Morrissey et al., 1987; Scarpati et al., 1987; Spicer et al., 1987). The cDNA sequence predicts a mature protein of 263 amino acids that can be divided into 3 domains: an extracellular domain (residues 1–219) containing 4 cysteine residues and 3 potential Asn-linked glycosylation sites; a hydrophobic putative transmembrane domain (residues 220–242); and a cytoplasmic domain (residues 243–263) which contains the fifth cysteine residue and 1 potential Asn-linked glycosylation site.

In this paper, we describe the immunoaffinity purification of the recombinant apoprotein produced in human kidney 293 cells as well as in *Escherichia coli*. Both forms of recombinant TF (rTF) are active in a TF chromogenic assay. Thus, it seems apparent that the proper glycosylation is not essential for activity in vitro. In addition, a variant has been constructed wherein the cysteine at position 245 located in the cytoplasmic domain has been changed to a serine residue by site-directed mutagenesis. The resulting immunoaffinity-purified four-cysteine variant (C245S) produced in *E. coli* is essentially free of dimers and also displays procoagulant activity upon relipidation. The purification of rTF and several variants of TF should provide significant quantities of purified protein for further structure/function studies.

Experimental Procedures

Materials

Cyanogen bromide (CNBr)-activated Sepharose 4B, protein A–Sepharose, and DEAE-Sepharose were from Pharmacia LKB. Tetramethylammonium chloride (TMAC) and CNBr were from Kodak. Lysozyme, rabbit brain thromboplastin, and iodoacetic acid were from Sigma. TPCK-trypsin was from Worthington. Trifluoroacetic acid (TFA) and BCA protein assay reagent were from Pierce Chemical Co. Acetonitrile was from Burdick and Jackson. All acrylamide reagents were from Bio-Rad. [^{35}S]Cysteine was from Amersham Corp. Dithiothreitol (DTT) was from Bethesda Research Laboratories. The mouse monoclonal antibody, D3, and rabbit antiserum, RD010, specific for human TF were made as described (Paborsky et al., 1988).

Methods

Relipidation of TF, Chromogenic TF Assay, and One-Stage Prothrombin Time Assay for TF. rTF apoprotein was reassociated with phospholipid as described by O'Brien et al. (1988). TF assays were performed as described (O'Brien et al., 1988). A standard curve was constructed by using rabbit brain thromboplastin reconstituted according to the manufacturer's instructions. One unit of TF activity was defined as that contained in 10 μL of this material. Dilution of the rabbit brain thromboplastin produced a curve parallel to that of purified human rTF.

Construction of Plasmids pTF2A12 and pTF111. The scheme for construction of pTF2A12 starting with the human growth hormone (hGH) expression plasmid phGH1 (Chang et al., 1987) is shown in Figure 1. This plasmid, pTF2A12, was designed to express mature TF in *E. coli* with the aid of the enterotoxin II (STII) signal sequence (Lee et al., 1983; Picken et al., 1983). The transcriptional and translational sequences required for expression are provided by the alkaline phosphatase promoter (Kikuchi et al., 1981) and the tryptophan (Trp) (Bennett et al., 1978) and STII Shine–Dalgarno sequences. This identical series of sequences was used to promote the high level of secretion of hGH in *E. coli* (Chang et al., 1987).

The expression plasmid, phGH1, was first digested with *Nsi*I and *Bam*HI to remove the coding sequence for mature hGH. The *Nsi*I restriction site lies at the junction of the STII signal

sequence and the beginning of the hGH coding sequence (Chang et al., 1987). Three fragments were then ligated into this expression vector, the first of which was a synthetic DNA duplex encoding the first 11 amino acids of mature TF. The second was a 608 base pair *Bbv*I–*Eco*RI restriction fragment from plasmid pCISTF1 (Fisher et al., 1987) coding for amino acids 12–216. The third was a 514 base pair *Eco*RI–*Bam*HI fragment from pCISTF1 which includes the sequences which encode the last 47 amino acids of TF followed by some 3′ untranslated region.

Plasmid pTF111, which has a serine residue in place of the cysteine at position 245, was made in three steps. The first two steps reconstruct the 3′ end of the gene where the mutation is made. In the first step, a 32 base pair *Eco*RI–*Fok*I restriction fragment from pCISTF1 encoding amino acids 217–227 of mature TF was ligated along with the synthetic DNA duplex

```
5'-TATTTGTGGTCATCATCCTTGTCATCAT
    3'-ACACCAGTAGTAGGAACAGTAGTA

CCTGGCTATATCTCTACACAAGT-3'

GGACCGATATAGAGATGTGTTCAGATC-5'
```

into an *Xba*I–*Eco*RI-digested cloning vector. The resulting subclone of this first step was plasmid pTF100-1. In the second step, the synthetic DNA duplex

```
5'-CTAGAAAGGCAGGAGTTGGCCAGAGCTGGAAGGA
    3'-TTTCCGTCCTCAACCGGTCTCGACCTTCCT

GAACTCCCCACTGAATGTTTCATAAGCTTG-3'

CTTGAGGGGTGACTTACAAAGTATTCGAACCTAG-5'
```

coding for the last 18 amino acids of TF was subcloned into an *Xba*I–*Bam*HI-digested cloning vector to generate plasmid pTF80-3. The final step required the ligation of four DNA fragments. The first was the previously described vector phGH1 which had been digested with *Nsi*I and *Bam*HI to remove the hGH sequences. The second was a 650 base pair *Nsi*I–*Eco*RI restriction fragment from pTF2A12 encoding amino acids 1–217 of mature TF. The third was a 80 base pair *Eco*RI–*Xba*I fragment from pTF100-1 encoding amino acids 218–245. The fourth was a 60 base pair *Xba*I–*Bam*HI insert from pTF80-3 encoding the last 18 amino acids.

Expression and Amplification of pCISTF1 in 293 Cells. A TF expression vector, pCISTF1 (Fisher et al., 1987), was used for the establishment of a stable human kidney 293 cell (Graham et al., 1977) clone expressing TF, 63.45, and will be described elsewhere (L. R. Paborsky, unpublished experiments). For this report, the SV40–dihydrofolate reductase (dhfr) transcription unit present on pCISTF was used for coamplification of the dhfr and TF genes. One clone was selected by passaging through successive rounds of 10, 25, and 50 nM methotrexate (MTX). Clones were assayed at each round of amplification for increasing TF activity. The clone amplified to 50 nM MTX is referred to herein after as 63.45.1.

293 Cell Culture. Stock cultures were grown in selective medium consisting of a 1:1 mixture of Ham's F-12 without glycine, hypoxanthine, or thymidine, and Dulbecco's-modified Eagle's medium without glycine (Gibco). Additions to the basal medium included 7.5% extensively diafiltered fetal bovine serum (Armour), 50.0 nM MTX (Lederle), 2.0 mM L-glutamine (Gibco), and 10.0 mM HEPES buffer (Research Organics).

Immunoaffinity Purification of Recombinant Human TF. A typical preparation was initiated with 8×10^9 cells of clone 63.45.1. The cells were washed 2 times with phosphate-buffered saline (PBS) and then resuspended in 350 mL of extraction buffer: 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1% Triton X-100. The cell suspension was rotated end over end for 1 h at 4 °C and then centrifuged at 800*g* for 20 min. The supernatant was batch-adsorbed on DEAE-Sepharose equilibrated in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.05% Tween 80. The unadsorbed rTF fraction was collected, adjusted to 0.5 M NaCl, and then applied at a flow rate of 0.5 mL/min to a D3-Sepharose affinity column (30 mg of protein A purified D3 IgG was attached to 10 mL of CNBr-activated Sepharose 4B according to the manufacturer's instructions). The column was first washed with the equilibration buffer (20 mM Tris-HCl, pH 7.5 150 mM NaCl, and 0.05% Tween 80) until the absorbance at 280 nm of the effluent returned to base line and was then washed with 1 M NaCl, 0.5 M TMAC, 20 mM Tris-HCl, pH 7.5, and 0.05% Tween 80. rTF was eluted with 0.1 M acetic acid, pH 3.0, containing 150 mM NaCl and 0.05% Tween 80. The eluate was immediately adjusted to pH 7.5 with 1 M Tris base.

Purification of E. coli Produced TF. rTF was isolated from *E. coli* K12 cells harboring plasmid pTF2A12 or pTF111. The cells were grown to stationary phase in a 10-L fermenter, harvested by centrifugation, and stored at −70 °C. Five grams of cell paste was thawed and resuspended in 250 mL of extraction buffer containing 1 mg/mL lysozyme. The extract was sonicated (10 pulses for 10 s each) and rotated end over end for 1 h at 4 °C. Poly(ethylenimine) (5% w/v), pH 7.5, was added to a final concentration of 0.1%. The extract was stirred for 5 min and then clarified by centrifugation at 800*g* at 22 °C for 20 min. rTF was purified by using DEAE-Sepharose and D3-Sepharose as described above.

Pulse–Chase Analysis of C245S rTF. E. coli cells harboring plasmid pTF111 were pulse-labeled for 5 min with 250 μCi of [^{35}S]cysteine in 10 mL of medium as described by Chang et al. (1987) except Hycase amino acids (Sheffield Products, Norwick, NY) and yeast extract (Fidco, White Plains, NY) were used as a complex nitrogen source. The cells were chased with 10 mg of cold cysteine, and at various intervals of chase (as indicated in the figure legends), the cells were pelleted. rTF was immunoprecipitated using a D3-Sepharose resin.

CNBr Cleavage. One nanomole of *E. coli* produced rTF was methanol precipitated by the addition of 3 volumes of cold methanol at 0 °C. Following centrifugation, the pellet was resuspended in 500 μL of 0.25 M Tris-HCl, pH 8.5, 5 mM EDTA, and 4 M urea containing 20 mM DTT. The sample was incubated overnight at room temperature. Iodoacetic acid (1 M in 1 M NaOH) was added to a final concentration of 100 mM. Incubation was at room temperature for 30 min, and the reaction was stopped by the addition of 100 mM DTT. The rTF was methanol precipitated again and resuspended in 70% formic acid. CNBr (60 mg) was added, and the sample was incubated in the dark, overnight at room temperature. The digest was dried, resuspended in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 80, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting, using the polyclonal serum RDO10.

Protein Sequence Analysis. Protein sequence analysis was carried out by using an Applied Biosystems 470A protein sequencer.

Trypsin Digestion. One nanomole of *E. coli* produced rTF was dialyzed into against 2 L of 10 mM Tris, pH 8.3, 100 mM sodium acetate, and 0.1 mM $CaCl_2$. TPCK-trypsin (trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl ketone) was added at a ratio of 1:100 (w/w) for digestion at 37 °C for 2.5

Table I: Purification of rTF

step	volume (mL)	protein[a] (mg/mL)	TF act.[b] (units)	sp. act. (units/mg)	yield (%)
(A) 293-Produced rTF					
extract	350	3.65	784000	615	100
DEAE-Sepharose	400	1.95	750000	975	95
D3-Sepharose	20	0.17	385000	115000	49
(B) *E. coli* Produced rTF					
extract	250	1.89	765000	1620	100
DEAE-Sepharose	300	1.03	575000	1850	75
D3-Sepharose	20	0.16	305000	95000	40
(C) *E. coli* Produced C245S rTF					
extract	250	2.90	1160000	1600	100
DEAE-Sepharose	300	1.45	987000	2270	85
D3-Sepharose	15	0.29	795000	185000	68

[a] Protein concentrations were estimated by using the BCA protein assay with BSA as the standard. [b] TF activity was measured by using chromogenic assays as described under Experimental Procedures.

h. A second 1:100 aliquot was then added and the digest continued for an additional 2.5 h at 37 °C. Ten microliters of TFA was added to stop the digestion.

HPLC. A Vydac C_4 column (4.6 × 250 mm) was equilibrated with 70% solvent A (0.1% TFA in water)/30% solvent B (0.08% TFA in acetonitrile) at 40 °C with a flow rate of 1.0 mL/min. Starting 5 min after sample injection, a linear gradient from 30% B to 80% B over 75 min was developed by a Hewlett-Packard Model 1090 HPLC. Fractions were collected manually. Tryptic digests were chromatographed by using the same system but with a different column (Waters Novapak C_{18}, 3.9 × 150 mm) and gradient (0–50% B over 100 min after a 2-min delay). The flow rate was 1.0 mL/min, with a temperature of 40 °C. Peak collection was performed manually.

Amino Acid Analysis. Aliquots of collected peaks were hydrolyzed for 24 h at 110 °C in 6 N HCl, in vacuo. Hydrolysates were analyzed with a Beckman 6300 amino acid analyzer. Quantitation was determined by peak area analysis using a Nelson Analytical M600 data system.

SDS–Polyacrylamide Gel Electrophoresis. Denaturing gel electrophoresis was performed according to Laemmli (1970), and the gels were stained with 0.25% Coomassie Brilliant Blue or silver stained according to Morrissey (1981).

Results

Purification of rTF. The results of the immunoaffinity purification of rTF produced in 293 cells are summarized in Table IA. Ten liters of 293 cells containing pCISTF1 and grown in 50 nM MTX was harvested by centrifugation. The detergent extract was batch-adsorbed onto DEAE-Sepharose. rTF does not bind under the conditions used, and the unadsorbed fraction was collected, adjusted to 0.5 M NaCl, and loaded directly onto the D3-Sepharose immunoaffinity column. The column was washed with buffer containing Tween 80 to replace the Triton X-100. rTF was eluted with acid, and the pooled fractions were neutralized. rTF produced in *E. coli* was immunoaffinity purified by essentially the same procedure that was used to purify material derived from 293 cells.

The purified 293 and *E. coli* produced rTF were analyzed by both reducing and nonreducing 7–17% SDS–PAGE as shown in Figure 2. The rTF expressed in 293 cells is glycosylated and migrates with an apparent molecular mass of 45 kDa under reducing conditions (Figure 2, lane 1). The rTF produced in *E. coli* is not glycosylated and migrates on SDS–PAGE under reducing conditions as a doublet of 33 and 35 kDa (Figure 2, lane 2). The calculated molecular weight of human TF on the basis of the amino acid sequence is 29593.

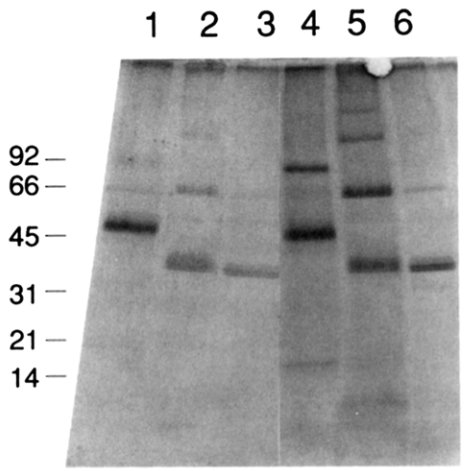

FIGURE 2: SDS–polyacrylamide gel electrophoresis of immunoaffinity-purified rTF produced in 293 cells and *E. coli*. rTF was purified from either *E. coli* or 293 cells as described under Experimental Procedures. rTF (0.5 μg) was loaded per lane and visualized by silver staining (Morrissey, 1981). Lane 1, 293 cell produced rTF, reduced; lane 2, *E. coli* produced rTF, reduced; lane 3, *E. coli* produced C245S rTF, reduced; lane 4, 293 cell produced rTF, nonreduced; lane 5, *E. coli* produced rTF, nonreduced; lane 6, *E. coli* produced C245S rTF, nonreduced. Molecular weight standards ($\times 10^{-3}$) are indicated on the left.

The difference in molecular weight between the calculated and apparent molecular weight of the nonglycosylated *E. coli* produced rTF may be due to other posttranslational modifications or, more likely, to aberrant migration of the protein on SDS–PAGE. The purified 293-produced rTF was also subjected to gel filtration on a Superose 12 column in 20 mM Tris-HCl, pH 9.0, 150 mM NaCl, and 0.05% Tween 80. Under these conditions, rTF migrates as a large aggregate with an approximate molecular mass of 340000 daltons (data not shown).

TF contains five half-cysteine residues, and, under nonreducing SDS–PAGE conditions, interchain disulfide-bonded TF dimers and aggregates are observed (Figure 2, lanes 4 and 5). The cytoplasmic domain which contains one cysteine residue is separated from the remaining four cysteine residues in the extracellular domain. The cysteine at position 245 was theorized to have a free sulfhydryl because it is the only cysteine residue located in the purported cytoplasmic domain and unlikely to be disulfide-bonded to a cysteine residue in the extracellular domain. In order to test this hypothesis, the purified *E. coli* produced rTF was reduced and alkylated and then digested with CNBr as described under Experimental Procedures. TF contains only one methionine residue (position 209), and treatment with CNBr results in the cleavage of the protein into two fragments: one of approximately 23500 daltons containing four cysteine residues and another of 6100 daltons containing the one cysteine residue in the cytoplasmic domain. The fragments were separated on a 7–17% SDS–polyacrylamide gel and transferred to nitrocellulose, and the nitrocellulose filter was probed with RDO10, an anti-human TF rabbit polyclonal antibody. CNBr digests of both reduced and alkylated (Figure 3, lane 2) and nonreduced and alkylated rTF (Figure 3, lane 3) result in the generation of a fragment of 23500 daltons. This demonstrates that there is no intrachain disulfide bond between a cysteine residue at position 245 and a cysteine residue in the extracellular domain. The disappearance of dimer following CNBr cleavage suggests that dimers are formed by intermolecular disulfide bonds involving the cysteine residue at position 245. Identical results were found with the rTF produced in 293 cells (data not shown).

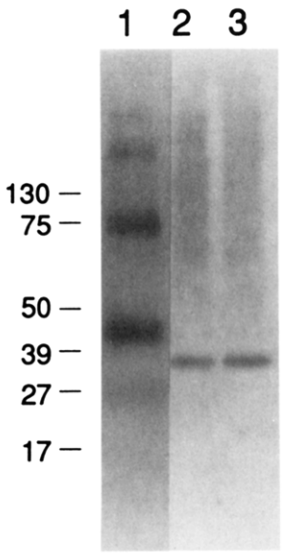

FIGURE 3: Immunoblot of CNBr digests of reduced and alkylated and of nonreduced and alkylated rTF. Wild-type *E. coli* produced rTF, reduced or nonreduced, was alkylated and digested with CNBr. The starting material and digests were separated on a 7–17% polyacrylamide gel, transferred to nitrocellulose, and probed with RDO10 as described under Experimental Procedures. The antibody–antigen complex was visualized using ^{125}I protein A. Lane 1, wild-type *E. coli* produced rTF; lane 2, reduced and alkylated *E. coli* produced rTF digested with CNBr; lane 3, nonreduced and alkylated *E. coli* produced rTF digested with CNBr.

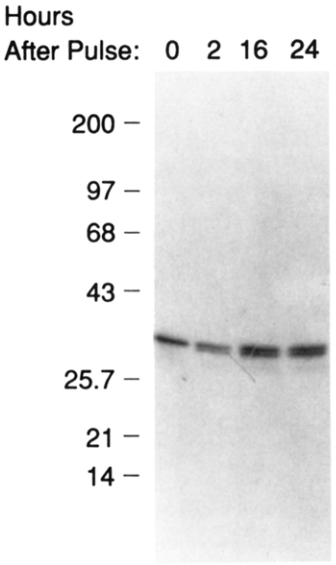

FIGURE 4: Pulse–chase analysis of immunoprecipitated *E. coli* produced C245S rTF. *E. coli* containing plasmid pTF111 was pulse-labeled for 5 min with 25 μCi/mL [^{35}S]cysteine and then chased for various intervals. One-milliliter samples were taken immediately after labeling or at various times indicated ("hours after pulse"). Lysates were prepared, and rTF was immunoadsorbed on D3-Sepharose, analyzed by 7–17% SDS–PAGE, and visualized by fluorography.

A variant wherein the cysteine residue at position 245 has been changed to a serine residue by site–directed mutagenesis was constructed. The controlling elements for expression, the alkaline phosphatase promoter, Trp, and STII Shine–Dalgarno sequences, and the STII leader sequence are identical with those used in plasmid pTF2A12. The resulting four-cysteine variant (C245S) was expressed in *E. coli* and purified by the same method as the wild-type rTF. This purification scheme, summarized in Table IC, resulted in a 200-fold purification with a yield of 68%. SDS–PAGE analysis of this variant is displayed in Figure 2 (lanes 3 and 6). The amount of dimer formation observed with the C245S rTF is dramatically reduced; however, a small amount of dimer formation is still observed under nonreducing conditions. The specific activity of this variant appears to be approximately 2-fold greater than that of the wild-type *E. coli* produced rTF. This variant also migrates as a large aggregate when subject to the gel filtration conditions described for the 293 cell produced rTF.

The amino-terminal sequence of the rTF was examined. The wild-type TF and C245S rTF produced *E. coli* have the NH_2-terminal sequence predicted for mature TF. In contrast, 40% of the rTF produced in the 293 cells is missing the first two amino acids, serine and glycine, consistent with previous studies of human brain TF (Morrissey et al., 1987; Scarpati et al., 1987; Spicer et al., 1987).

The *E. coli* produced rTF appears to be a doublet (Figure 2, lanes 2 and 3), whereas the rTF purified from 293 cells is a single band on reducing SDS–PAGE, as is native TF purified from human placenta and human brain (Broze et al., 1985; Guha et al., 1986; Morrissey et al., 1988; Rao & Rapaport, 1987). To determine how the *E. coli* produced protein was being processed, pulse–chase analysis was performed. A 10-mL shaker flask of *E. coli* containing plasmid pTF111 was pulse-labeled for 5 min with 25 μCi/mL [^{35}S]cysteine and then chased with 10 mg of cold cysteine. Immediately following the labeling and at various intervals of chase (indicated in the figure legend to Figure 4), cells were pelleted and stored on ice. rTF was extracted and immunoprecipitated with D3-Sepharose as described under Experimental Procedures. These results are displayed in Figure 4. rTF is synthesized as a single polypeptide but is clipped to a doublet within 2 h. The amount of the lower molecular weight protein band does not appear to accumulate with time, with the amount of each band remaining at an approximate 50:50 ratio, even after a 24-h chase period.

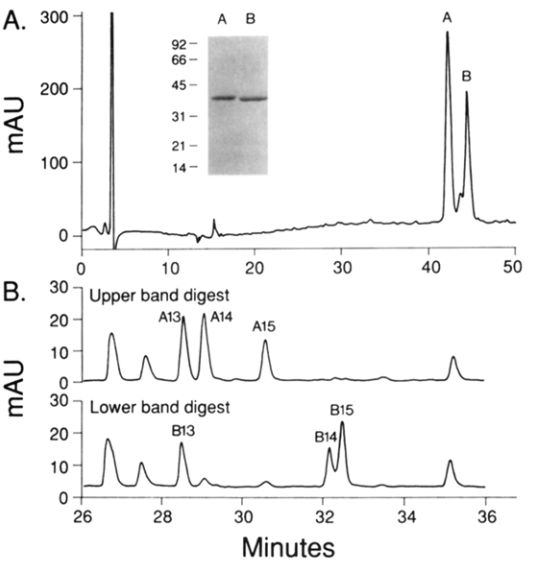

FIGURE 5: Reverse-phase HPLC of *E. coli* produced C245S rTF. Column, solvents, and flow rate were as given under Experimental Procedures. (A) *E. coli* produced C245S rTF. Insert: 7–17% SDS–PAGE of upper (A) and lower band (B) of C245S rTF. The gel is stained with Coomassie Brilliant Blue. (B) Tryptic digests of lower band (A) and upper band (B) of *E. coli* produced C245S rTF doublet. The peptides were detected by measuring the absorbance at 214 nm.

The C245S rTF doublet was chromatographed on a C_4 reverse-phase HPLC column as described under Experimental Procedures. The chromatogram is displayed in Figure 5A. The upper (A) and lower (B) bands separate under these conditions as demonstrated following analysis on a 7–17%

Table II: Identification of Tryptic Peptides of Upper (A) and Lower (B) Bands of *E. coli* Produced C245S rTF

peak	nmol	residues	deduced sequence
A13	0.69	123–131	VNVTVEDER
	0.19	247–255	KAGVGQSWK
A14	0.41	248–255	AGVGQSWK
A15	0.65	256–263	ENSPLNVS
B13	1.13	123–131	VNVTVEDER
B14	0.23	247–254	KAGVGQSW
B15	0.51	248–254	AGVGQSW

SDS–polyacrylamide gel (Figure 5A, insert). These fractions were digested with trypsin, and the digests were chromatographed on a C_{18} column. Figure 5B displays only the section of the tryptic digests where the peaks are nonidentical. The tryptic peptides were identified by amino acid analysis, and these results are summarized in Table II. The absence of the C-terminal tryptic peptide (peak A15) and the loss of peaks A13 and A14 demonstrate that residues 255–263 are missing from the lower band. Proteolytic processing of rTF by *E. coli* results in a truncated form ending at Trp-254. Presently, different growth conditions and *E. coli* hosts are being examined in an attempt to minimize this cleavage.

Discussion

Recombinant human TF was produced in both *E. coli* and human kidney 293 cells and immunoaffinity purified by using a TF-specific monoclonal antibody. The mammalian-produced rTF appears as a single 45-kDa band on reduced SDS–PAGE. The *E. coli* produced material is a doublet of 33 and 35 kDa under the same conditions.

The five-cysteine wild-type *E. coli* produced rTF appears to form a series of multimers. When analyzed on nonreducing SDS–PAGE, this "ladder" disappears following CNBr digest, which separates the cytoplasmic cysteine residue from the remaining four cysteine residues in the extracellular domain. The cause of the multimerization is not known at present. The fact that the multimerization is not observed with the 293-produced rTF may indicate that glycosylation can be a factor. Both the *E. coli* and 293 cell produced rTF are functional in the TF chromogenic assay as well as the one-stage prothrombin time assay, indicating that the glycosylation is not required for procoagulant activity in vitro and any conformational differences do not affect function.

Our result demonstrating that the cysteine at position 245 is not involved in intrachain disulfide bonding is in agreement with results recently published by Bach et al. (1988), who demonstrated that the four cysteine residues in the extracellular domain form two disulfide bonds and the fifth cysteine in the cytoplasmic region can be acylated by palmitic acid and stearic acid. Their results indicated that formation of dimers in human brain TF is due to deacetylation during isolation. Immunoprecipitation of rTF from 293 cells metabolically labeled with [^{3}H]palmitic acid indicates that the rTF produced in these cells is also acylated (L. R. Paborsky, unpublished observations). In order to eliminate the formation of interchain disulfide bonds, the free cysteine residue located in the cytoplasmic domain was mutagenized to a serine residue. The observation that the resulting *E. coli* produced C245S rTF is functional suggests that the palmitic and steric acids are not required for function in vitro, which is in agreement with the findings of Bach et al. (1988).

The C-terminal cleavage that is observed with the *E. coli* produced rTF has never been observed with the TF purified from 293 cells nor has it been observed with the purification of native TF. This suggests that the protease responsible for this cleavage is probably not a TF-specific protease but an artifact of the *E. coli* expression system. We have not been able to separate the two bands by a method that retains functionality. However, we have constructed a variant that is lacking the cytoplasmic domain (residues 244–263), and this purified variant is fully functional (L. R. Paborsky, unpublished results), further proving that cleavages in the cytoplasmic domain of TF do not affect its relipidated activity.

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Registry No. TF, 9035-58-9; cysteine, 52-90-4; serine, 56-45-1.

References

Bach, R., Nemerson, Y., & Konigsberg, W. (1981) *J. Biol. Chem. 256*, 8324–8334.

Bach, R., Konigsberg, W. H., & Nemerson, Y. (1988) *Biochemistry 27*, 4227–4231.

Bennett, G. N., Schweingruber, M. E., Brown, K. D., Squires, C., & Yanofsky, C. (1978) *J. Mol. Biol. 121*, 113–137.

Bom, V. J., Ram, I. E., Alderkamp, G. H., Reinalda-Poot, H. H., & Bertina, R. M. (1986) *Thromb. Res. 42*, 635–693.

Broze, G. J., Jr., Leykam, J. E., Schwartz, B. D., & Miletich, J. P. (1985) *J. Biol. Chem. 260*, 10917–10920.

Carson, S. D., Bach, R., & Carson, S. M. (1985) *Blood 66*, 152–156.

Chang, C. N., Rey, M., Bochner, B., Heyneker, H., & Gray, G. (1987) *Gene 55*, 189–196.

Fisher, K. L., Gorman, C. M., Vehar, G. A., O'Brien, D. P., & Lawn, R. M. (1987) *Thromb. Res. 48*, 89–99.

Graham, F. L., Smiley, J., Russell, W. C., & Nairn, R. (1977) *Gen. Virol. 36*, 59–77.

Guha, A., Bach, R., Konigsberg, W., & Nemerson, Y. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 299–302.

Kikuchi, Y., Yoda, K., Yamasaki, M., & Tamura, G. (1981) *Nucleic Acids Res. 9*, 5671–5678.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lee, C. H., Moseley, S. L., Moon, H. W., Whipp, S. C., Gyles, C. L., & So, M. (1983) *Infect. Immun. 42*, 264–268.

Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.

Morrissey, J. H., Fakhrai, H., & Edgington, T. S. (1987) *Cell 50*, 129–135.

Morrissey, J. H., Revak, D., Tejada, P., Fair, D. S., & Edgington, T. S. (1988) *Thromb. Res. 50*, 481–493.

Nemerson, Y., & Bach, R. (1982) *Prog. Hemostasis Thromb. 6*, 237–261.

O'Brien, D. P., Giles, A. R., Tate, K. M., & Vehar, G. A. (1988) *J. Clin. Invest. 82*, 206–211.

Osterud, B., & Rapaport, S. I. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5260–5264.

Picken, R. N., Mazaitis, A. J., Maas, W. K., Rey, M., & Heyneker, H. (1983) *Infect. Immun. 42*, 269–275.

Pitlick, F. A., & Nemerson, Y. (1970) *Biochemistry 9*, 5105–5113.

Rao, L. V. M., & Rapaport, S. I. (1987) *Anal. Biochem. 165*, 365–370.

Scarpati, E. M., Wen, D., Broze, G. J., Jr., Miletich, J. P., Flandermeyer, R. R., Siegel, N. R., & Sadler, J. E. (1987) *Biochemistry 26*, 5234–5238.

Spicer, E. K., Horton, R., Bloem, L., Bach, R., Williams, K. R., Guha, A., Kraus, J., Lin, T.-C., Nemerson, Y., & Konigsberg, W. H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5148–5152.