Tissue Factor Cytoplasmic Domain Peptide Is Multiply Phosphorylated in Vitro†

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ABSTRACT: Human tissue factor was phosphorylated when incubated with lysates of U87-MG cells or fractions from preparative isoelectric focusing of the lysates. The cytoplasmic domain peptide, isolated following chemical cleavage at cysteine 245, focused on PhastGel IEF near pH 3.4, indicating the presence of three phosphate groups. A peptide corresponding to the carboxyl-terminal cytoplasmic domain (residues 245−263) was synthesized and shown to be a protein kinase substrate when incubated with lysates of U87-MG cells and radiolabeled ATP. As found with full-length tissue factor, the $TF_{245-263}$ peptide was phosphorylated at all three serines, but a diphosphate form was also identified. $TF_{245-263}$ was phosphorylated in the absence of calcium as well as in the presence of calphostin C, indicating that phosphorylation can be independent of protein kinase C. These results reveal that tissue factor can be multiply phosphorylated in vitro, and that the synthetic $TF_{245-263}$ cytoplasmic domain peptide serves as a model substrate.

Tissue factor (TF)[1] is a transmembrane glycoprotein which serves as an essential cofactor for factor VII in the physiological initiation of blood coagulation. It consists of 3 principal domains: a large extracellular domain of 219 amino acid residues, a single transmembrane domain of 23 residues, and a cytoplasmic domain of 21 residues (Spicer et al., 1987; Ruf & Edgington, 1994). The extracellular domain is responsible for factor VII binding and subsequent procoagulant activity (Broze, 1982; Drake et al., 1989). Although tissue factor is believed to associate with cytoskeletal structures (Muller et al., 1993; Carson et al., 1996) and transduce calcium-flux signals in response to factor VII binding (Rottingen et al., 1995), functions for the cytoplasmic domain remain to be established. Tissue factor can be palmitylated and phosphorylated and can form cysteine-linked homodimers, all via the cytoplasmic domain (Bach et al., 1988; Zioncheck et al., 1992; Roy et al., 1991). Published precedents for regulatory roles in membrane function exist for each of these modifications. Intermediary proteins participating in the modifications and potential regulatory interactions involving the TF cytoplasmic domain remain largely unstudied.

The importance of protein kinases in signal transduction and cellular recognition has been well recognized, and reversible phosphorylation is a major intracellular regulatory mechanism controlling receptor activity and cellular processing. Moreover, phosphorylation of the cytoplasmic domain of a number of membrane proteins has been shown to affect their structural and functional properties, consequently signalling specific cellular events (Smith et al., 1994; O'Toole et al., 1995; Stratford et al., 1984; Hoxie et al., 1986; Shin et al., 1991; Mitchell et al., 1994; Crowe et al., 1993). Generally, the phosphorylation of specific residues is determined by patterns (motifs) of amino acid residues located near the target site (Edelman et al., 1987). Hence, synthetic peptides bearing the necessary phosphorylation site and recognition motif have been used as effective model substrates (Woodgett, 1991). Since these peptide substrates are amenable to various post-phosphorylation procedures that make study easier than if the native macromolecular substrate were employed, they are useful surrogates for study of phosphorylation mechanisms and control (Krebs et al., 1986).

A considerable body of knowledge has accumulated from studies of protein kinase activity required for tissue factor expression in monocytes and endothelial cells (e.g., Crossman et al., 1990; Carr et al., 1990). The consensus of these studies is that protein kinase C (PKC) is required for tissue factor upregulation at the mRNA level. Additional roles for PKC are implied from other studies, which have shown that phorbol myristate acetate (PMA), a potent activator of PKC, can suppress tissue factor expression in monocytes, and can increase the direct phosphorylation of tissue factor in a recombinant expression system (Zioncheck et al., 1992; Janco & Morris, 1985; Brozna & Carson, 1988). Additional evidence that other protein kinases (e.g., cAMP-dependent protein kinase, PKA) also influence tissue factor expression is also accumulating (Lyberg, 1984; Crutchley et al., 1992; Ollivier et al., 1993; Brozna et al., 1994). The work of Zioncheck et al. (1992) has shown that the cytoplasmic domain of recombinant human TF expressed in transfected cells is phosphorylated on a serine residue by a PKC-dependent mechanism. We initiated the studies reported here to identify the site(s) of tissue factor phosphorylation and to

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[1] Abbreviations: TF, tissue factor; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; HPLC, high-pressure liquid chromatography; DTT, dithiothreitol; PMA, phorbol 12-myristate 13-acetate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IEF, isoelectric focusing; PKC, protein kinase C; PKA, protein kinase A; PKG, protein kinase G; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; pNPP, *p*-nitrophenyl phosphate; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; PS, phosphatidylserine; DAG, diacylglycerol; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; BSA, bovine serum albumin.

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begin studies that will elucidate the role of this modification in tissue factor regulation.

EXPERIMENTAL PROCEDURES

Materials. Phast IEF and SDS−PAGE gels were from Pharmacia−LKB Biotechnology. Peptides were synthesized in the University of Nebraska Medical Center peptide synthesis facility and purified by preparative reverse phase HPLC. Homogeneity of the peptide was demonstrated by analytical HPLC, and its identity was confirmed by amino acid composition analysis and fast atom bombardment mass spectrometry. Human placental tissue factor was purified by immunoaffinity chromatography (Carson et al., 1987).

U87-MG glioblastoma cells (ATCC HTB 14) were maintained in DMEM supplemented with 10% fetal bovine serum, 1 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Lysates were prepared from U87-MG cells cultured in 175-cm^2 flasks for 48 h to approximately 70% confluence. Cells were washed twice with ice-cold TBS (0.05 M Tris, 0.1 M NaCl, and 0.01% NaN_3, pH 7.6) and 1.0 mL of lysis mixture [50 mM β-glycerophosphate (pH 7.3) containing 15 mM $MgCl_2$, 1 mM PMSF, 0.2 mM Na_3VO_4, 1% Triton X-100, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 30 mM *p*-nitrophenyl phosphate]. The cell lysate was vortexed briefly, kept on ice for 5 min, and centrifuged at 12000*g* for 30 min at 4 °C in a microcentrifuge.

Phosphorylations were carried out in kinase buffer [25 mM MOPS (pH 7.2) containing 50 mM β-glycerophosphate, 50 μg of substrate, 10 μM (50−100 μCi) [^{32}P]ATP (7000 Ci/mmol), 3 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM DTT, 0.1 μM microcystin LR, 40 mM pNPP, and either 2 mM EGTA or 0.5 mM $CaCl_2$, 200 μg/mL phosphatidylserine, and 20 μg/mL 1,2-dioleoyl-*sn*-glycerol (DAG)]. The last two ingredients were suspended by sonicating in kinase buffer for 5 min prior to the reaction. Typical reactions used 50 μg of peptide substrate in a volume of 20−50 μL. In some experiments, assay tubes containing calphostin C were illuminated for 30 min with the enzyme (lysate) prior to the addition of the substrate. All kinase assays were performed at 30 °C using fresh cell extracts. Except where noted, reactions were terminated by addition of ice-cold trichloroacetic acid at a final concentration of 20% and left on ice for 10 min prior to centrifugation for 10 min in a microcentrifuge at 4 °C. The resulting pellet was rinsed twice with ice-cold 20% TCA and once with ice-cold acetone and dried under vacuum. Reactions containing complete TF protein were terminated by adding 6 volumes of ice-cold acetone. Tubes were then centrifuged at 12000*g* for 30 min at 4 °C. The supernatant was aspirated, and the pellet was washed twice in ice-cold acetone and dried.

For proteolytic digestion, $TF_{245-263}$ was incubated with trypsin in 50 mM ammonium bicarbonate buffer (pH 8.4) at 37 °C overnight. The reaction tubes were briefly centrifuged and evaporated to dryness in a Savant speedvac. The dry digested sample was dissolved in sterile distilled water and analyzed by IEF.

Cyssor digestion of phosphorylated tissue factor protein was carried out according to the method of Holmes and Lawton (1976). The phosphorylated TF, after acetone precipitation of the kinase assay mixture, was repurified by immunoaffinity chromatography on monoclonal antibody HTF1 against the extracellular domain of TF (Carson et al., 1987) coupled to Affi-Gel 10. The affinity-purified TF was suspended in 0.1 N acetic acid containing 0.5% SDS, 1 mM EDTA, and 1 mM Cyssor reagent (2-methyl-N^1-benzenesulfonyl-N^4-bromoacetylquinonediimide) and incubated at room temperature for 16 h, followed by heating at 80 °C for 1 h. The cleavage products were TCA-precipitated, and the dry pellet was resuspended in 0.1% TFA in water and isolated on a C8 column.

Phosphopeptides were purified by HPLC on a reverse-phase C8 column fitted to a C8 guard column using a ternary gradient programmer and a detection wavelength of 214 nm. Solvent A was 0.1% TFA in distilled water, and solvent B was 0.1% TFA in 80% aqueous acetonitrile. Both solvents were degassed prior to use. The solvent gradient profile was as follows: 0−5 min, 0% B; 5−35 min, 0−30% B; 35−45 min, 30−100% B; 45−55 min, 100% B; 55−65 min, 100−0% B; 65−70 min, 0% B. All samples were mixed in a maximum volume of 1.0 mL of solvent A prior to loading. The flow rate was 1.0 mL/min. Fractions were collected at 1 min intervals and counted for Cerenkov radiation. The recovery of ^{32}P was 90−95% based on rechromatography of isolated radiolabeled peptide. Fractions corresponding to retention times of the phosphopeptides were dried in a vacuum evaporator (SpeedVac) and resuspended in sterile distilled water.

For reduction and alkylation, samples were made to a final concentration of 10 mM DTT and incubated for 5 min at 70 °C, followed by the addition of 20 mM iodoacetamide and further incubation at 70 °C for 15 min in the dark. Samples were TCA-precipitated to remove excess reagents.

Prior to gel electrophoresis, samples were prepared either without reduction or with reduction in sample solvent containing 2% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, and 10 mM dithiothreitol. Samples were analyzed in 8−25% polyacrylamide gels with Bio-Rad mini-electrophoresis equipment, or in 8−25% PhastGels using the PhastSystem (Pharmacia−LKB) according to the company protocol adapted from Laemmli (1970). Molecular weights were determined by comparison with prestained molecular weight standards. After electrophoresis, PhastGels were used for passive transfer to PVDF membranes or air-dried and used for autoradiography. For quantitative experiments, radioactivity on the dried gel or blot was measured in a BetaScope or by densitometric analysis of autoradiograms and expressed in terms of counts per minute (cpm) or relative optical density times the unit area, respectively.

The PhastSystem and PhastGel IEF (pH 3−9) were used for characterization of peptide phosphorylation reaction products. A p*I* calibration kit was used to determine the pH gradient for each individual IEF run. All samples were dissolved in sterile distilled water prior to being loaded on IEF gels.

For Western blotting, proteins were electrophoretically transferred from the SDS minigels to nitrocellulose in 10 mM Tris−acetate buffer (pH 8.25), and blocked in Carnation milk (6%) dissolved in TBS, or with 2% BSA in TBS, for 1 h prior to probing with the primary antibody diluted in TBS. Rabbit anti-mouse HRP-conjugated IgG (Dako) was used to detect primary monoclonal IgG bound to the antigen on the blots. The blots were developed with 4-chloro-1-naphthol (Hawkes et al., 1982) and photographed with Kodalith film. Final figures were composed using Microsoft Power Point.

Peptides were transferred to Immobilon. Peptide blots were probed with monoclonal IgG against the tissue factor carboxyl terminal followed by the Vectastain ABC reagents from Vector Labs. After excess ABC reagents were washed out, the blots were developed with diaminobenzidine in the presence of 100 μg/mL nickel chloride.

Western blot analysis of phosphopeptides resolved on IEF gels required specific procedures to avoid significant loss of the peptides during washes of the transfer membranes. The phosphopeptides from IEF gels were transferred to buffer-equilibrated PVDF membranes by passive diffusion at 15 °C for 30 min. After transfer, the blots were immersed in 10% acetic acid for 1 min, rinsed with a large excess of 100% methanol for 5−10 s, and dried in a 27 °C dry air oven for 1 h. The hydrophobic property of the membrane was exploited in subsequent procedures, after modification of the method described by Mansfield in the application note of Millipore Corp. titled "Rapid immunodetection of blotted proteins without blocking". The dried membranes containing the phosphopeptides were incubated in TBS containing 10 μg/mL primary monoclonal antibody (C28 2.1) which recognizes the terminal nine residues of the cytoplasmic domain of TF (Carson & Yoder, 1992) for 15 min at room temperature. This was followed by gentle washing in ice-cold TBS and incubation with 5 μg/mL biotinylated horse anti-mouse IgG (Vector Labs) and 1 μg/mL rabbit anti-mouse HRP-conjugated IgG in TBS for 15 min at room temperature. The blots were again washed in ice-cold TBS and incubated with preformed avidin−biotin complex (Vectastain ABC kit) for 15 min. The blots were thoroughly washed and developed with diaminobenzidine and nickel chloride.

Fractions enriched in protein kinase activity from cell lysates were prepared by Rotofor preparative IEF. Fresh extract from U87-MG cells was prepared in 5.0 mL of lysis buffer and mixed with 50 mL of 1% (w/v) Ampholine (pH 3−10) and 1 mM PMSF. Samples were focused at a constant 10 W at 4 °C for 4−5 h. Fractions were collected, and their pH was determined. Each fraction was then adjusted to pH 7.0 with NaOH or HCl prior to assaying kinase activity.

RESULTS

We demonstrated phosphorylation of purified human placental TF using U87-MG cell lysates and a lysate fraction focused on the Rotofor at pH 7.4. TF (2.3 μg in 5 μL) was incubated in 25 μL of kinase buffer with 20 μL of the cell extract for 1 h at 30 °C, and then repurified by immunoaffinity chromatography. The reisolated, phosphorylated TF was analyzed by Western blotting and autoradiography, as shown in Figure 1. The reisolated tissue factor localized with HTF1 anti-tissue factor monoclonal antibody (lanes C and D) migrated with the same electrophoretic mobility as the starting material (lane E), and autoradiography (lanes A and B) showed that the tissue factor-associated phosphate colocalized with the antigen. To demonstrate that the phosphates were located in the cytoplasmic domain of the TF, we carried out cysteine-selective modification and cleavage of phosphorylated TF using the Cyssor reagent. After Cyssor digestion and HPLC isolation of the liberated carboxyl-terminal peptide, 38% of the initial radioactivity was recovered with the carboxyl-terminal peptide. Radioactive HPLC fractions corresponding to the elution times for

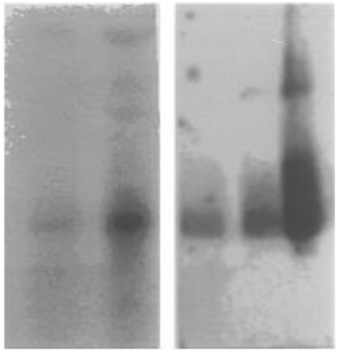

FIGURE 1: SDS−PAGE (8−25% acrylamide) analysis of human placental tissue factor repurified from kinase reactions using Rotofor-enriched kinase(s) (lanes A and C) or crude cell lysates (B and D). The panel containing lanes A and B is from autoradiography of the Western blot (lanes C−E). Lane E contained untreated placental tissue factor.

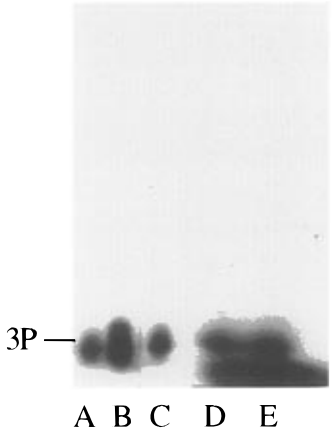

FIGURE 2: Autoradiogram of phosphorylated tissue factor cytoplasmic domain synthetic peptide, $TF_{245-263}$ (lanes A−C), and cytoplasmic domain released from tissue factor protein by chemical cleavage at Cys245 (lanes D and E) after repurification and analytical isoelectric focusing. Lanes A through C were from HPLC fractions eluted at 21, 24, and 28 min, respectively. Fractions eluted at 21 min and 24 min (lanes D and E) were the only radioactive peptide fractions obtained from the digested tissue factor. 3P indicates the triphosphate form of the peptide, focused at pH 3.4.

$TF_{245-263}$ were analyzed by PhastGel IEF. In this experiment, shown in Figure 2, lanes D and E, the cytoplasmic fragment cleaved from TF focused near pH 3.4, consistent with the presence of three phosphates. The exposed area at the bottom of lanes D and E was beneath the anode strip where free phosphate is concentrated. For comparison, this figure contains $TF_{245-263}$ with three phosphates (described below; Figure 7) run in lanes A, B, and C, confirming that the cytoplasmic domain isolated from phosphorylated TF contained three phosphate groups, presumably one at each of the three serine residues.

The cytoplasmic domain of human tissue factor consists of residues 243 through 263:

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H K C R K A G V G Q S W K E N S P L N V S
|             |       |       |
245           253     258     263
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in which the last nine amino acids contain two of the serines that are potential phosphorylation sites, and contain the epitope recognized by monoclonal antibodies C28 1.1 and

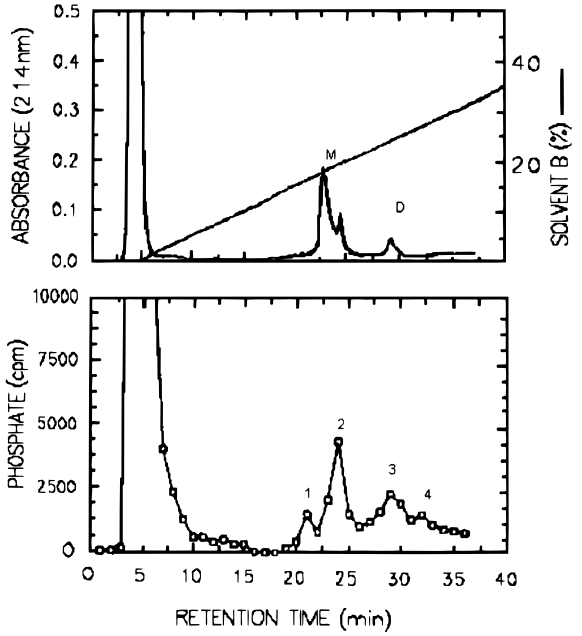

FIGURE 3: Isolation of $TF_{245-263}$ by reverse-phase HPLC (solvent B was acetonitrile). The upper panel shows the elution profile, monitored at 214 nm, obtained with the synthetic peptide following the kinase reaction. Peaks M and D correspond to monomeric and dimeric peptide. The optical profile of the pure peptide before treatment revealed the dominant M peak, and little or no D peak. The lower panel presents the radioactivity elution profile for $TF_{245-263}$ after incubation with cell lysates and the kinase buffer. 1 through 4 designate peaks discussed in the text.

C28 2.1. Initial attempts to phosphorylate the nine residue peptide were not successful. We therefore examined the more complete cytoplasmic domain, $TF_{245-263}$, as a substrate for protein kinase(s) in lysates of U87-MG cells, which were shown above to phosphorylate full-length TF. To reduce the background of phosphorylated cellular proteins and labeled ATP, $TF_{245-263}$ was reisolated from the kinase reaction mix using reverse-phase HPLC. On a C8 column, $TF_{245-263}$ resolved into two absorbance peaks (M and D), as shown in Figure 3 (upper panel). The retention time for peak M was consistently near 24 min, and that of peak D was consistently near 28 min. Reduction and alkylation of isolated peak D, followed by rechromatography, gave rise to a new peak M and a loss of peak D. We concluded that peak M fractions contained monomeric peptide, whereas dimeric peptide was present in fractions corresponding to peak D.

The optical absorbance profile of the peptide reisolated from the kinase reaction revealed broadened peaks compared to those obtained from HPLC chromatography of the unmodified $TF_{245-263}$. Cerenkov analysis of fractions over this range showed four radioactive peaks (lower panel, Figure 3). Following the kinase reaction, radioactive peaks 1 and 2 overlapped absorbance peak M, the peptide monomer, while radioactive peaks 3 and 4 overlapped peak D, the peptide dimer. Fractions corresponding to radioactive peaks 1 through 4 were pooled together from multiple chromatographic isolations and analyzed by SDS−PAGE. The pooled sample gave two radioactive bands (Figure 4, lane A), but only a single band after reduction (lane B). These radioactive bands coincided with bands detected by silver staining (Figure 4, lane C), and bands detected with antibody C28 2.1 on Western blots (Figure 4, lane D). These experiments

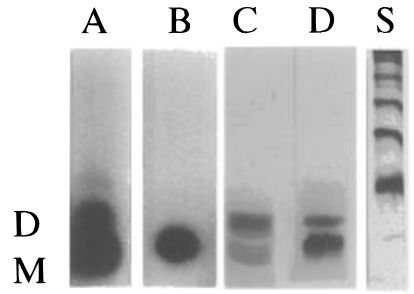

FIGURE 4: SDS−PAGE (20% acrylamide) analysis of combined HPLC peaks 1 through 4 (Figure 3) from HPLC repurification of phosphorylated $TF_{245-263}$. Only lane B contains reduced sample. Lanes A and B are autoradiographs of samples run without reduction (A) or after reduction (B), showing that dimeric peptide (left margin D) reduced to monomeric peptide (M). Lane C was silver-stained, showing only monomeric and dimeric peptide in the pooled fractions. Lane D shows a Western blot, probed with antibody C28 2.1 against the tissue factor carboxyl-terminal peptide, confirming that all of the material in the pooled fractions was $TF_{245-263}$. Lane S contained molecular weight markers, the fastest of which was molecular weight 8600.

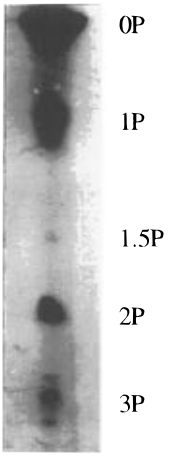

FIGURE 5: Western blot of pooled HPLC peaks 1 through 4 (Figure 3) resolved on isoelectric focusing probed with monoclonal antibody C28 2.1. The antigen bands focused at approximately pH 10 (0P, adjacent to the cathode), pH 7.2 (1P), pH 5.8 (1.5P), pH 5.1 (2P), and pH 3.4 (3P). The 3P spot was typically 1−3 mm removed from the anode.

confirmed that the fractions within radioactive peaks 1−4 contained phosphorylated monomeric $TF_{245-263}$ and dimers formed at Cys245. Further analyses confirmed that nonphosphorylated monomeric $TF_{245-263}$ coeluted with the radioactive peak 2, corresponding to peak M, shown in Figure 3. No phosphorylation of the peptide was detected in the absence of added cell extract. No absorbance or radioactive peaks were detected at these elution times when cell extracts were chromatographed in the absence of added $TF_{245-263}$.

We used calculated isoelectric points and isoelectric focusing to resolve differentially phosphorylated forms of $TF_{245-263}$. The calculated p*I* of the nonphosphorylated peptide was determined to be 10, whereas the monophosphopeptide (lP), the diphosphopeptide (2P), and the triphosphopeptide (3P) had calculated p*I* values of 7.2, 5.1, and 3.4, respectively. The peptide was phosphorylated using the crude cell extract, reisolated by HPLC, and analyzed by PhastSystem IEF. Figure 5 shows a Western blot of the pooled fractions corresponding to the M and D elution times (peaks 1−4) after isoelectric focusing. All of the theoretical phosphorylated forms of $TF_{245-263}$, as well as a minor band

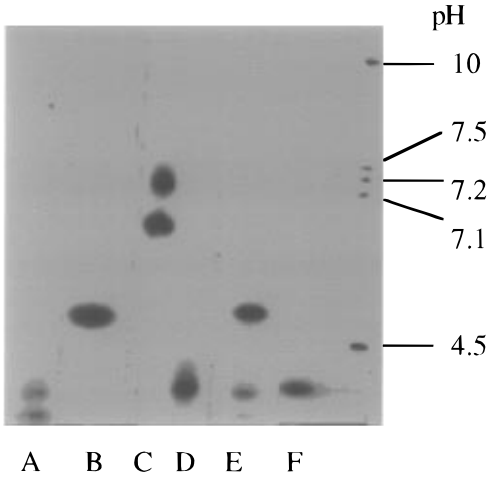

FIGURE 6: Autoradiographic exposure of isoelectric focusing analysis of phosphorylated $TF_{245-263}$ resolved by HPLC into peak 1 (lane A), peak 2 (lane B), peak 3 (lane C), and peak 4 (lane D). Lane E contained the peak 3 fraction after reduction and alkylation, and lane F contained the peak 4 fraction after reduction and alkylation. Isoelectric markers were labeled with phosphorescent strips, and their pH values are as indicated.

corresponding to 1.5 phosphates per peptide, were resolved (Figure 5). We reasoned that the 1.5P form must correspond to dimeric peptide containing a total of three phosphates, and conducted additional experiments to more fully characterize the phosphorylated forms of $TF_{245-263}$. The results are presented in Figure 6. Lane A contained the fraction eluted at 21 min, corresponding to monomeric peak 1 (Figure 3), and shows that this fraction contained monomeric triphosphopeptide focused near pH 3.4. Lane B contained the fraction eluted at 24 min, which produced a spot on autoradiography corresponding to the monomeric diphosphopeptide focused near pH 5.1. The fraction eluted at 29 min (dimeric peptide elution zone, peak 3) produced two focused bands (Figure 6, lane C), which had p*I* values appropriate for 1.5P and 1P monomeric peptide. Since we had previously shown that these fractions contained dimeric $TF_{245-263}$, we reanalyzed the fraction after reduction and alkylation. As seen in lane E, reduction led to new bands corresponding to diphopshate and triphosphate forms of monomeric peptide. We concluded that the monophosphopeptide spot in lane C contained a dimer of unphosphorylated $TF_{245-263}$ and diphosphopeptide, and the 1.5P band was due to unphosphorylated peptide dimerized with triphosphopeptide. The peak 4 fraction (eluted at 32 min) was focused in lane D, and in lane F after reduction and alkylation. This dimeric peptide must have contained three phosphates on each monomer. We did not observe any monomeric peptide containing a single phosphate.

To confirm this interpretation, fraction 29 was reduced and alkylated, and then reanalyzed on HPLC. Following reduction and alkylation, the peptide elution shifted from 29 to 25 min (i.e., from radioactive peak 3 to peak 2), indicating monomerization of the peptide. We concluded, therefore, that the fraction eluted at 29 min contained two phosphorylated forms of dimeric peptide. The band focused near pH 7.2 consisted of 0P−2P dimers, and the band focused near pH 5.8 consisted of 0P−3P dimers. Following reduction and alkylation, the retention time of the fraction eluted at 32 min on HPLC shifted to 21 min, and IEF analysis of the reisolated peptide gave a band focused near pH 3.4, again

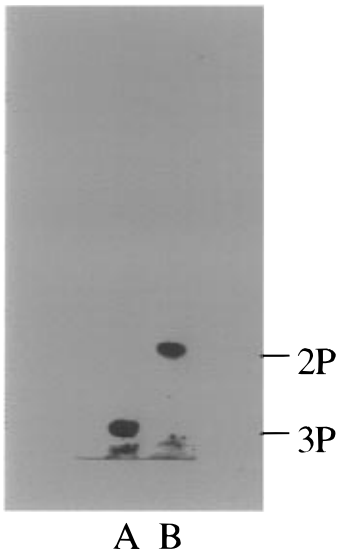

FIGURE 7: Autoradiographic exposure of isoelectric focusing analysis of rechromatographed fractions containing monomeric diphosphopeptide and triphosphopeptide (eluted at 21 and 24 min, respectively), showing isolation of the species.

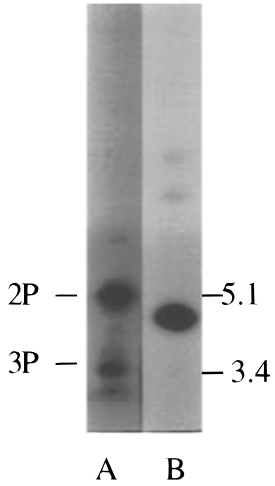

FIGURE 8: Autoradiogram of an isoelectric focusing gel containing both monomeric 2P and 3P $TF_{245-263}$ (lane A) and isolated diphosphopeptide (Figure 7, lane B) which had been digested with trypsin. Phosphorylated species are labeled on the left, and the pH values are indicated on the right.

appropriate for the 3P monomer. Figure 7 shows that we were able to cleanly isolate the 2P and 3P forms of the monomeric peptide by rechromatography after reduction and alkylation.

In order to determine which serine residues are phosphorylated in the diphosphopeptide, we carried out complete trypsin digestion of the isolated 2P monomer (Figure 7). The proteolytic fragments were analyzed on IEF gels (Figure 8). The loss of radioactive diphosphopeptide focused at p*I* 5.1 (Figure 8, lane A) from the lane containing trypsin-treated peptide indicates that all of the monomeric peptide was digested. Among the expected potentially phosphorylated fragments produced by trypsin digestion (AGVGQSWK and ENSPLNVS), only phosphorylation of Ser253 in the former would give a band focused near p$I = 4$ as seen in Figure 8, lane B, while the latter, with even a single phosphate, would focus under the anode. We have not yet been successful in identifying the second site phosphorylated in the diphosphopeptide.

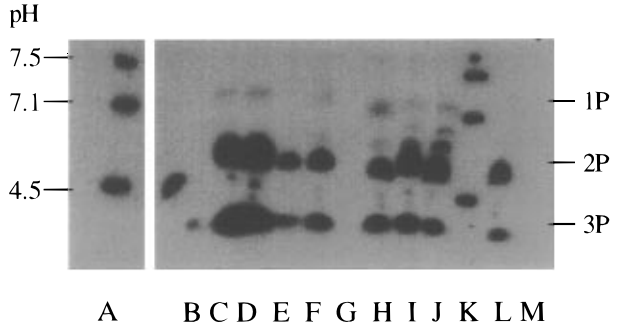

FIGURE 9: $TF_{245-263}$ was phosphorylated by the Rotofor-enriched kinase fraction of cell lysates in the presence of varied protein kinase cofactors and inhibitors. Peaks 1 and 2 (Figure 3) corresponding to monomeric peptide were pooled and analyzed by isoelectric focusing. This autoradiogram shows phosphorescent pH markers in lanes A and K, and lane G contained no sample. Other lanes contained $TF_{245-263}$ phosphorylated in the presence of EGTA (I) or EGTA and (B) cAMP, (C) cGMP and calphostin, (D) cAMP and calphostin, (E) H7, (F) cGMP, and (J) phosphatidylserine and diacylglycerol. (Lane B skewed left during the run; the dominant band corresponds to 2P.) The reaction analyzed in lane (L) contained calcium, and that in lane (H) contained calcium, phosphatidylserine, and diacylglycerol. Lane M contained fractions corresponding to peaks 1 and 2 (Figure 3) from HPLC separation of a kinase reaction containing the enriched kinases, EGTA, cAMP, cGMP, phosphatidylserine, and diacylglycerol, from which $TF_{245-263}$ had been omitted.

To begin initial characterization of the kinase(s) responsible for phosphorylation of the peptide, we used a Rotofor cell for preparative liquid IEF to enrich kinase activity from the cell lysate. The focused fractions were assayed for kinase activity using the peptide as substrate. The kinase reactions were done both with and without calcium and phospholipid. Under both conditions, $TF_{245-263}$ was phosphorylated by fractions focused over the pH range of 5.9−8.6 with peak activity near pI = 7.4. Using the fraction focused at pH 7.4, we tested the effects of known cofactors and inhibitors of various kinases on the phosphorylation of $TF_{245-263}$. The peptide was reisolated from reaction mixtures by HPLC, and fractions corresponding to peptide monomer were examined by IEF. As shown in Figure 9 (lane M), no radiolabel was recovered in these fractions when peptide was omitted from the reaction. In other reactions, the incubations lacking the enriched kinase(s) also produced no peptide-associated label. The presence of calcium in the kinase reaction (lane L) did not increase peptide phosphorylation over that obtained in the presence of EGTA (lane I), and frequently resulted in diminished radiolabel. Addition of diacylglycerol and phosphatidylserine to the reaction containing calcium (lane H) restored peptide phosphorylation to near the level obtained in the absence of calcium. Additional reactions were done in the presence of EGTA. We consistently found that 200 μM H7 diminished the amount of peptide-associated phosphate (lane E). cAMP or cGMP failed to increase phosphorylation relative to that obtained in the presence of EGTA (lanes B and F). Addition of 0.5 μM calphostin to these reactions resulted in the highest levels of peptide phosphorylation observed (lanes C and D). At the concentrations used, we expect that calphostin should predominantly inhibit protein kinase C, while H7 should inhibit a number of protein kinases, including protein kinase C, cAMP-dependent protein kinase, cGMP-dependent kinase, and myosin light chain kinase. From these experiments, we conclude that inhibition of protein kinase C increased the amount of phosphorylated $TF_{245-263}$ recovered from the kinase reactions.

DISCUSSION

Although multiple studies have examined the effects of modulating protein kinase activity on induction of tissue factor expression (e.g., Crossman et al., 1990; Carr et al., 1990; Janco & Morris, 1985; Brozna & Carson, 1988; Lyberg, 1984; Crutchley, 1992; Ollivier, 1993; Brozna et al., 1994), no progress on phosphorylation of the protein itself has been published since the initial report of Zioncheck et al. (1992). In contrast to the transfected expression system they used to establish that the protein can be phosphorylated when expressed by cells, we elected to study the phosphorylation by cell lysates as a prelude to identification of the specific protein kinases capable of phosphorylating tissue factor. We have confirmed that tissue factor, notably its cytoplasmic domain, is phosphorylated when incubated with lysates of U87-MG cells and ATP. Moreover, the recovered cytoplasmic domain was phosphorylated at three residues. Since serine is the most likely residue to be phosphorylated in the tissue factor cytoplasmic domain, and the only phosphorylated residue identified by Zioncheck et al.(1992), we have presumed that the phosphorylation achieved in our experiments occurs on serine residues. This presumption is consistent with the maximum phosphorylation of three residues and the presence of three serines in the cytoplasmic domain.

To facilitate biochemical studies of cytoplasmic domain phosphorylation, we established that the synthetic peptide $TF_{245-263}$, corresponding to the final 19 amino acids of the cytoplasmic domain, could be reisolated from reactions containing cell lysates by HPLC and characterized with respect to phosphate content by analytical isoelectric focusing. Our inability to obtain phosphorylated $TF_{255-263}$, containing only two serines, suggests that residues from Cys245 to Trp254 contain information required for substrate recognition by the protein kinase(s) which phosphorylate the more complete cytoplasmic domain peptide, and imply a degree of substrate specificity in our results.

Although interpretation of the results was initially compounded by the appearance of multiple chromatographic peaks and phosphopeptides focused at unanticipated isoelectric points (e.g., the 1.5P peptide), we established by multiple criteria that the peaks corresponded to variably phosphorylated monomeric peptide and its disulfide-linked dimer. The peptides identified by isoelectric focusing, and confirmed by Western blotting, corresponded to stoichiometrically phosphorylated $TF_{245-263}$ and dimeric combinations. Monophosphorylated peptide was never observed in either monomers or dimers. This latter observation is notable since serine or threonine is highly conserved at positions 253 and 258 in human, bovine, and rabbit tissue factor (Spicer et al., 1987; Ruf & Edgington, 1994; Andrews et al., 1991; Takayenoki et al., 1991). Mouse TF contains lysine at position 253 and threonine at position 258, and serine at position 260 (Hartzell et al., 1989). We were able to directly confirm phosphorylation of Ser253 in the diphosphorylated peptide, but the identity of the second phosphorylation site remains to be determined. Based on the noted sequence conservation, we speculate that it will be found to be residue 258.

Our initial step to fractionate the protein kinase(s) from the cell lysate using preparative isoelectric focusing provided

maximum phosphorylation of $TF_{245-263}$ with the pH 7.4 fraction. This fraction also phosphorylated human placental tissue factor, from which we isolated the cytoplasmic domain and established the presence of three phosphates. The peptide kinase activity of this fraction was largely independent of calcium, markedly inhibited by H7, and reproducibly increased in the presence of calphostin. Although this latter finding clearly reveals that $TF_{245-263}$ phosphorylation can be independent of protein kinase C, it remains unclear why inhibition of protein kinase C increases peptide phosphorylation. It is reasonable to speculate that protein kinase C may activate a protein phosphatase, and the reduced phosphorylation observed under conditions where protein kinase C is active may be the result of this phosphatase acting on the peptide. Whatever the mechanism of the calphostin effect, the result underscores that the relative levels of peptide phosphorylation observed must be the net effect of multiple activities.

Following the work of Paborsky et al. (1991), which determined that recombinant forms of tissue factor lacking its cytoplasmic domain could be functionally expressed by transfected cells, few laboratories pursued a role for the cytoplasmic domain while considerable effort was dedicated to structure−function studies of the extracellular domain, generally utilizing a soluble construct truncated before the membrane-spanning sequence (Waxman et al., 1992, 1993; Neuenschwander et al., 1992; Harios et al., 1994; Banner et al., 1996). More recent reports describing tissue factor-mediated cell signalling (Rottingen et al., 1995), tissue factor association with elements of the cytoskeleton, partitioning among membrane microdomains (Carson et al., 1996; Carson, 1996; Sevinsky et al., 1996), and a potential role for the cytoplasmic domain in the malignant phenotype of transfected cells (Zhang et al., 1994; Bromberg et al., 1995) have renewed interest in the function of this part of the tissue factor protein. While the roles of fatty acid acylation of Cys245 and phosphorylation of serine residues in mediating these events will most likely be solved using site-directed mutants of the cytoplasmic domain, the results we have reported here will facilitate biochemical studies of the enzymes which mediate these posttranslational modifications.

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REFERENCES

Andrews, B. S., Rehemtulla, A., Fowler, B. J., Edgington, T. S., & Mackman, N. (1991) *Gene 98*, 265−269.

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

Banner, D. W., D'Arcy, A., Chene, C., Winkler, F. K., Guha, A., Konigsberg, W. H., Nemerson, Y., & Kirchhofer, D. (1996) *Nature 380*, 41−46.

Bromberg, M. E., Konigsberg, W. H., Madison, J., Pawashe, A., & Garen, A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8205−8209.

Broze, G. J., Jr. (1982) *J. Clin. Invest. 70*, 526−535.

Brozna, J. P., & Carson, S. D. (1988) *Blood 72*, 456−462.

Brozna, J. P., Forman, M., & Carson, S. D. (1994) *Blood Coagulation Fibrinolysis 5*, 929−938.

Carr, B. D., Slausen, D. O., Dore, M., & Suyemoto, M. M. (1990) *Inflammation 14*, 681−689.

Carson, S. D. (1996) *Blood Coagulation Fibrinolysis 7*, 303−313.

Carson, S. D., & Yoder, S. C. (1992) *Blood Coagulation Fibrinolysis 3*, 779−787.

Carson, S. D., Ross, S. E., Bach, R., & Guha, A. (1987) *Blood 70*, 490−493.

Carson, S. D., Kuszynski, C. A., & Pirruccello, S. J. (1996) *Blood Coagulation Fibrinolysis 7*, 314−324.

Crossman, D. C., Carr, D. P., Tuddenham, E. G. D., Pearson, J. D., & McVey, J. H. (1990) *J. Biol. Chem. 265*, 9782−9787.

Crowe, P. D., Van Arsdale, T. L., Goodwin, R. G., & Ware, C. F. (1993) *J. Immunol. 151*, 6882−6890.

Crutchley, D. J., Solomon, D. E., & Conanan, L. B. (1992) *Arterioscler. Thromb. 12*, 664−670.

Drake, T. A., Ruf, W., Morrissey, J. H., & Edgington, T. S. (1989) *J. Cell Biol. 109*, 389−395.

Edelman, A. M., Blumenthal, D. K., & Krebs, E. G.(1987) *Annu. Rev. Biochem. 56*, 567−613.

Harios, K., Martin, D. M. A., O'Brien, D. P., Jones, E. Y., Stuart, D. I., Polikarpov, I., Miller, A., Tuddenham, E. G. D., & Boys, C. W. G. (1994) *Nature 370*, 662−666.

Hartzell, S., Ryder, K., Lanahan, A., Lau, L. F., & Nathans, D. (1989) *Mol. Cell. Biol. 9*, 2567−2573.

Hawkes, R., Niday, E., & Gordon J. (1982) *Anal. Biochem. 119*, 142−147.

Holmes, T. J., Jr., & Lawton, R. G. (1976) *J. Am. Chem. Soc. 99*, 1984−1986.

Hoxie, J. A., Matthews, D. M., Callahan, K. J., Cassel, D. L., & Cooper, R. A. (1986) *J. Immunol. 137*, 1194−1201.

Janco, R. L., & Morris, P. J. (1985) *Blood 65*, 545−552.

Krebs, E. G. (1986) *Enzymes (3rd Ed.) 17*, 3−20.

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

Lyberg, T. (1984) *Haemostasis 14*, 393−399.

Mitchell, M. A., Huang, M. M., Chien, P., Indik, Z. K., Pan, X. Q., & Schreiber, A. D. (1994) *Blood 84*, 1753−1759.

Muller, M., Flossel, C., Haase, M., Luther, T., Albrecht, S., Nawroth, P. P., & Zhang, Y. (1993) *Virchows Arch. B: Cell Pathol. 4*, 265−269.

Neuenschwander, P. F., & Morrissey, J. H. (1992) *J. Biol. Chem. 267*, 14477−14482.

Ollivier, V., Houssaye, S., Ternisien, C., Leon, A., de Verneuil, H., Elbim, C., Mackman, N., Edgington, T. S., & de Prost, D. (1993) *Blood 81*, 973−979.

O'Toole, T. E., Ylanne, J., & Culley, B. M. (1995) *J. Biol. Chem. 270*, 8553−8558.

Paborsky, L. R., Caras, I. W., Fisher, K. L., & Gorman, C. M. (1991) *J. Biol. Chem. 266*, 21911−21916.

Rottingen, J.-A., Enden, T., Camerer, E., Iversen, J.-G., & Prydz, H. (1995) *J. Biol. Chem. 270*, 4650−4660.

Roy, S., Paborsky, L. R., & Vehar, G. (1991) *J. Biol. Chem. 266*, 4665−4668.

Ruf, W., & Edgington, T. S. (1994) *FASEB J. 8*, 385−390.

Sevinsky, J. R., Rao, L. V. M., & Ruf, W. (1996) *J. Cell Biol. 133*, 293−304.

Shin, J., Dunbrack, R. L., Jr., Lee, S., & Strominger, J. L. (1991) *J. Biol. Chem. 266*, 10658−10665.

Smith, J. E., Sheng, Z. F., & Kallen, R. G. (1994) *DNA Cell Biol. 13*, 593−604.

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.

Stratford May, W., Jacobs, S., & Cuatrecasas, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2016−2020.

Takayenoki, Y., Muta, T., Miyata, T., & Iwanaga, S. (1991) *Biochem. Biophys. Res. Commun. 181*, 1145−1150.

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.

Waxman, E., Laws, W. R., Laue, T. M., Nemerson, Y., & Ross, J. B. A. (1993) *Biochemistry 32*, 3005−3012.

Woodgett, J. R. (1991) *Methods Enzymol. 200*, 169−178.

Zhang, Y., Deng, Y., Luther, T., Muller, M., Ziegler, R., Waldherr, R., Stern, D. M., & Nawroth, P. P. (1994) *J. Clin. Invest. 94*, 1320−1327.

Zioncheck, T. F., Roy, S., & Vehar, G. A. (1992) *J. Biol. Chem. 267*, 3561−3564.

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