The Cytoplasmic Domain of Tissue Factor Is Phosphorylated by a Protein Kinase C-dependent Mechanism*

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Tissue factor (TF) is an integral membrane glycoprotein that serves as a cellular receptor and cofactor for the activation of the plasma protease factor VII. TF activity in both monocytes and endothelial cells is regulated by various cytokines and mitogens, including the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA). Three TF constructs (full-length human, a cytoplasmic domain deletion mutant, and a human-rat TF chimera), expressed in a human kidney cell line, were used to examine the in vivo phosphorylation state of TF after PMA treatment. The cytoplasmic domains of both rat and human TF were rapidly phosphorylated after cells were treated with 10–100 nM PMA. This response was completely abolished by preincubating cells with staurosporine, the potent PKC inhibitor, prior to PMA treatment. Localization of the phosphorylation site(s) to the cytoplasmic domain was demonstrated using a deletion mutant of TF and by CNBr digestion at the single methionine residue (Met-210) in the TF sequence. The receptor cytoplasmic domain was phosphorylated to a higher specific activity than the human TF cytoplasmic domain. Phosphoamino acid analysis of the chimeric TF revealed both phosphothreonine and phosphoserine, whereas human TF contained only phosphoserine. Thus both potential phosphoacceptor sites are phosphorylated in the rat TF cytoplasmic domain. Alignment of TF cDNA sequences of mouse, rat, rabbit, and man revealed that the phosphoacceptor site (X-S*/T*-P-X, where asterisk indicates the phosphorylated residue) in the cytoplasmic domain has been conserved through evolution.

Tissue factor (TF) is a 46-kDa transmembrane glycoprotein that serves as a cellular receptor and cofactor for the plasma protease factor VII. Tissue damage or membrane perturbation exposes TF to circulating factor VII; this ultimately results in the activation of factor VII and the initiation of the extrinsic blood clotting cascade. In addition to being a critical molecule controlling hemostasis, TF has also been implicated in the control of thrombosis and inflammation (for reviews, see Refs. 1–3). Significantly, in vitro experiments have shown quiescent endothelial cells and macrophages to be devoid of cell surface TF activity. It is only after physical perturbation or stimulation by exogenous growth factors and mitogens such as fibroblast growth factor, platelet-derived growth factor (4), thrombin (5), interleukin-1 (6), bacterial lipopolysaccharide (7), tumor necrosis factor (8), and PMA (7–12) that these cells become competent in catalyzing a procoagulant response. Current dogma suggests that TF activity is recruited from an intracellular compartment or is synthesized de novo in response to a mitogenic stimulus (4).

In this report, we provide evidence for the in vivo phosphorylation of TF in response to PMA treatment. This result, taken together with the many examples of phosphorylation regulating intermediary metabolism, suggests that TF may be regulated via phosphorylation in vivo.

The recent cloning and expression of TF cDNA have facilitated the understanding of its structure, function, and regulation (1–3). The predicted primary sequence indicates that it is a multidomain protein composed of a hydrophilic extracellular domain, a membrane-spanning region, and a short cytoplasmic tail consisting of 21 amino acid residues. Carbohydrate composition analysis has confirmed the presence of N-linked sugars and an apparent absence of the O-linked variety (13). There is evidence that the intracellular cysteine residue (Cys-245 in the human sequence) is thioester-linked to palmitic acid, helping to anchor TF to the plasma membrane (14). Recent chemical cross-linking studies have revealed that the TF molecule, like other membrane-bound receptors, is capable of forming dimers on the cell surface (15).

Analysis of the human TF protein sequence revealed a putative phosphorylation site (X-S*/T*-P-X) in the cytoplasmic domain. Subsequent cloning of the mouse (16), rabbit (15), and rat TF cDNAs indicates that this phosphorylation site has been conserved through evolution (see Fig. 1), suggesting a potential functional role. Similar sequences in other proteins have been reported to be phosphorylated by a proline-directed protein kinase (17) and the p34<sup>cdc2</sup> kinase (18). Since protein phosphorylation has been shown to be a key event regulating receptor activity, signaling, dimerization, and protein turnover, we sought to determine whether TF was phosphorylated in vivo. In this report, we have used a human kidney cell line stably transfected with human TF (HuTF), a human TF cytoplasmic domain deletion mutant, and a human-rat TF chimera (HuRTF) to demonstrate the phosphorylation of the cytoplasmic domain of TF in response to PMA.

**MATERIALS AND METHODS**

**Reagents—**The mouse monoclonal antibody (D3) to human TF is currently characterized and described (19). Protein G-Sepharose was obtained from Pharmacia LKB Biotechnology Inc. PMA, MPMA, and staurosporine were obtained from Calbiochem. Carrier-free [32P]orthophosphate was purchased from Amersham Corp. Elec-

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The abbreviations used are: TF, tissue factor; HuTF, recombinant human tissue factor; HuRTF, recombinant human-rat chimeric tissue factor; PMA, phorbol 12-myristate 13-acetate; MPMA, phorbol 4-O-methyl 12-myristate 13-acetate; MeSO, dimethyl sulfoxide; PKC, protein kinase C; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

† K. Fisher and K. Schwartz, personal communication.
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**FIG. 1.** Amino acid sequence of the C-terminal region of human TF (amino acids 209-263) aligned with the rat, mouse, and rabbit TF sequences. The hydrophobic transmembrane region and serine/threonine residues in the cytoplasmic domain are boxed. The conserved proline at position 259 is shown. For the construction of the HuRTF chimera, the amino acids in the human TF sequence and cytoplasmic domains. The cytoplasmic domain deletion mutant of human TF lacks residues C-terminal to histidine 243.

trophoblast was carried out on precast gradient mini-gels from Bio-Rad. Thin-layer cellulose plates (100 μm) were purchased from EM Science, Inc. Phosphoamino acids, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, p-nitrophenyl phosphate, and NaF were obtained from Sigma. Mouse monoclonal antibodies to phosphorytrosine were purchased from ICN Biomedicals and Upstate Biotechnology, Inc. The rat TF cDNA was provided by K. Schwartz (Genentech, Inc.).

Vector Construction—The mammalian expression vectors pCISTFl (20), directing the synthesis of full-length HuTF, and pRK5TFdes245 (15), directing the synthesis of a TF variant with a truncation of the C-terminal 20 amino acids (Fig. 1), have been described.

The rat TF cDNA was isolated from a rat brain cDNA library using the human cDNA (coding region) as a probe. The complete amino acid sequence of rat TF was determined by translation of the cDNA nucleotide sequence. The plasmid pRK5TFHR directing the synthesis of a chimera consisting of most of the extracellular domain of HuTF fused to the rat TF transmembrane and cytoplasmic domains was made as follows. The restriction enzyme EcoRI was used to digest HuTF cDNA at the nucleotide sequence ...AAA GGG GAC TTTG...encoding the amino acids KGEF... (Fig. 1). The 5' overhang was then removed by digestion with mung bean nuclease. The rat TF cDNA was similarly digested with the restriction enzyme AapLI and mung bean nuclease at the nucleotide sequence ...AAG TGC ACT GAG...encoding the residues ...KCTE... (Fig. 1). Blunt end ligation between the cDNA fragment coding for most of the extracellular domain of HuTF to the cDNA fragment encoding the rat TF transmembrane and cytoplasmic domains results in the nucleotide sequence ...AAA GGG GCT GAG... (encoding ...KGEF...) at the junction of the human-rat chimeric cDNA. This chimeric HuRTF cDNA was cloned in the pRK5 expression plasmid (21). The constructions were verified by DNA sequencing.

Cell Culture—The human kidney cell line 293 was used for all transfection experiments. The stable clones expressing full-length recombinant human TF, a human cytoplasmic domain deletion mutant, or a human-rat TF chimera were metabolically labeled with [32P]orthophosphate for 4 h. The cells were then subjected to effector (100 nM PMA) for 30 min, prior to immunoprecipitation with a monoclonal antibody directed against the extracellular domain of TF. A phosphorylated protein with the same apparent molecular mass as TF (~46 kDa) was immunoprecipitated from lysates of PMA-treated cells expressing HuTF or the HuRTF chimera (Fig. 2, lanes 4 and 8, respectively). This phosphorylated protein was not observed in control experiments that used the untransfected parent cell line (Fig. 2, lanes 1 and 2) or in a cell line expressing the cytoplasmic domain deletion (Fig. 2, lanes 5 and 6). In darker autoradiographic exposures, the phosphorylated protein appeared as a very faint band in the absence of PMA, indicating a low basal level of phosphorylation. The 46-kDa protein was phosphorylated after exposure of HuTF or HuRTF expressing cells to 100 nM PMA for 30 min; however, this phosphorylation event was observed as early as 10 min with concentrations as low as 10 nM PMA (data not shown). The 46-kDa phosphoprotein was not observed in immunoprecipitations when excess exogenous purified HuTF was added to cell lysates prior to immunoprecipitation (data not shown). These data suggest that TF was the immunoprecipitated 46-kDa phosphoprotein.

The HuRTF chimera routinely resulted in a higher degree of phosphorylation than HuTF after PMA treatment (Fig. 2, lanes 4-7). In immunoprecipitations with excess exogenous phosphorylated HuTF or HuRTF chimera were cut out from a dried unfixed gel using an exposed autoradiograph as a template. Phosphorylated TF was then electroeluted from rehydrated gel slices using a Bio-Rad electroeluter apparatus. The electroelute was diluted to 2 ml with water and concentrated in a Centricon-30 device (Amicon). The concentrate was then added to 2 ml of 10 nM orthophosphate and the process repeated three times. After the final concentration step, formic acid was added to 70% and CNBr cleavage was carried out overnight as described (23).

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described (24). Briefly, [32P]-labeled proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membrane (Immobilon, Millipore). The radioactive bands were cut out, and subjected to hydrolysis with 6 N HCl at 110 °C for 1 h. The hydrolysate was analyzed by two-dimensional electrophoresis on 100-μm cellulose plates along with phosphoserine, phosphothreonine, and phosphotyrosine internal standards. Separated phosphoamino acids were visualized with ninhydrin and the relative positions of [32P]-labeled phosphoamino acids were determined by autoradiography.

RESULTS AND DISCUSSION

Phosphorylation of Tissue Factor in 293 Cells—In order to determine if TF was phosphorylated in vivo, 293 cells stably expressing full-length recombinant human TF, a human cytoplasmic domain deletion mutant, or a human-rat TF chimera were metabolically labeled with [32P] for 4 h. The cells were then subjected to effector (100 nM PMA or MeSa) for 30 min, prior to immunoprecipitation with a monoclonal antibody directed against the extracellular domain of TF. A phosphorylated protein with the same apparent molecular mass as TF (~46 kDa) was immunoprecipitated from lysates of PMA-treated cells expressing HuTF or the HuRTF chimera (Fig. 2, lanes 4 and 8, respectively). This phosphorylated protein was not observed in control experiments that used the untransfected parent cell line (Fig. 2, lanes 1 and 2) or in a cell line expressing the cytoplasmic domain deletion (Fig. 2, lanes 5 and 6). In darker autoradiographic exposures, the phosphorylated protein appeared as a very faint band in the absence of PMA, indicating a low basal level of phosphorylation. The 46-kDa protein was phosphorylated after exposure of HuTF or HuRTF expressing cells to 100 nM PMA for 30 min; however, this phosphorylation event was observed as early as 10 min with concentrations as low as 10 nM PMA (data not shown). The 46-kDa phosphoprotein was not observed in immunoprecipitations when excess exogenous purified HuTF was added to cell lysates prior to immunoprecipitation (data not shown). These data suggest that TF was the immunoprecipitated 46-kDa phosphoprotein.

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RESULTS AND DISCUSSION

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compare lanes 4 and 8). This result is not related to differences in the expression levels of the two proteins, for the stable cell lines express all of the TF constructs at similar levels (as determined by enzyme-linked immunosorbent assay and Western blotting of total cell lysates). Furthermore, cells expressing the three different constructs all have the same relative activity in a chromogenic TF assay (data not shown).

In addition to the 46-kDa protein being phosphorylated, a phosphoprotein of approximately 50 kDa was observed in the immunoprecipitations from all cell lines and appeared to be of equal intensity regardless of whether the cells were treated with PMA or Me2SO. Unlike the 46-kDa band, the 50-kDa band was not immunoreactive in subsequent Western blotting experiments and could not be competed out with exogenously added TF in immunoprecipitation experiments. The identity of this nonspecifically immunoprecipitated phosphoprotein is presently unknown.

Localization of the Phosphorylation Site in Tissue Factor to the Cytoplasmic Domain—HuTF and the HuRTF chimera contain only one methionine residue (Met-210, Fig. 1). Thus, cleavage with CNBr would result in the generation of a C-terminal polypeptide fragment composed of 53 and 59 amino acids, respectively (with a calculated molecular mass of approximately 6 kDa). When phosphorylated TF from HuTF or HuRTF expressing cells was extracted from an SDS-PAGE gel, digested with CNBr and re-electrophoresed, only one phosphopeptide (<15 kDa) was observed (in addition to the residual undigested parent molecule, Fig. 3). Moreover, the cytoplasmic domain deletion mutant was not phosphorylated in cells exposed to PMA (Fig. 2, lane 4). These data suggest that the in vivo phosphorylation site(s) are located in the C-terminal cytoplasmic domain of the TF molecule.

Phosphoamino Acid Analysis of Tissue Factor after PMA Stimulation—HuTF and HuRTF were subjected to phosphoamino acid analysis after being phosphorylated in vivo. HuTF contained phosphoserine only (Fig. 4, panel A). HuRTF was phosphorylated primarily on a threonine residue, however a relatively small amount of phosphoserine was also observed (Fig. 4, panel B). This indicated that both phosphoacceptor sites flanking the conserved proline in the rat TF cytoplasmic domain (Fig. 1) were utilized. The appearance of phosphotyrosine was not observed and would not be expected because tyrosine is absent in the cytoplasmic domains of both rat and human TF. The phosphorylation of tyrosine residues was also ruled out by carrying out Western blots using two different anti-phosphotyrosine monoclonal antibodies (data not shown).

The involvement of PKC in Tissue Factor Phosphorylation—To characterize the role of PKC in mediating the in vivo phosphorylation of TF, HuTF expressing cells were pretreated with the potent PKC inhibitor, staurosporine, prior to PMA stimulation. Staurosporine (100 nM for 30 min) completely abolished the phosphorylation of HuTF in cells that were subsequently treated with 100 nM PMA (lanes 2 and 3) for 30 min. Cells were washed and lysed with RIPA buffer prior to immunoprecipitation with monoclonal antibody D3. Phosphorylated proteins in the immune complex were visualized by SDS-PAGE on a 4–15% gradient gel followed by autoradiography.

At present it is unclear if PKC acts directly or through the action of other cellular kinases. Preliminary experiments using partially purified rat PKC and purified HuTF suggest that the cytoplasmic domain of TF may not serve as a substrate for PKC in vitro. This does not rule out PKC as a potential candidate for the in vivo phosphorylation of TF, inasmuch as a particular in vivo conformation of TF or a cofactor may be required for PKC mediated phosphorylation.

3 T. F. Zioncheck, S. Roy, and G. A. Vehar, unpublished observations.
to occur. Ser-253 in the cytoplasmic domain of HuTF (and the corresponding serine in the rabbit sequence) may be considered a weak PKC phosphorylation site, as it conforms to the core PKC consensus phosphorylation site S'/T'-X-K/R (25). However, this site lacks additional basic residues N- and C-terminal to the target serine that are required to enhance the \( V_{\text{max}} \) and \( K_0 \) parameters for the phosphorylation. Moreover, the functional relevance of this phosphorylation site in vivo is questionable in light of the fact that the mouse and rat TF sequences lack a corresponding serine/Throneine (Fig. 1).

PKC may also phosphorylate Ser-258 in HuTF and the corresponding threonine in the rat sequence directly, or indirectly through the action of other cellular kinases. The fact that the rat TF serves as a better substrate for phosphorylation in vivo may be due to the additional N- and C-terminal basic residues surrounding the site, which are lacking in the human TF sequence.

In view of the conserved Pro-259 in all known TF sequences, one may also consider the recently described proline-directed protein kinase (which phosphorylates the consensus sequence X-S'/T'-P-X, Ref. 26) or p44\(_{206}\)-kinase (consensus phosphorylation sequence: X-S'/T'-P-K/R, Ref. 18) as candidates for phosphorylating Ser-258 in the human sequence (K-E-N-S'-P-L-N-V) and the corresponding threonine in the rat sequence (R-K-N-T'-P-S-R-L). These kinases are thought to phosphorylate structural proteins or critical substrates involved in cytoskeletal reorganization as a cell goes through transition from interphase to mitosis (18). Based on phosphoamino acid analysis, the threonine residue in the rat TF cytoplasmic domain is the major in vivo phosphorylation site. Due to the conservation of this site, it is quite possible that this site is also being phosphorylated in the human sequence.

In summary, reports describing the effects of cytokines and mitogens on TF expression and activity are numerous (1–12). In an effort to begin to understand the mechanism of this regulation, we investigated the possibility that TF was phosphorylated in response to the tumor promoting phorbol ester PMA, a potent activator of PKC. In this report, we have used a human cell system that expresses high levels of full-length HuTF, a cytoplasmic domain deletion mutant of TF, and a HuRTF chimera to provide evidence that the cytoplasmic domain of both rat and human TF rapidly become phosphorylated in vivo after cells expressing TF are stimulated with PMA. The evidence to support this conclusion is as follows: 1) a phosphoprotein with the same apparent molecular weight as TF was immunoprecipitated from lysates of cells expressing HuTF and HuRTF with a monoclonal antibody that recognizes the extracellular domain of TF; 2) this phosphoprotein was only observed in cell lines that express full-length HuTF or a chimeric HuRTF, and not in the untransfected (TF minus) parent cell line; 3) CNBr digestion of phosphorylated TF (at the only methionine in the TF primary sequence; Met-210) only liberates one C-terminal phosphopeptide, composed of the transmembrane and cytoplasmic domain; 4) a cytoplasmic deletion mutant of TF was not phosphorylated in vivo. These data support the conclusion that the in vivo phosphorylation site(s) reside entirely within the cytoplasmic domain.

The functional significance of TF phosphorylation remains to be elucidated. Although various changes in TF activity have been reported when monocytes or saphenous-vein endothelial cells were treated with PMA in culture, we have not observed significant changes in TF activity in our HuTF-transfected cell system up to 1 h after PMA treatment. Significantly, other investigators have reported alterations in monocyte and endothelial cell TF activity in response to both positive and negative regulators of PKC. For example, PMA has been reported to enhance TF synthesis and activity in endothelial cells (7, 8) and monocytes (11). In addition to PMA, two more physiologically relevant cofactors for PKC activation are Ca\(^{2+}\) and phosphatidylserine (27, 28). The calcium ionophore A23187 or phosphatidylinositol rapidly enhances the cell surface expression of TF activity (29). Treatment of monocytes with sphingosine, a competitive inhibitor of PKC (30), decreased TF activity by lowering the binding affinity and number of binding sites for factor VII (31). Phosphorylation could act to indirectly modulate TF activity by altering internalization and degradation (32), or subcellular localization (33). Phosphorylation of the cytoplasmic domains of various integral membrane proteins have also been shown to regulate protein–protein interactions with associated peripheral membrane proteins (34, 35). A closer examination of these possibilities will give further insight into the mechanism of TF regulation and cellular signaling.

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