**DNA Binding of TEF/ATTS Domain Factors Is Regulated by Protein Kinase C Phosphorylation in Human Choriocarcinoma Cells**

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Transcription enhancer factor 1 (TEF-1) controls the expression of a diverse set of genes. Previous studies implicated protein kinase C (PKC)-mediated signal transduction in modulating TEF function. We demonstrate that in human choriocarcinoma BeWo cells, the PKC activator 12-O-tetradecanoyl phorbol 13-acetate and PKC inhibitor bisindolylmaleimide reciprocally down- and up-regulate, respectively, TEF-mediated GGAATG core enhancer activity. In **vitro** TEF-1 phosphorylation with several PKC isozymes and phosphoamino acid analysis confirmed that TEF-1 is a potential PKC substrate. TEF-1-DNA complexes formed by BeWo nuclear extracts are supershifted by phosphoserine- and phosphothreonine- but not phosphotyrosine-specific antibodies, indicating that TEF-1 is phosphorylated in **vivo** at serine and threonine residues. The TEF-1 phosphorylation domain was localized to the third α-helix of the DNA binding domain and adjacent hinge region by phosphopeptide analysis. TEF-1 phosphorylation significantly reduced its DNA binding activity both in **vitro** and in **vivo**, providing a possible mechanism for the inhibitory action of PKC. Finally, BeWo cells contained abundant levels of γ and δ PKC isoforms, and their overexpression resulted in even greater inhibition of GGAATG core enhancer activity after 12-O-tetradecanoyl phorbol 13-acetate treatment. These data strongly suggest that PKC-mediated phosphorylation is a key factor controlling TEF function.

Cells possess a remarkable capability for responding to external signals. In most cases, a cascade of intermediate signal transduction mechanisms is activated in response to external stimuli. These signal transduction cascades end in the activation or repression of factors controlling gene expression. Protein phosphorylation provides cells with a major signal transduction pathway, and numerous transcriptional factors are the final substrates for phosphorylation modification by protein kinases. Phosphorylation and dephosphorylation control the function of transcription factors by modulating their nuclear localization, DNA binding, protein-protein interaction, and trans-activation and -repression activities (1, 2).

TEF-1 is the prototype of the eukaryotic TEF/ATTS family of transcription factors (3, 4). These factors are composed of several evolutionarily conserved domains, including the TEF/ATTS DNA binding domain, proline-rich, serine-threonine-tyrosine-rich, and zinc finger-like domains. The 68-amino acid TEF/ATTS domain is located toward the amino terminus of the protein and contains three putative α-helices, two of which have been demonstrated to be important for DNA binding by mutational analysis (5). In mammalian tissues, based on amino acid sequence alignment, TEF/ATTS transcription factors can be classified into four groups, which correspond to human TEF-1, TEF-3, TEF-4, and TEF-5 (3). Generally, these factors have overlapping but distinct tissue-specific expression patterns, similar but distinguishable DNA binding activities, and related but different transcriptional activities (3, 4, 6, 7).

Several lines of evidence indicate involvement of the PKC signal transduction pathway in TEF-regulated gene expression. First, TEF-1 exhibits high-affinity binding to the GT-IIC enhancer from the SV40 72-base pair repeat unit that regulates SV40 early gene expression (4, 8, 9). Early studies indicated that although the SV40 enhancer is highly responsive to TPA treatment, the AP-1 site within the 72-base pair repeat was not required for the effect, indicating that non-AP-1 cise-active elements, including the GT-IIC site, may mediate the TPA response (10). Second, in cultured rat cardiac myocytes, a 20-base pair sequence containing an enhancer core was found to mediate β-myosin heavy chain gene promoter induction by α-adrenergic and PKC stimulation (11–13). Subsequent studies identified the core element, TGTTGTTATG, as a TEF binding site (14), suggesting that TEF-1 may be a conserved signal transducer of the cardiac growth program and is a likely target of PKC phosphorylation. Also, phosphorylation involvement in TEF function is suggested by sequence and functional analysis of two TEF-1 isoforms, TEF-1β and TEF-1γ, which we cloned recently (7). Compared with the original TEF-1 (the α isoform) the β and γ isoforms contain 11 amino acid substitutions or insertions at the end of the TEA/ATTS DNA binding domain and the hinge region. These relatively limited changes, which are most likely the result of alternative splicing events, caused substantial alterations on DNA binding and transcriptional activities. Inspection of the amino acid sequences found that 4 of the amino acid alterations involve removal or addition of the serine and threonine residues, and 3 of these residues are potential PKC phosphorylation sites, indicating a possible link between PKC modification and TEF-1 function (7).

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The abbreviations used are: TEF, transcription enhancer factor; hCS, human choriionic somatomammotropin; PKC, protein kinase C; TPA, 12-O-tetradecanoyl phorbol 13-acetate; LUC, luciferase; CMV, cytomegalovirus; GT-IIC, GGAATG core; AP, activator protein; GF109203X, bisindolylmaleimide; DAG, diacylglycerol; PAGE, polyacrylamide gel electrophoresis; CSp, choriionic somatomammotropin promoter.
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In the current study we present data that demonstrate PKC involvement in the regulation of TEF-1 transcriptional activity. Functional assays coupled to DNA binding assays showed that PKC phosphorylation of TEF-1 inhibits its DNA binding, and therefore its activity, to a GT-IIC enhancer in BeWo cells. We further demonstrate that TEF-1 can be phosphorylated by a mixture of PKC isoenzymes in vitro. Phosphoamino acid and phosphopeptide analysis confirms that serine and tyrosine residues within the TEF/ATTs DNA binding domain and the immediately following hinge region are the phosphorylated moieties. These results establish TEF-1 as a natural target of the PKC signal transduction pathway. Because TEFs control the expression of numerous genes in various tissues, the pathway may play active roles in many TEF-controlled physiological processes, such as cardiac myogenesis, nervous system development, pregnancy, and early zygotic gene expression (3, 4, 6, 11, 13, 15, 16).

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized in the Molecular Biology Core Facility of the Mayo Clinic. GF109203X was purchased from Procion Biopharma, Inc. (Orval, Quebec, Canada). A human PKC panel containing PKC isotypes α, β1, γ, δ, ε, and η was obtained from PanVera Co. (Madison, WI). TPA, DAG (in the form of Diolein, dithiothreitol, poly(dI-dC), and phenylmethylsulfonyl fluoride were purchased from Sigma. Bio-Gel P-100 and 30% acrylamide/bis-acrylamide (29.1) solution were purchased from Bio-Rad. [γ-32P]ATP (5000 Ci/mmol) was obtained from Amersham Pharmacia Biotech.

Cell Culture, Transfection, and Luciferase Assays—Human choriocarcinoma BeWo cells obtained from ATCC were maintained at 37 °C in an atmosphere containing 5% CO2 and 100% humidity. The culture medium RPMI 1640 (Life Technologies, Inc.) was supplemented with 10% fetal bovine serum (BioWhittaker), 100 units/ml penicillin (Life Technologies), 100 g/ml streptomycin (Life Technologies) and 2 mM L-glutamine (Life Technologies). Cells were grown to 80% confluence in T175 flasks (Becton Dickinson). After rinsing with 10 ml of phosphate-buffered saline containing 5 ml of trypsin (1 mg/ml) were mixed with 2 ml of complete medium. A human PKC panel (17, 18).

RESULTS

PKC Phosphorylation Pathway Regulates GT-IIC Enhancer Activity in BeWo Cells—To investigate TEF-1 functional regulation by PKC in BeWo cells, we used the reporter (GT-IIC)12-Csp.LUC, which contains an enhancer composed of GT-IIC tandem repeats fused to the hCS promoter. This enhancer was capable of stimulating the promoter activity up to 15-fold in BeWo cells (6, 7). Thus, by comparing the TPA induction on the reporter activities of (GT-IIC)12-Csp.LUC and the Csp.LUC control, we can eliminate the TPA effects on the hCS promoter alone and can assess how PKC modulates TEF-mediated enhancer function. Treatment of transfected BeWo cells did not change the hCS promoter activity (Fig. 1). Treatment by GF109203X, a PKC-specific inhibitor that exhibits minimal effects on non-PKC kinases (23), increased hCS promoter activity by a modest 50%. In contrast to Csp.LUC, TPA reduced the (GT-IIC)12-Csp.LUC activity by >50%, and, more dramatically, GF109203X treatment resulted in an ~3-fold increase of reporter activity on the same plasmid. Although the two copies of the CMV enhancer, cloned at the same position as the GT-IIC enhancer, yielded even higher enhancer activity

Phosphoamino Acid and Phosphopeptide Analysis—To obtain large amounts of phosphorylated TEF-1, the in vitro protein kinase reaction described above was scaled up 10-fold. After SDS-PAGE, the position of TEF-1 was determined by directly exposing the wet gel to Eastman Kodak Co. x-ray film at 4 °C for 4 h. The radioactive band representing phosphorylated TEF-1 was extracted from the excised gel by homogenization with a Dounce homogenizer in water. The gel slurry was washed twice with 5 ml of 0.2% SDS, and the eluate was lyophilized. This material was resuspended and precipitated in 85% ethanol for at least 4 h at ~20 °C. The precipitate was pelleted at 20,800 × g for 30 min. Pellets were rinsed with 0.5 ml of ice-cold ethanol and dried in a vacuum centrifuge. Samples were then solubilized in 60 μl of 0.2% SDS, and to each sample was added 140 μl of formic acid containing 2.5 mg of CnBr. The mixture was incubated for 24 h at room temperature in the dark. The sample was then reduced under vacuum and washed twice with water in preparation for electrophoresis. Products of cyanogen bromide cleavage were separated on a 10% NuPAGE gel using 2-[N-morpholino]ethanesulfonic acid running buffer (NoveX, San Diego, CA) and visualized by autoradiography. For phosphoamino acid analysis, gel-purified TEF-1 was subjected to one-dimensional TLC analysis using the method of Nairn and Greengard (21).

Gel Shift Assays—Whole-cell extracts were isolated by the modified microscale method (22). The synthetic oligonucleotides used in gel shift assays were 5′ end labeled with [γ-32P]ATP and TPA nucleotidease (5, 6). After binding of the probe, native gel and native gel electrophoresis were performed as described in detail by Jiang and Eberhardt (18). To study how PKC phosphorylation modulates TEF-1 DNA binding activity, TEF-1 coated on glutathione-Sepharose beads was treated with PKC and recovered as described above, except that nonradioactive ATP was used in the reaction. For negative controls, kinase or ATP was left out of the reaction. To confirm the specificity of the PKC action on TEF-1, GF109203X was included in one tube. After PKC treatment, TEF-1 was recovered by thrombin digestion, and its DNA binding activity was evaluated in gel shift experiments.

Western Blot Analysis—Whole-cell extract (50 μg) prepared from BeWo cells by the microscale method (22) was resolved by 4–15% gradient SDS-PAGE (Bio-Rad) and electrophoresed onto 0.2 μm nitrocellulose membrane (Transblot; Bio-Rad). After blocking in 3% gelatin for 1 hr in 20 m Tris-HCl, pH 7.5, and 500 mM NaCl, mouse monoclonal anti-PKC antibodies specific for isoforms β1, β2, and γ or rabbit polyclonal antibodies specific for isoforms α and δ and secondary alkaline phosphatase-conjugated antibodies (all from Sigma), were applied in the same buffer. Antibody binding and subsequent color development using an Immuno-Bidot (Bio-Rad) were performed following the manufacturer’s instructions.

Data Analysis—All the transfection results are expressed as relative luciferase light units/μg of protein. Data were subjected to analysis of variance. For experimental groups satisfying the initial analysis of variance criterion, post hoc comparisons were performed using Bonferroni t tests, and significance was established at the p < 0.05 level.

RESULTS

PKC Phosphorylation Pathway Regulates GT-IIC Enhancer Activity in BeWo Cells—To investigate TEF-1 functional regulation by PKC in BeWo cells, we used the reporter (GT-IIC)12-Csp.LUC, which contains an enhancer composed of GT-IIC tandem repeats fused to the hCS promoter. This enhancer was subject to one-dimensional TLC analysis using the method of Nairn and Greengard (21).
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**Fig. 1.** PKC pathway regulates GT-IIC enhancer activity in BeWo cells. Human placental choriocarcinoma BeWo cells were transfected with the reporter plasmids CSp.LUC control (A), the enhancer containing (GT-IIC)12_CSp.LUC (B), and (CMVEn)12_CSp.LUC (C) and treated with the PKC activator TPA or PKC inhibitor GF109203X (GF). Control groups without drug treatment are included in each experiment. +, post hoc Bonferroni p < 0.05 relative to the control. Although the multivariate analysis of variance comparison of the data in A was significant (p = 0.0363), the individual post hoc comparisons were not. GF109203X increased significantly the (GT-IIC)12_CSp.LUC activities. Note that in (CMVEn), CSp.LUC, TPA conferred opposite effects, and GF109203X did not affect the CMV enhancer. The reporter activities are expressed as light units/μg protein. The data represent the average values and S.E. from three independent transfections.

than the GT-IIC enhancer, both TPA and GF109203X conferred opposite effects. This result strongly suggests that the alterations on GT-IIC enhancer activity by TPA and GF109203X are due to specific actions of the PKC pathway. This view was further strengthened by a separate set of experiments in which TPA treatment repeatedly resulted in increased reporter activities from CMV-β-galactosidase in transfected BeWo cells (data not shown).

The PKC-mediated regulation of the GT-IIC enhancer was verified in dose-response experiments (Fig. 2, A and B). The dose-response curves of CSp.LUC and (GT-IIC)12_CSp.LUC were established by treating transfected BeWo cells with various concentrations of TPA and GF109203X, ranging from 10 to 160 ng/ml and 0.2–20 μM, respectively. Although TPA treatment did not affect CSp.LUC expression, (GT-IIC)12_CSp.LUC expression exhibited progressive negative regulation. GF109203X treatment caused a two-phase response on both CSp.LUC and (GT-IIC)12_CSp.LUC. From 0.2 to 5 μM, GF109203X dramatically increased (GT-IIC)12_CSp.LUC reporter activity but had much smaller effects on the CSp.LUC control. The sharp decline of reporter activities at 20 μM GF109203X treatment with both constructs was most likely due to a nonspecific effect, because at these high concentrations most cells exhibited abnormal morphologies, including cell detachment and large cytoplasmic vacuoles, which indicate severe cytotoxicity (24). Because the TEF-mediated transcriptional control of the GT-IIC enhancer in BeWo cells has been firmly established, these results suggest that TEF function is subject to the regulation of the PKC phosphorylation pathway.

**PKC in Vitro Phosphorylation of TEF-1 and Phosphoamino Acid Analysis—**As the first step to investigate the mechanism by which PKC regulates GT-IIC enhancer function, we tested whether the prototype of TEF, TEF-1, is an appropriate PKC phosphorylation substrate. In vitro phosphorylation experiments were performed using a mixture of PKC isoforms, including PKC α, β1, δ, θ, and ε isoforms, and purified TEF-1 (25). In the presence of radioactive [γ-32P]ATP and DAG, activation of PKC resulted in a strong radioactive 53-kDa TEF-1 band after autoradiography (Fig. 3A, left lane), indicating that TEF-1 was phosphorylated by PKC.

To identify which amino acid residues were modified by PKC, phosphorylated TEF-1 was subjected to phosphoamino acid analysis (Fig. 3B). The relatively strong serine, somewhat weaker threonine, and absent tyrosine signals support the concept that multiple phosphorylation sites involving both serine and threonine but not tyrosine residues were modified by PKC.

**Endogenous TEFs Are Phosphorylated on Serine and Threonine Residues in BeWo Cells—**We next examined whether native TEFs are naturally phosphorylated in BeWo cells, which express both TEF-1 and TEF-5 (6). Taking advantage of the fact that the DNA-protein interaction between TEFs and GT-IIC sequences has been well characterized and easy to identify in gel shift assays (18, 22), we performed supershift experiments using increasing amounts of the phosphoamino acid-specific antibodies (Fig. 4). In BeWo cell extracts, supershifted bands were observed with both phosphoserine- and phosphothreonine-specific antibodies but not with the phosphotyrosine antibody, although the experiment did not distinguish between PKC phosphorylation of TEF-1 and TEF-5, because both of these proteins may have contributed to the gel shifts observed in Fig. 4. Nevertheless, the data establish that TEA/ATTS domain members represent an in vitro phosphorylation target. Importantly, the supershift data reveal a threonine-serine phosphorylation pattern, consistent with the results from in vitro PKC phosphorylation experiments (Fig. 3, A and B).
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PKC Phosphorylation Reduces TEF-1 DNA Binding Activity in Vitro—It has been well documented that for many transcriptional factors, phosphorylation modulates their DNA binding activities (26–29). To determine whether such a correlation occurred with TEF-1, we compared the GT-IIC binding abilities of unphosphorylated and phosphorylated forms of TEF-1 in gel shift experiments. Because we and others have demonstrated that TEF-1 was able to bind DNA as a monomer or dimer depending on the structure of the DNA elements (3, 8, 30), two types of DNA probes were used to examine the phosphorylation effects on the formation of both monomeric (probe H) and dimeric (probe S3) complexes. As expected, in the nonkinase control, TEF-1 bound strongly to the two probes, H and S3, as monomer or dimer, respectively (Fig. 5). After in vitro treatment with PKC in the presence of ATP, TEF-1 binding to probe S3 was significantly reduced, and binding to probe H was virtually eliminated. The effect, however, was not observed in negative controls, for which PKC treatment was performed in the absence of ATP or the presence of GF109203X, providing evidence that the decrease in DNA binding activity is caused by PKC phosphorylation on TEF-1.

TPA Down-regulates TEA/ATTS Domain Factors Binding to the GT-IIC Enhancer in BeWo Cells—After the in vitro studies on PKC modulation of TEF-1 DNA binding activities, we proceeded to determine how PKC regulates TEF DNA binding in vivo. Although TPA treatment of BeWo cells led to slightly reduced levels of the TEF-GT-IIC complex, GF109203X treatment resulted in an ~2-fold increase in the DNA-protein complex (Fig. 6). In a parallel experiment, PKC regulation of the DNA-protein complex levels in TPA- and GF109203X-treated BeWo cells remained the same even when protein synthesis was inhibited by cycloheximide, indicating that de novo protein syntheses is not required, and the alterations in TEF-GT-IIC complex levels are due to the direct modification of existing proteins (Fig. 6). Thus PKC-mediated down-regulation of PKC DNA binding activity is established both in vitro and in vivo.

PKC-δ and -γ Regulate TEF Function in BeWo Cells—It has been reported that PKC isozymes have different substrate and cellular localization preferences (31–33). We wanted to establish which PKC isozyme(s) regulate TEF functions in BeWo cells. Using Western blot analysis, we examined the expression of several PKC isoforms in BeWo cells (Fig. 7A). We found that the γ and δ PKC isoforms were particularly abundant, and the relative expression of the isoforms that we examined in decreasing order was γ > δ ~ β2 > α > β1. One way to examine the regulatory role of individual PKC isoforms is by cotransfection experiments (31). For this purpose, PKC effects on (GT-IIC)12-CSp.LUC reporter gene activity were monitored in the presence of overexpression of individual DAG-reactive PKC isoforms (α, β, γ, and δ). As expected, none of the PKC isoforms significantly altered TPA regulation of hCS promoter (CS-p.LUC) activity (Fig. 7B). In the case of the enhancer-contain-
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PKC-γ and δ isoforms regulate TEF function in BeWo cells. Western blots (see "Experimental Procedures") from BeWo cell lysates were developed with the α, β, γ, and δ antibodies. BeWo cells were cotransfected with reporter plasmids (CSpLUC or (GT-IIC)12-CSpLUC) and either CMV-β-galactosidase (Control) or the individual PKC α, β, γ, and δ isoforms. Transfected cells were divided into two groups with and without TPA treatment, and TPA induction on CSpLUC and (GT-IIC)12-CSpLUC was measured. Cotransfection of PKC isoforms did not change the nonresponsiveness of CSpLUC to TPA treatment. PKC-γ and δ cotransfection increased the inhibitory effects of TPA on GT-IIC enhancer activity.

**DISCUSSION**

PKC-mediated TPA induction of AP-1 is one of the best-studied signal transduction pathways (1, 2). After TPA stimulation, expression of AP-1, a heterodimer composed of jun and fos subunits, is quickly elevated. In addition, TPA-activated PKC directly phosphorylates the jun subunit, which leads to increased AP-1 trans-activation (34). Subsequent studies revealed that in some TPA-responsive promoters and enhancers, AP-1 binding sites were unable to confer TPA regulation (35, 36). Instead, different regulatory elements, including binding sites for AP-2, AP-3, PEA3, nuclear factor κB, and SRF, were found to be responsible for the TPA response (37–39). These studies demonstrated that PKC modification may influence a variety of transcriptional factors. In the current study, we demonstrate that TEF can serve as major transducer for TPA-mediated signal transduction. Interestingly, unlike its action on AP-1 and most other transcription factors, TPA confers negative effects on TEF-1 DNA binding and GT-IIC enhancer activity. Indeed, in our studies, TPA strongly activated the CMV enhancer in the same cells and under the same experimental conditions, underscoring the specificity of the TPA-mediated effect on TEF-controlled enhancer activity. The fact that both PKC activation and inhibition resulted in significant alterations of GT-IIC enhancer activity, albeit in opposite directions, indicates that there is a basal level of TEF phosphorylation, and its activity is controlled by both phosphorylation and dephosphorylation processes. Accordingly, the balance between kinase and phosphatase activities may provide mechanisms for both up- and down-regulation of target gene expression. Given the wide distribution of PKC isoforms and their fundamental importance in controlling viral and muscle-specific gene expression and nervous system development (3, 4), PKC-mediated phosphorylation of TEFs may have a major role in regulating these important physiological processes.

Although all the PKC isozymes can modify the same consensuses sequence, isoform-specific preference on given peptides has been documented (32, 33, 40). The isoform preference was believed to be imperative in maintaining the selectivity and diversity for intracellular signal transduction (32, 40). In this study, we assessed PKC isoform expression in BeWo cells by Western blots and examined the isoform specificity on TEF function by measuring the relative expression of selected PKC isoforms. Among the isoforms we examined, the PKC γ and δ isoforms were the most abundant (Fig. 7A). Cotransfection of each of these isoforms along with the GT-IIC reporter gene resulted in significantly more TPA-mediated inhibition of TEF function, whereas overexpression of PKC-α and -β failed to affect GT-IIC enhancer activity (Fig. 7B). In related experiments, we confirmed that TEF-1 was efficiently modified by PKC-δ and γ in vitro (data not shown), providing further evidence about their involvement in TEF phosphorylation regulation. Significant γ and δ isoform expression has also been reported in human term placenta along with α and β isoforms (41, 42). In JEG-3, a choriocarcinoma cell line similar to BeWo, expression of the α, δ, and γ isoforms has been reported (43). Taken together, these results strongly support the concept that the DAG-activated PKC isozymes δ and γ may be physiological regulators of TEF transcriptional activities in placental syncytiotrophoblasts. Further studies are required to understand how different substrate specificity and subcellular distribution of various PKC isoforms affect TEF function.

Because many DNA binding domains are basic, and positive
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**Fig. 8. Multiple PKC phosphorylation sites are present in the CnBr fragments F1 and F2 of hTEF-1.** The 6.7-kDa peptide F1, representing the major phosphorylation peptide, is located in the carboxy-terminal region of the TEA/ATTs DNA binding domain and the hinge region. The more weakly phosphorylated 4.6-kDa peptide F2 is located within the STY-rich domain. Multiple potential PKC phosphorylation sites are found in the F1 and F2 sequences and are shown in bold.

Note that Ser102, Thr112, Ser113, Thr117, and Ser122 are absent in several of the individual TEF isoforms.

Charges contribute to their affinities to a DNA duplex, phosphorylation at sites within or near DNA binding domains frequently attenuates DNA binding, perhaps by direct electrostatic effects. For example, the PKA phosphorylation of NGFI-B, an orphan member of the nuclear receptor family, at Ser350 within the DNA binding domain results in decreased binding to the NGFI-B-responsive element (27). Similarly, PKA and PKC phosphorylation of C/EBPβ at Ser346 within the DNA binding domain leads to inhibition of DNA binding (28). Also, the phosphorylation of jun inhibits DNA binding as the dephosphorylation of three sites adjacent to the DNA binding domain potentiates its DNA binding (44). In this respect, specific PKC phosphorylation in the carboxy-terminal region of the TEA/ATTs DNA binding domain is of particular interest. Protein modeling has predicted that the TEA/ATTs domain forms three α-helices, and α-helix-3, which is contained in the F1 phosphopeptide, has been shown to be important for DNA binding activity (5). Helix-3 harbors 4 potential PKC phosphorylation sites, Thr31, Thr84, Thr36, and Ser91. Because these sites are conserved across all the TEA/ATTs domain family members, PKC phosphorylation on these sites may provide a general regulatory mechanism for all TEF members, including TEF-1 (α, β, and γ isoforms) and TEF-5, all of which are expressed in placental cells (6, 7, 47).

Although the hinge region itself is not known to participate in direct DNA contacts (5), its close location to the TEA/ATTs domain could contribute to changes in the conformation of the nearby α-helix structures and could modulate DNA binding. Most interestingly, although generally the four TEF family members share high amino acid sequence similarities, the hinge region is particularly diversified. This is especially true for the three TEF-1 isoforms, α, β, and γ, that most likely arise from alternative splicing of the primary transcripts encoded by a single gene (7). These isoforms match each other perfectly in nucleotide and amino acid sequences, except for the hinge region, in which 30 nucleotide changes cause 7 amino acid substitutions and a 4-amino acid insertion. Strikingly, these alterations involve three changes in their potential PKC phosphorylation profiles. As a result, each of the four TEA/ATTs domain family members and three TEF-1 isoforms has a unique potential PKC phosphorylation profile (Fig. 8). This raises the possibility that the function of each TEF family member may be differentially regulated by PKC phosphorylation. Among the four TEF family members and three TEF-1 isoforms characterized so far, each of them has shown different DNA binding and trans-activation and -repression functions. Accordingly, unlike the PKC sites in the TEA/ATTs domain helix-3, which is conserved among the different TEF family members, PKC phosphorylation in the hinge region may convey unique member-specific regulatory signals.

The viewpoint that member-specific phosphorylation regulates TEF family members uniquely is exemplified by a close correlation between the function and PKC phosphorylation potentials of the TEF-1 isoforms. We have demonstrated that amino acid divergence in the hinge region caused significant alterations in DNA binding and trans-repression function (7). In terms of the PKC phosphorylation sites, the TEF-1 β and γ isoforms lack the Ser102 site that is present in TEF-1α. In addition, TEF-1γ is further distinguished from TEF-1β by a 4-amino acid insert, which harbors 2 additional potential PKC phosphorylation sites, Thr112 and Ser122. In relation to their function, the TEF-1 β and γ isoforms possess higher DNA binding activity than TEF-1α (7). Because the TEF-1 isoforms used in our previous studies were generated by in vitro translation (7), and the reticulocyte lysates are known to contain PKC activities (45), it is possible that the differences in DNA binding activity observed among these isoforms were due to differential phosphorylation. If this is the case, then Ser102, the PKC site distinguishing the TEF-1 β and γ isoforms from TEF-1α, may play a key role for TEF-1 function.

It is important to point out that although we characterized the PKC regulation on TEF DNA binding activity, PKC modulation on other TEF functions, e.g., PKC trans-activation and -repression and nuclear localization activities, has yet to be assessed. On the basis of results that the native TEF from BeWo cells was only supershifted by phosphoserine- and phosphothreonine-specific antibodies, TEF serves as a target for serine and threonine kinases. Although our data clearly demonstrate that PKC agonists and antagonists regulate TEF function, possibly by altering its ability to bind DNA, we cannot exclude non-PKC-mediated mechanisms. Indeed, recent studies have shown that TEF-1 is a substrate for protein kinase A-dependent phosphorylation and that this phosphorylation is involved in the cAMP-dependent stimulation of the α-myosin heavy chain gene promoter in cardiac myocytes (46). TEF-1 was phosphorylated by protein kinase A at Ser102, and phosphorylation inhibited the ability of TEF-1 to bind DNA, providing support for the importance of this residue as discussed above. Sequence analysis indicates that TEF-1 is a potential substrate for other serine/threonine protein kinases, including casein kinase II, calcium-dependent protein kinase II, and cGMP-dependent kinase. Further work will be required to understand the roles that these alternative phosphorylation pathways may play in TEF activation in placenta and tissues.

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