Protein Kinase C Isozymes Differentially Regulate Promoters Containing PEA-3/12-O-Tetradecanoylphorbol-13-acetate Response Element Motifs*

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To investigate the regulation of promoters containing classical phorbol ester response sequences (PEA-3/12-O-tetradecanoylphorbol-13-acetate response element motifs) by protein kinase C (PKC) isozymes, co-transfections were performed in human dermal fibroblasts with a plasmid containing either the human collagenase promoter or the porcine urokinase plasminogen activator (uPA) promoter linked to the chloramphenicol acetyltransferase gene and a plasmid expressing an individual PKC isozyme. Using this experimental design, seven PKC isozymes were analyzed for their ability to transactivate the collagenase and uPA promoters. Our results demonstrate that only PKC δ, ε, and η trans-activate the collagenase promoter and that binding of Ap-1 family members to the collagenase 12-O-tetradecanoylphorbol-13-acetate response element (TRE) was not controlled for the isozyme-specific trans-activation. In contrast, the uPA promoter was stimulated by all of the PKC isozymes examined (PKC α, βI, γ, δ, ε, ζ, and η). These results indicate that PKC isozymes differentially regulate promoters containing PEA-3/TRE motifs and suggest that individual isozymes play unique roles within the cell.

Protein kinase C (PKC) is a serine/threonine kinase that was first characterized by its dependence on calcium, phospholipid, and diacylglycerol for in vitro activation (Nishizuka, 1992; Asakawa et al., 1992). PKC is also activated by phorbol esters and is considered the major phorbol ester receptor in the cell (Nishizuka, 1984). Once activated, PKC plays a key regulatory role in a variety of cellular functions such as stimulation or repression of growth, changes in morphology, and modulation of gene expression (reviewed in Nishizuka 1986; Nishizuka 1989). Extensive molecular cloning and biochemical studies have revealed that PKC is a family of related polypeptides. Eleven mammalian genes have been isolated and cloned that code for 12 different PKC isozymes identified by the following Greek symbols α, β, γ, δ, ε, ζ, η, θ, i, λ, and μ (reviewed in Dekker and Parker, 1994).

Structurally, the PKC isozymes are heterogeneous and can be divided into four subfamilies based on their primary structure and activation requirements. The conventional isozymes (α, βI, βII, and γ) contain two constant domains, C1 and C2, within their regulatory element and require calcium for activation, whereas the novel isozymes (δ, ε, η, and θ) lack a C2 region in their regulatory domain and do not require calcium for activation. The atypical isozymes (ζ, ι, and λ) lack a C2 region and have only one cysteine-rich stretch in their C1 region. These isozymes do not require calcium, diacylglycerol, or phorbol esters for activation (Dekker and Parker, 1994; Liscovitch and Cantley, 1994). The fourth subfamily has only one member (PKC μ). The unique feature of this isozyme is its long N-terminal region that may function as a transmembrane domain. Like the novel and atypical PKC isozymes, PKC μ lacks the C2 domain and does not require calcium for activation (Johannes et al., 1994; Johannes et al., 1995).

In addition to structural differences, the PKC isozymes also display functional heterogeneity due to, in part, differences in tissue distribution, subcellular localization, and substrate selectivity. For example, some of the isozymes are present in the majority of tissues investigated (α, δ, ζ) whereas others (γ, η, and θ) are expressed in a tissue-specific manner (Dekker and Parker, 1994; Hug and Sarre, 1993). Additionally, it has been reported that in resting cells PKC α, βI, and ζ are found in the cytosol; PKC θ is predominantly found in the particulate fraction, and PKC η is located in the nucleus. The subcellular location of PKC δ and ε is cell type-specific (reviewed in Hug and Sarre, 1993). Furthermore, substrate selectivity of the PKC isozymes has been demonstrated. Goode et al. (1992) have shown that glucogen synthase kinase-3B (GSK-3B), an enzyme involved in the activation of c-jun (a member of the Ap-1 transcription factor family), is a much better substrate for PKC α, βI, and γ than it is for PKC βII, whereas PKC ε is unable to significantly phosphorylate GSK-3B. This isozyme hierarchy for substrate phosphorylation is seen with a number of PKC substrates including the epidermal growth factor receptor (Ido et al., 1987), the neuron-specific protein F1/GAP-43 (Sheu et al., 1990), and the vitamin D receptor (Hisle et al., 1991). This high degree of structural and functional heterogeneity found among the isozymes suggests that each isozyme plays a unique role within the cell.

PKC has been shown to be associated with enhanced expression of both the collagenase and urokinase plasminogen activator (uPA) genes (Rajabi et al., 1992; Juarez et al., 1993; Sudbeck et al., 1994; Rondeau et al., 1990; Hamilton et al., 1991; Niedbala and Stein-Picarella, 1993; Diersk-Venting et al., 1989; He et al., 1992). Due to the large heterogeneity that exists among the PKC isozymes as described above, it is pos-
sible that the isozymes regulate the collagenase and uPA promoters differently. To test this idea, co-transfections were performed in human dermal fibroblasts (HDF) with a plasmid that expresses an individual PKC isozyme and a plasmid containing either the porcine uPA or human collagenase promoter linked to the chloramphenicol acetyltransferase (cat) gene. Our results indicate that these two promoters are regulated differently by the PKC isozymes and suggest a biological explanation for the heterogeneity that exists within the PKC family.

MATERIALS AND METHODS

Plasmids—The plasmid containing the human collagenase promoter linked to the cat gene, −1200 to +63 Cat (Angel et al., 1987a) was kindly provided by Hans-Joob Rahmsdorf. pCAT-4660 (Cassady et al., 1991), a plasmid containing the porcine uPA promoter sequences linked to the cat gene, was a gift from David A. Humke. The expression vector p5-VHNX-neo was constructed from the parent plasmid p5V2HNBX that contains the β-lactamase gene and a prokaryotic origin of replication from pBR322 (pBR322 sequences 2064–4361), the SV40 origin of replication and early promoter (SV40 sequences 270–5190), a 16-bp polylinker containing unique SphI, HincII, HindIII, BglII, and NdeI sites, the SV40 origin of replication, and the SV40 early region polyadenylation signal (SV40 sequences 2770–1782). pSVHNX-neo was then constructed by the following steps. The neomycin resistance gene was isolated from pSV2-neo (Southern and Berg, 1982) on an NdeI to BamHI fragment, and the ends were made blunt with T4 DNA polymerase. The BamHI fragment plus dCTP, dGTP, and TTP. This fragment was then inserted in the unique BamHI site of pSV2HNBX that previously had been blunted as described above. An oligonucleotide containing an Nhel site flanked by XhoI sites was then ligated into the unique XhoI site creating a unique Nhel site. The individual PKC cDNAs were then ligated (either cohesive or blunt-ended) into the Nhel site isolation and cloning of these cDNAs has been described (Basta et al., 1992; Aris et al., 1993; Barbee et al., 1993; Hoevar et al., 1993; Rice et al., 1993). A plasmid containing the PEA-3 and TRE of the collagenase promoter, pTRET-K-CAT, was constructed as follows. The thymidine kinase promoter from herpes simplex virus type 1 was isolated from pHSV-106 (Life Technologies, Inc.) and a 17-bp, double-stranded oligonucleotide encompassing the TRE motif from the human collagenase promoter. The sequence of this oligonucleotide is as follows, AAGCATGAGTCGAC. The oligonucleotide was labeled at the 5′ ends with T4 polynucleotide kinase (Life Technologies, Inc.) and γ-[32P]ATP (DuPont NEN). Nuclear extracts (1 μg) were incubated under conditions similar to those described by Carthew et al. (1985) at 25°C for 30 min with 0.03 pmol (approximately 10,000 cpm) of probe. The 10-μl reaction mixture also contained 200 ng of poly(dI-dC)-poly(dI-dC) (Pharmacia Biotech Inc.), 15% glycerol, 20 mM HEPES (pH 7.9), 100 mM KC1, 5 mM MgCl2, 0.2 mM EDTA, and 0.5 mM dithiothreitol. Samples were subjected to electrophoresis on either a 4% or 4–20% gradient, non-denaturing polyacrylamide gel. The quantity of probe in each band was determined by visualizing the dried gel with a Betascope 603 Blot Analyzer (Betagen Corp.) or by analyzing the autoradiogram with an Ultrascan XL Laser Densitometer (Pharmacia).

RESULTS

Promoters Containing PEA-3 and TRE Motifs Are Regulated Differently by PKC Isozymes—To investigate PKC isozyme regulation of promoters containing classical phorbol ester-responsive sequences, co-transfections were performed in HDF with a plasmid containing either the human collagenase promoter or the porcine uPA promoter linked to the cat gene (−1200 to +63 Cat or pCAT-4660, respectively) and either the control vector (pSVHNX-neo) or a plasmid expressing an individual PKC isozyme or the control vector pSVHNX-neo. Four hours after transfection, 1.0 ml of 15% glyceral in Dubelco’s modified Eagle’s medium (Sigma) was placed on the cells for 60 s. The cells were washed twice with phosphate-buffered saline, and then fresh culture medium with either 10 or 1% FBS was added to the dishes. Forty-eight hours after transfection, the cells were washed twice with phosphate-buffered saline and then removed from the dishes by scraping in TE buffer (40 mM Tris, pH 7.5, 1.0 mM EDTA, 150 mM NaCl). After centrifugation, the pellet of cells was resuspended in 100 μl of phosphate-buffered saline containing 1.0 mM phenylmethylsulfonyl fluoride. A cellular lysate was prepared by three consecutive cycles of freeze-thaw, and the protein concentration was determined for each lysate using the BCA protein concentration assay (Pierce). The amount of Cat activity in the lysate or the control vector (pSVHNX-neo) was measured at 405 nm with a Dynatech MR 5000 (Dynatech Laboratories) microplate reader. Cat protein concentrations were extrapolated from a standard curve prepared with Cat enzyme supplied in the kit. The degree of trans-activation of the uPA or collagenase promoter by the PKC isozymes was calculated and expressed as fold activation. Fold activation was calculated by dividing the percentage of acetylation (TLA method) or the amount of Cat protein produced (ELISA method) from each co-transfection by that value generated from the co-transfection containing the control plasmid, pSVHNX-neo. Statistics—Statistical analyses were performed with the statistical software JMP (SAS Institute Inc.), version 3.0. To determine if a given isozyme had activity significantly greater than the control, a one sample t test was performed on the logarithm of the fold activation for each isozyme. The logarithmic transformation was used because fold activation was determined on a ratio scale. One-sided p values are reported because only increased activity is of interest. A p value of <0.05 was considered significant.

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Fig. 1. Schematic representation of pSVHNX-neo and a description of the individual PKC cDNAs and the resulting PKC expression plasmids. Construction of pSVHNX-neo and the PKC expression plasmids is described under “Materials and Methods.” Briefly, pSVHNX-neo contains the β-lactamase gene (AmpRes) and a prokaryotic origin of replication from pBR322. The neomycin resistance gene (neo), derived from pSV2neo (Southern and Berg, 1982), is regulated by the SV40 early promoter (SV40 EP) and has downstream SV40 processing sequences (Intron, SV40poly(A)). The PKC cDNAs were ligated either cohesive or blunt-ended into the unique NheI site located in the polylinker (PL) of pSVHNX-neo. The PKC cDNAs are regulated by SV40 sequences (SV40 EP, Intron, SV40poly(A)). The resulting PKC expression plasmids each express an individual isozyme.

Fig. 2. Effect of individual PKC isoforms on the full-length collagenase (A) or uPA (B) promoter in 10% FBS. A, co-transfections were performed with −1200/+63 CAT and either pSVHNX-neo (control vector, CV) or a plasmid that expresses an individual PKC isozyme. Fold activation describes the degree of trans-activation by the individual PKC isoforms. This value was calculated by dividing the percentage of acetylation (TLC) or the amount of Cat protein produced (ELISA) from each co-transfection by that value generated from the co-transfection with the control vector. The significance of the fold activation for each isozyme was determined as described under “Materials and Methods.” The data represent the average from three to six different co-transfections (mean ± S.E.). B, co-transfections were performed with pCAT 4660 and either pSVHNX-neo (CV) or a plasmid expressing an individual PKC isozyme. Fold activation was calculated, and the significance of these values was determined as described above. The data shown represent the average of from three to four separate co-transfections (mean ± S.E.).

The observation that only PKC ε and η strongly trans-activated the collagenase promoter (Fig. 3A), and only PKC ε weakly stimulated the uPA promoter above that seen with the control vector (Fig. 3B). Therefore, these data suggest that a substance present in the FBS was responsible for activating the exogenous isoforms that resulted in the data shown in Fig. 2.

Fig. 4A. Schematic representation of pSVHNX-neo and a description of the individual PKC cDNAs and the resulting PKC expression plasmids. Construction of pSVHNX-neo and the PKC expression plasmids is described under “Materials and Methods.” Briefly, pSVHNX-neo contains the β-lactamase gene (AmpRes) and a prokaryotic origin of replication from pBR322. The neomycin resistance gene (neo), derived from pSV2neo (Southern and Berg, 1982), is regulated by the SV40 early promoter (SV40 EP) and has downstream SV40 processing sequences (Intron, SV40poly(A)). The PKC cDNAs were ligated either cohesive or blunt-ended into the unique NheI site located in the polylinker (PL) of pSVHNX-neo. The PKC cDNAs are regulated by SV40 sequences (SV40 EP, Intron, SV40poly(A)). The resulting PKC expression plasmids each express an individual isozyme.

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PKC Isozyme Selective Gene Regulation—PKC isoforms have been implicated in altering the DNA binding activity of c-jun, a member of the Ap-1 family of transcription factors (Boyle et al., 1991; Jackson, 1992; Goode et al., 1992). In addition, all the PKC isoforms have been shown to regulate transcription of the collagenase promoter at the TRE site (Angel et al., 1987b; Angel et al., 1988; Chiu et al., 1988). Because PKC ε enhanced transcription of the collagenase promoter at the TRE site, a gel retardation assay was performed to determine if Ap-1 family members are bound to the collagenase TRE when exogenous PKC ε is present. A 17-bp oligonucleotide probe representing the native collagenase TRE, nuclear extracts prepared from cells transfected with pSPKCE, and varying concentrations of unlabeled competitor oligonucleotides representing either the native or a mutant
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FIG. 3. The effect of individual PKC isozymes on the full-length collagenase (A) or uPA (B) promoter in the presence of 1% FBS. A, the plasmid +1200/+63CAT was transfected with either pSVHNX-neo (control vector, CV) or a plasmid that expresses an individual PKC isozyme in medium containing 1% FBS as described under "Materials and Methods." Fold activation was calculated as described previously, and the significance of these values was determined using the statistical software program MP, version 3.0. The data represent the average of three separate co-transfections (mean ± S.E.). B, a plasmid containing the full-length uPA promoter, pCAT 4660, was transfected with either pSVHNX-neo (CV) or a plasmid that expresses an individual PKC isozyme in medium containing 1% FBS. Fold activation for each isozyme was calculated, and the significance of the values was determined as described previously. The data represent the average of two separate co-transfections (mean ± S.E.).

TRE sequence were used in the gel mobility retardation assay. The mutant sequence has previously been shown to have very little TRE activity (Angel et al., 1987b). As seen in Fig. 5A, a single band was produced in all lanes suggesting that a single protein or a single protein complex is bound to the TRE. The amount of bound probe in each lane was determined as described under "Materials and Methods." As seen in Fig. 5B, the native TRE sequence competed for Ap-1 factor binding in a dose-dependent manner, whereas the mutant TRE oligonucleotide efficiently competed for binding only at the 100-fold concentration. These results demonstrate that an Ap-1 family member is specifically bound to the TRE sequence when exogenous PKC ε is present in the nuclear extracts.

Although a single band was produced in the gel mobility retardation assay described above, subtle changes in the form of the protein or complex of proteins bound to the TRE may have occurred in response to exogenous PKC ε. We have found that better resolution of protein-DNA complexes from gel mobility retardation assays can be obtained on gradient, nondeaturing polyacrylamide gels (Birch et al., 1996). Therefore, additional gel mobility retardation assays were performed to further investigate the molecular mechanism for PKC ε-selective gene regulation. As seen in Fig. 6A, two distinct bands (B1 and B2) were consistently produced when binding reactions containing nuclear extracts prepared from cells transfected with the control vector, pSPKCε, or pSPKCζ and incubated with the 17-bp TRE probe were resolved on 4–20% gradient gels. These two bands could represent either two different Ap-1 dimers or two forms, perhaps phosphorylation alterations, of the same dimer bound to the TRE. As mentioned above, PKC isozymes have been shown to alter the DNA binding activity of Ap-1 family members (Boyle et al., 1991; Jackson, 1992; Goode et al., 1992); therefore, the density of B1 and B2 in each lane was determined as described under "Materials and Methods." Only small variations in the total amount of bound probe (B1 + B2) were detected (Fig. 6B); however, the relative amount of probe present in B1 compared with B2 was altered. The amount of probe bound in B1 increased while that in B2 decreased when nuclear extracts prepared from cells transfected with pSPKCε or pSPKCζ were compared with cells transfected with the control vector, pSVHNX-neo (Fig. 6C). These results suggest that the total amount of protein bound to the TRE was altered only slightly by exogenous PKC ε isozenes, whereas the protein or form of protein bound was significantly altered. Surprisingly, the binding pattern and the quantity of bound probe were very similar for extracts prepared from cells transfected with pSPKCε or pSPKCζ. These observations suggest that trans-activation of the collagenase promoter by PKC ε is not due to alterations in the binding of Ap-1 family members to the TRE motif.

FIG. 4. Schematic representation of pTRETK-CAT (A) and the effect of PKC ε, ζ, and η on the human collagenase PEA-3/TRE motifs (B). A, pTRETK-CAT contains a 71-bp oligonucleotide representing sequences from -111 to -40 of the human collagenase promoter. This region of the promoter contains both a PEA-3 and a TRE motif. The 71-bp oligonucleotide was placed upstream of the herpes simplex virus type I thymidine kinase promoter. This regulatory region was then linked to the cat gene. Construction of this plasmid is described under "Materials and Methods." B, pTRETK-CAT was transfected with pSVHNX-neo (control vector, CV), pSPKCε, pSPKCζ, or pSPKCη. The results represent the average of five separate co-transfections (mean ± S.E.).

DISCUSSION

Due to the heterogeneity that exists among the PKC family of isozenes and reports that suggest that individual isozenes may have distinct roles in biological functions (Nishizuka, 1988; Borner et al., 1992; Dekker and Parker, 1994), we designed the study presented here to investigate trans-activation of two phorbol ester-responsive genes by individual PKC isozenes. Our finding that the collagenase promoter is regulated only by the novel PKC isozenes δ, ε, and η clearly dem-
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A concatenated TRE cluster is trans-activated by PKC. Kariya (1994) presented data demonstrating isozyme-selective gene regulation. Other investigators have also demonstrated that some promoters are regulated by specific PKC isozymes and supports the idea that individual isozymes have unique functions within the cell. Other investigators have also shown that two phorbol ester-responsive promoters are regulated better by PKC isozymes. Our data with the collagenase promoter (Fig. 2B) suggest that all of the isozymes are functional, and the inability to trans-activate the collagenase promoter by some of the isozymes (PKC ε and γ) is due to a unique aspect of this promoter.

**Fig. 5. Identification of protein(s) bound to the collagenase TRE motif.** A, gel mobility retardation assays were performed as described under “Materials and Methods” with 0.03 pmol of native TRE oligonucleotide as probe, 1 μg of nuclear extract prepared from cells transfected with pSPKCε, and 0, 10-, 50-, and 100-fold excess unlabeled, competitor oligonucleotide (mutant or native). The native and mutant TRE oligonucleotides differ only by the two bases that are shown in bold in the mutant TRE sequence. Bound probe was separated from the free probe on a 4% nondenaturing polyacrylamide gel. B, the percent of native TRE sequence that was bound (% of probe bound) was determined from this representative gel by scanning the bands on the autoradiogram with a densitometer as described under “Materials and Methods.” The percent of bound probe was then plotted versus the amount of competitor oligonucleotide that was added to the reaction.

Demonstrates that some promoters are regulated by specific PKC isozymes and supports the idea that individual isozymes have unique functions within the cell. Other investigators have also presented data demonstrating isozyme-selective gene regulation. Kariya et al. (1991) have shown that the β-myosin heavy chain promoter is regulated better by PKC β than by α. In addition, the primary response gene, ST2, has recently been shown to be regulated by PKC β, γ, ε, and δ, or δ (Kiers et al., 1995), and Hata et al. (1993) have shown that a concatenated TRE cluster is trans-activated by PKC α, βII, and ε but not by γ. Although the results by these investigators are interesting, our data may more accurately reflect PKC isozyme regulation as we have used full-length cellular promoters, transiently expressed PKC isozymes, and physiological stimulation.

In addition to isozyme-selective gene regulation, we have shown that two phorbol ester-responsive promoters are regulated differently by the PKC isozymes (compare Fig. 2A and 2B). The TRE in both the human collagenase promoter (Angel et al., 1987a) and the porcine uPA promoter (Rorth et al., 1990) is required for induction of gene expression by tumor-promoting agents. In addition, the PE A-3 site on these promoters acts synergistically with the TRE to achieve maximal levels of induction (Gutman and Wasylyk, 1990; Rorth et al., 1990). Although the PE A/TRE motifs found in these promoters are similar, their sequences, the spacing between the PE A-3 and TRE, and their location within the promoter are unique to each gene (Angel et al., 1987a; Cassady et al., 1991). While it remains unknown whether these differences are responsible for the diverse trans-activation profiles seen in this study, it is possible that substrates for the different PKC isozymes differentiate the two promoters at these sites.

Similar to our finding that serum concentrations greater than 1% are necessary to achieve trans-activation (Figs. 2 and 3), other investigators have shown that a substance present in serum activates PKC (Abdel-Ghani et al., 1989). In fact, Ohno et al. (1994) report that serum selectively activates the PKC isozymes. Our data with the collagenase promoter (Fig. 2A) is consistent with the data of Ohno et al. (1994), i.e. serum activation leads to selective trans-activation by the novel PKC isozymes. However, our findings with the uPA promoter (Fig. 2B) suggest that all of the isozymes are functional, and the inability to trans-activate the collagenase promoter by some of the isozymes (PKC α, βII, γ, and ε) in Fig. 2A is due to a...
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