Phosphorylation of CCAAT-enhancer Binding Protein by Protein Kinase C Attenuates Site-selective DNA Binding*

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Four DNA-recombinant proteins, corresponding to the DNA-binding domain of CCAAT/enhancer binding protein (C/EBP), were phosphorylated in vitro by protein kinase C (PKC). High-performance liquid chromatography-peptide mapping of 32P-labeled C/EBP indicated the presence of three major 32P-labeled peptides: S277 (P)RD, AKKS277 (P)VDK, and GAAGLP-GPGGS248 (P)LK. Phosphorylation of C/EBP by PKC or M-kinase resulted in an attenuation of binding to a 32P-labeled CCAAT oligodeoxynucleotide. Three other truncated forms of C/EBP, C/EBP87, C/EBP87-S277, and C/EBP60, were studied to define the sites of phosphorylation affecting DNA binding. Phosphorylation of the C/EBP87, containing sites Ser206 and Ser277, and C/EBP60, containing only site Ser206, by PKC also resulted in attenuation of DNA binding. In contrast, phosphorylation of C/EBP87-C, which retained Ser277 but had a Cys in place of Ser206, had no effect on DNA binding. Ser206 could not be phosphorylated by PKC if the protein is already bound to specific DNA. Phosphorylation of intact C/EBP from liver nuclear extract by PKC or M-kinase occurred at Ser206 and Ser277 and at an additional site, as demonstrated by immunoprecipitation and peptide mapping.

Protein kinase C (PKC),* coded for by a family of homologous genes, α, β, γ, δ, ε, η, and ζ (for reviews see Refs. 1 and 2), has been implicated in numerous trans-cell membrane signal transduction processes (for reviews see Refs. 3–5). Many of the cellular events regulated by PKC involve changes in gene transcription. For example, genes containing 12-O-tetradecanoylphorbol-13-acetate responsive elements, such as collagenase, jun/AP1, c-fos, c-myc, and metallothionein genes (6–10), all exhibit altered transcription rates in a phorbol ester-dependent fashion. Although PKC has been shown to be the cellular receptor for the tumor-promoting phorbol esters (11, 12), the mechanism of signal transduction from the phorbol esters to the transcriptional process remains elusive. Recently, evidence for a nuclear PKC (rat liver) was reported (13), suggesting a possible PKC-mediated phosphorylation of nuclear transcription factors. Although many transcription factors are potential PKC substrates, e.g. SP1, SREF, HBP-1, GCN4, and CPC-1 (14–18), based on the presence of Thr or Ser residues flanked by basic residues, there is little evidence to date that they can be phosphorylated by PKC.

CCAAT/enhancer binding protein (C/EBP) is a member of the bZIP family of DNA-binding proteins. These proteins are characterized by a leucine repeat-dimerization interface immediately preceded by a region rich in basic amino acids that is believed to constitute the DNA contact surface (for reviews see Refs. 19–23). The presence of potential PKC phosphorylation sites in C/EBP, coupled with the colocalization of C/EBP and PKC in rat liver nuclei raised the possibility that C/EBP may be a substrate of PKC. We report here the in vitro phosphorylation by PKC of proteins corresponding to the DNA-binding domain of C/EBP and holo-C/EBP. We have identified the sites of PKC-mediated phosphorylation of C/EBP and have studied the subsequent effect that modification exerts on sequence-specific DNA binding. Our results suggest that site-selective phosphorylation at Ser206 of C/EBP results in attenuation of the DNA binding.

EXPERIMENTAL PROCEDURES

Materials—Commercially available reagents were purchased: histone-I, II-S, CNBr, poly(L-lysine)/agarose, and CAMP-dependent protein kinase (PKA) from Sigma; calf thymus DNA (Promega); 1,2-dioleoylglycerol (Avanti Polar Lipids); cellulose thin-layer chromatography plates (20 × 20 cm) from Kodak; hydroxyapatite and protein determination reagents from Bio-Rad; and all reagents from Life Technologies-Bethesda Research Labs. NICK (Sephadex G50, DNA grade) and Mono Q columns from Pharmacia LKB Biotecnology Inc.; endoproteinase Lys-C from Boehringer-Mannheim; Sep-Pak NH2 columns and Immobilon (polyvinylidene difluoride) membranes from Waters/Millipore; Centricon 10 concentrators from Amicon; and myosin light chain (11–23) peptide from Peninsula. The GAP43 (1–20) peptide was synthesized by Dr. H. Nakabayashi (National Institutes of Health) and glycogen synthase(1–12) peptide was generously provided by Dr. C. Londos (National Institutes of Health).

Methods—PKC was purified from rat brain by the procedure of Huang et al. (24). Catalytic fragment (M-kinase) of trypsinized type II PKC was purified by chromatography on a Mono Q column as previously described (25). PKC activity was measured in vitro in final volumes of 20–40 μl and incorporation of

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The abbreviations used are: PKC, protein kinase C; C/EBP, CCAAT/enhancer binding protein; EGT, [ethylenebis(oxyethyl)tetraacetic acid; DTT, dithiothreitol; PS, phosphatidylycerine; DAG, diacylglycerol; HPLC, high-performance liquid chromatography; PDBu, phorbol 12,13-dibutyrate; T9, retention time; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PKM, protease-degraded PKC.
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25^P was determined, as previously described, using 30% trichloroacetic acid containing 25 mM ATP as the stop solution and 20% trichloroacetic acid containing 0.2 M KCl as the chromatographic solvent (26). In the case of the smaller C/EBP proteins (C/EBPβ77, C/EBPβ78, and C/EBP60), incorporation of 25^P was determined by using 5B1 phosphocellulose paper (27). In the case of gel retardation or DNase I protection assays, phosphorylation reactions were stopped by dilution with an equal volume of 20 mM EDTA, pH 7.5, containing 20% glycerol. SDS-polyacrylamide gel electrophoresis was by the procedure of Laemmli (28).

Polyptides fragments containing the DNA-binding domain of C/EBP, consisting of residues M1^14-A458 (C/EBP145), G72^72-A80 (C/EBP87), G72^72-A80 (C/EBP87-), and N70^340 (C/EBP60) (Fig. 1) were synthesized in Escherichia coli strain B21 (DE 3) polyS with the use of the phage T7 expression system (29) and purified from the bacteria according to Shuman et al. (30). Numbering of the amino acid residues is according to Landschulz et al. (31).

Truncated C/EBP proteins (3 µM), phosphorylated with PKC or M-kinase using [γ-32P]ATP, were precipitated with 2 volumes of 30% trichloroacetic acid containing 25 mM ATP. Pellets were washed with 20% trichloroacetic acid, resuspended in 0.2 M NaOH, and resuspended with 20% trichloroacetic acid, and washed with ice-chilled ether. Protein pellets were then successively cleaved with CNBr and endoproteinase Lys-C as follows. CNBr cleavage (100-fold molar excess over total Met) was carried out in 70% formic acid for 16–24 h at room temperature (22–40 µl final volume). The reaction was terminated by evaporating the formic acid with a stream of air in a fume hood, the peptide mixture was washed with 22 µl of formic acid and vacuum-evaporation was repeated; residual CNBr was then sublimated (70 °C, 5 min). CNBr-cleaved protein was digested with endoproteinase Lys-C (1/10, w/w) in 100 mM NH_4HCO_3, pH 9.0, for 4 h at room temperature (22 µl final volume). The reaction mixture was directly analyzed by HPLC. The peptide mapping was carried out employing a Vydac 218TP54 (C-18, 5 µm, 300 A, 25 × 0.46 cm) column and elution was by a 0.25%/min gradient from solvent A (0.1% trifluoroacetic acid) to solvent B (0.1% trifluoroacetic acid in CH_3CN) at a flow rate of 1 ml/min. Fractions (1 ml) were collected and counted in a liquid scintillation counter.

Phosphorylation of C/EBP (Fig. 1), was phosphorylated by PKC in a manner dependent on PKC isozyme type. Types II and III PKC phosphorylated C/EBP at a rate and extent that were comparable to phosphorylation of histone-IIIS on a molar basis (35). The rate of C/EBP phosphorylation was relatively insignificant as compared to phosphorylation of PKC isozyme type. Types II and III PKC phosphorylated C/EBP at similar rates and to a similar extent, whereas type I PKC exhibited a ~5-fold lower rate and an ~3-fold lower extent of phosphorylation. A maximum of 1 mol of 32P/mol protein monomer could be incorporated into C/EBP145. Under these assay conditions auto-phosphorylation of PKC was relatively insignificant as compared to phosphorylation of C/EBP. Several quantitative methods for measuring phosphorylation, instant thin-layer chromatography (26), phosphocellulose paper (27), and SDS-polyacrylamide gel electrophoresis (28) yielded similar measurements of phosphate incorporation into C/EBP by PKC. The K_m values for the C/EBP145, using type II PKC, were ~10–20 µM (data not shown) which is similar to that for histone-IIS on a molar basis (~1 µM) (35). Additionally, C/EBP145 and histone IIS are comparable substrates for PKC as they exhibit similar V_max values, as well as values for Ca^2+ and PS/DAG as activating factors (Fig. 2B). Phos-
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Fig. 1. Amino acid sequence and schematic representation of the C/EBP145, C/EBP87, C/EBP87'-C, and C/EBP60 truncated C/EBP proteins. Truncated C/EBP proteins corresponding to the C-terminal half of the protein contained the encoded residues M\(^{214}\)A\(^{250}\) (C/EBP145), G\(^{272}\)A\(^{286}\) (C/EBP87'), G\(^{275}\)A\(^{289}\) (C/EBP87'-C), and N\(^{281}\)G\(^{295}\) (C/EBP60) with leader sequences of GKA- and MGS-(for the latter three), respectively. A, amino acid sequence of C/EBP145. Sequence of isolated \(^{32}\)P-labeled peptides (stippling), phosphorylated Ser residues (capsules), basic regions A and B (cigar shapes), and the leucine zipper residues (boxed) are as indicated. B, schematic representation of truncated C/EBP proteins. Phosphorylation sites (Ser\(^{90}\), Ser\(^{175}\), and Ser\(^{290}\)) (S), the basic DNA binding regions A and B (BR and stippling), and leucine zipper (LZ and stippling) are as indicated. C/EBP87'-C has a Cys residue in place of Ser\(^{90}\).

The effect of phosphorylation of truncated C/EBP proteins on DNA binding was studied by using a \(^{32}\)P-labeled synthetic oligodeoxynucleotide probe containing a high affinity C/EBP binding site. C/EBP145 phosphorylated by PKCII in the presence of 400 \(\mu\)M Ca\(^{2+}\) and PS/DAG bound the oligodeoxynucleotide probe with decreased affinity relative to the nonphosphorylated starting material (Fig. 3). DNA binding by both the non- and phosphorylated forms of C/EBP145 was dependent on the protein concentration. Increasing the KCl concentrations from 50 to 500 mM resulted in decreased

Fig. 2. Phosphorylation of C/EBP145 by PKC. A, C/EBP145 (60 \(\mu\)g/ml) was phosphorylated with type I (squares), II (circles), or III (triangles) PKC (50 ng) in the presence of 400 \(\mu\)M CaCl\(_2\), 40 \(\mu\)g/ml PS, and 8 \(\mu\)g/ml DAG at 30°C in a final volume of 40 \(\mu\)l. B, C/EBP145 was phosphorylated by PKC (type III, 25 ng) in the presence of 400 \(\mu\)M EGTA and absence of PS/DAG (lanes 1–5); in the presence of 400 \(\mu\)M CaCl\(_2\) and absence of PS/DAG (lanes 6–10); and in the presence of 400 \(\mu\)M CaCl\(_2\), 40 \(\mu\)g/ml PS, and 8 \(\mu\)g/ml DAG (lanes 11–15) at 30°C in a final volume of 20 \(\mu\)l. Each set of five lanes (1–5, 6–10, and 11–15) represent time points of 1, 2, 3, 4, and 10 min, respectively. At various times, reaction mixtures were quenched with SDS sample buffer and analyzed on 14% SDS-polyacrylamide gels.

Proteins (\(^{32}\)P-labeled) were detected by autoradiography.

Fig. 3. Attenuation of binding of C/EBP145 to the CCAAT probe by phosphorylation with PKC. C/EBP145 (3 \(\mu\)M) was incubated in the absence (A) or presence of (B) PKCII under standard assay conditions. Incubations were carried out until 1 mol of \(^{32}\)P/mol of protein was incorporated as determined in a parallel experiment in the presence of [\(^{\gamma}\)\(^{32}\)P]ATP. Nonphosphorylated (A) and phosphorylated (B) C/EBP145 were incubated with CCAAT probe (0.28 ng of \(^{32}\)P-labeled 20-mer) in molar ratios (C/EBP145/CCAAT probe) of 0 (lanes 1, 4, 7, and 10), 0.9 (lanes 2, 5, 8, and 11), and 69 (lanes 3, 6, 9, and 12). KCl concentrations of the binding reactions were 50 (a), 190 (b), 250 (c), or 500 (d) mM. Binding of the \(^{32}\)P-labeled probe to C/EBP145 was analyzed by electrophoresis on a 5% native polyacrylamide gel. Free and retarded \(^{32}\)P-labeled CCAAT probe were detected by autoradiography.
binding of C/EBP145 to the 32P-labeled 20-mer probe, indicating that ionic interactions play a significant role in the interaction of C/EBP145 with probe. Gel retardation experiments in the presence of 25 μg/ml poly(dI-dC) yielded similar results as in its absence which is consistent with a specific interaction between the CCAAT oligodeoxynucleotide and C/EBP145.

In order to determine the effect of each phosphorylation site on DNA binding, several smaller C/EBP fragments, which contained the leucine zipper and adjacent upstream basic region, were produced. These proteins contained either two (C/EBP87, Ser299 and Ser277) or one (C/EBP87'-C, Ser277 only, and C/EBP60, Ser299 only) phosphorylation site(s). C/EBP87, C/EBP87'-C, and C/EBP60 were phosphorylated by PKC to near stoichiometric levels of 2.0, 0.75, and 0.7 mol of 32P/mol of protein monomer, respectively (Fig. 4). In all cases, modification proceeded in a Ca2+- and lipid-dependent manner. Peptide mapping (isoelectric focusing and reversed-phase HPLC) of 32P-labeled C/EBP87, C/EBP87'-C, and C/EBP60 indicated the presence of two, one, and one 32P-labeled peptides, respectively, each corresponding to the expected Ser residue(s). Phosphorylation of C/EBP87 and C/EBP60 to near stoichiometric levels with PKC resulted in an attenuation of DNA binding (Fig. 5), similar to that obtained with C/EBP145 (Fig. 3). In contrast, phosphorylation of C/EBP87'-C showed no apparent effect on site-specific DNA binding (Fig. 5). Analogous results were obtained using a DNase I protection assay (Fig. 6). Thus, negative effects on phosphorylation of Ser299. The maximum incorporation of 1 mol of 32P/mol of C/EBP145 monomer was distributed among the three major characterized sites, Ser299, Ser277, and Ser248 in the approximate ratio of 0.50:0.33:0.15, respectively, i.e. ~0.50 mol of P/mol of Ser299 site/mol of protein monomer (see Fig. 9B). It is not yet certain whether the E. coli-produced C/EBP145 is folded in the same manner as its smaller M counterparts such that each polypeptide chain can be phosphorylated by PKC. The dramatic weakening of DNA binding by maximally phosphorylated C/EBP145 could be due to phosphorylation of a single polypeptide chain within the C/EBP dimer to a level of ~1.0 mol of P/mol of Ser299 site, or alternatively, only 50% of the recombinant protein is in the active conformation at Ser299 to be recognized by PKC.

The observation that the phosphorylation of Ser299 weakens binding of C/EBP to its DNA substrate raised the possibility that this portion of the protein might be involved in intimate association with the DNA. If so, prior interaction of the protein with the DNA substrate might mask the Ser299 site from subsequent modification by PKC. To test this hypothesis, C/EBP60 was exposed to the specific oligodeoxynucleotide probe and analyzed by phosphorylation with PKC (Fig. 7A). Increasing mole equivalents of the DNA substrate probe per mol of C/EBP60 resulted in progressively greater inhibition of phosphorylation by PKC, with >75–80% inhibition being obtained at 0.93 mol eq of DNA probe dimer per mol active C/EBP60 dimer. To test whether the observed effect was due to site-specific interaction of the protein with its DNA substrate, we repeated the experiment using a nonspecific oligodeoxynucleotide probe (Fig. 7B). In this case, no significant effect on PKC-catalyzed phosphorylation was obtained with up to 1.49 mol eq of nonspecific DNA per mol of C/EBP60. Hence C/EBP60, when bound in a site-specific manner to its DNA substrate, could not serve as a substrate for PKC.

Since oligonucleotide could potentially inhibit PKC activity by somehow competing for ATP or otherwise by poisoning the catalytic activity of PKC, control peptides, GAP43 (1–20), glycogen synthase(1–12), and myosin light chain(11–23) peptide, were incubated with specific C/EBP DNA substrate.
followed by phosphorylation with PKC (Fig. 7C and data not shown). All of the control peptide substrates showed no inhibition of phosphorylation by PKC with up to 1.38 mol eq of DNA probe/mol of peptide, indicating that the specific DNA substrate for C/EBP had no effect on PKC-catalyzed phosphorylation of these PKC substrates.

Since many protein kinases have overlapping substrate specificities, PKA and PKG, which have similar substrate specificities as PKC, were tested for their ability to phosphorylate C/EBP145. PKA (bovine heart) and its catalytic subunit could incorporate up to 0.1 mol of "Pi per mol of C/EBP145; increasing the enzyme concentration or reaction time resulted in increased autophosphorylation levels but no further incorporation into the C/EBP145 (data not shown).

PKG phosphorylated the C/EBP145 to a considerably lower extent than PKA, as demonstrated by SDS-polyacrylamide gel analysis. Phosphoamino acid analysis of C/EBP145 that had been 32P-labeled with PKA indicated that only Ser residues were phosphorylated. Reverse-phase HPLC peptide mapping of C/EBP145 that had been 32P-labeled with PKA, electroblotted to Immobilon membrane, cut out, and successively cleaved with CNBr and endoproteinase Lys-C, indicated insignificant levels of phosphorylation of the peptide eluting at the position of the Ser22-containing peptide. Other 32P-labeled material eluted at 4' and 17', similar to 32P-labeled Ser200 and Ser277 containing peptides obtained with PKC, suggesting that PKA can phosphorylate Ser200 and Ser277 but not Ser22 in C/EBP145. Phosphorylation of the C/EBP145 with PKA had a slight attenuating effect on its ability to bind to the specific DNA substrate relative to nonphosphorylated protein (data not shown). Although PKA and PKG could phosphorylate C/EBP145, the low stoichiometries of phosphorylation of this protein casts doubt upon a physiological role for these two protein kinases in regulating C/EBP function.

Phosphorylation of holo-C/EBP by PKC was tested by employing liver nuclear extract. Liver nuclear extract, that had been heat-treated and concentrated, was phosphorylated with [γ-32P]ATP and either EGTA or Ca2+, phosphatidylserine, and phorbol 12,13-dibutyrate (PDBu), as demonstrated by immunoprecipitation of 32P-labeled C/EBP (42 kDa) (Fig. 8). A significant stimulation in the phosphorylation of C/EBP was apparent in the presence of Ca2+, PS, and PDBu as compared to basal phosphorylation.
We found that making use of PKM, which does not require lipid cofactors, resulted in a better recovery of the immunoprecipitated \[^{32}P\]-labeled C/EBP than with PKC. Phosphorylation of \[^{32}P\]-labeled holo-C/EBP, that had been immunoprecipitated, resulted in a better recovery of the immunoprecipitated protein, derived from 0.75 g of adult liver, that had been heat-treated with the exception of an additional major \[^{32}P\]-labeled peptide cleaved with CNBr and endoproteinase Lys-C, yielded a similar \[^{32}P\]-labeled peptide map as the truncated C/EBP proteins, with the exception of an additional \[^{32}P\]-labeled peptide at 12\(^{a}\) and a weak phosphorylation at Ser\(^{289}\) (60\(^{a}\)) (Fig. 9A). The immunoprecipitated \[^{32}P\]-labeled 30-kDa protein is putatively a fragment of C/EBP and the ratio of \[^{32}P\]-labeled 42-kDa C/EBP to 30-kDa material varied somewhat from preparation to preparation (39). Hence it appears that an additional PKC phosphorylation site is present in holo-C/EBP in addition to Ser\(^{289}\), Ser\(^{277}\), and Ser\(^{294}\) sites. Because of the relatively low abundance of PKC and C/EBP in liver, we have not yet successfully demonstrated \textit{in vivo} phosphorylation of C/EBP by PKC. In order to obtain specific immunoprecipitation of the \textit{in vitro} labeled C/EBP it was necessary to concentrate the heat-treated nuclear extract for phosphorylation by exogenously added PKM or PKC.

**DISCUSSION**

The DNA-binding domain of C/EBP can be phosphorylated by PKC in a manner dependent on Ca\(^{2+}\), lipid activators, and isoform type. Both type II and III PKC were observed to be more active than type I enzyme in the phosphorylation of the truncated C/EBP proteins. Since type II PKC is localized in rat liver nuclei (13), it is possible that this particular PKC isoform might phosphorylate C/EBP \textit{in vivo}. Three major PKC phosphorylation sites, Ser\(^{299}\), Ser\(^{277}\), and Ser\(^{294}\), were identified in C/EBP145. Intact C/EBP from liver nuclear extract was phosphorylated by PKCII in a Ca\(^{2+}\)- and lipid-dependent manner, and this protein was modified by M-kinase at Ser\(^{299}\) and Ser\(^{277}\), to a lower extent at Ser\(^{294}\), as well as at an additional unidentified site (Fig. 9). Although the former are sites of modification by PKC \textit{in vitro}, they are not typical PKC consensus sites. A consensus PKC phosphorylation site is immediately flanked by basic residues with at least an adjacent nonbasic residue between the Ser/Thr acceptor and the adjacent basic residues. However, despite being an imperfect match to the consensus PKC substrate sequences, these phosphorylation sites in C/EBP served as unusually good substrates.

A model for the interaction of the DNA binding domain of C/EBP with its specific recognition sequence has recently been proposed (20). This "scissors-grip" model hypothesizes that a dimenested set of \(\alpha\) helices (the leucine zipper) bifurcates in such a manner as to project positively charged \(\alpha\) helices in opposite directions into the major groove of DNA. Phosphorylation of Ser\(^{299}\) in C/EBP resulted in significant attenuation in DNA binding affinity. According to the scissors-grip model, Ser\(^{299}\) is positioned within the major groove of the DNA. One might therefore predict the observed effect. Since Ser\(^{299}\) is thought to come in close contact with the negatively charged DNA substrate, it is not surprising that modification...
of this residue by phosphorylation would interfere with DNA binding. The added negative charge resulting from phosphorylation might interfere with DNA binding by ionic charge repulsion. Alternatively phosphate modification might interfere with specific interactions made between Ser299 and the base pairs in the DNA recognition site. Since the scissors-grip model predicts that Ser299 will be buried within the major groove of DNA when C/EBP is bound to its DNA substrate, it might have been anticipated that DNA binding would inhibit phosphorylation of Ser299 by any protein kinase. Consistent with this interpretation, we have observed that specific C/EBP DNA substrates block the capacity of PKC to phosphorylate Ser299 of C/EBP.

Although Ser777 lies in a region of net positive charge (Fig. 1), mutagenesis studies replacing these positively charged residues with uncharged amino acids resulted in only a 2-4-fold decrease in DNA binding affinity (40). Similar site-directed mutagenesis experiments in which the basic residues in the BR-A and BR-B regions (Fig. 1) were mutagenized indicated that these residues are critical for specific interaction with DNA. The lack of an effect on DNA binding when only Ser299 is phosphorylated (Fig. 5, C/EBP Ser299(5)) is consistent with the notion that the basic region surrounding Ser777 is not of critical importance for DNA binding. We likewise infer that the detrimental effects of phosphorylation on Ser299 indicate that it and its surrounding amino acid residues come in close contact with DNA. The observation that protein kinase C exerts a negative effect on the function of C/EBP is consistent with our current understanding of the biology of both C/EBP and PKC. Umek et al. (41) have shown that functional expression of C/EBP in a rapidly dividing cell line results in cessation of proliferation, an experimental result consistent with the observation that C/EBP is found only in differentiated tissues. In addition, the expression of PKC in several tissues is more prominent after differentiation (42). The coexistence of PKC and C/EBP in the nucleus of differentiated tissues suggests that PKC may play a role in controlling C/EBP function in these tissues.

According to our observations activation of PKC should inhibit C/EBP. Phorbol esters tend to induce cell proliferation, whereas C/EBP inhibits cell proliferation. It is possible that PKC may reverse the growth-suppressive effects of C/EBP under conditions, for example, of liver regeneration. Under such conditions, Fox and Jun are induced (43). Although these two proteins are bZip proteins like C/EBP, perhaps they would not be negatively regulated by PKC because they have Cys at the position within the basic region corresponding to Ser299 in C/EBP. We conclude by emphasizing the fact that our observations have resulted from assays carried out exclusively in vitro. Until in vivo evidence is obtained, the relationship between PKC and C/EBP is entirely speculative. Despite this significant shortcoming, we do recognize that C/EBP is a very good substrate for PKC and that the effects of phosphorylation at Ser299 are consistent with the scissors-grip model. Thus, although the ultimate connection between PKC and C/EBP has yet to be made, useful information has resulted from these studies.

Based upon sequence alignment comparisons of 11 bZIP proteins, and conservation of a Ser at position 299 (Fig. 10), we predict that GCN4, v-Jun, CPC-1, HBP-1, TGA-1, and Opaque 2 are also PKC substrates and that c-Jun, Fox, YAP-1, and Cys-3 are not. CREB has been shown to be a PKC substrate (44), however, rat brain and human placental CREB lack Ser at positions corresponding to Ser299, Ser317, and Ser338 in C/EBP (45, 46). The amino acid sequences of the 11 transcription factors indicate limited conservation of Ser299, Ser317, and Ser338.

Fig. 10. Amino acid sequences of the Ser299, Ser277, and Ser777 region of C/EBP and several bZip transcription factors. Ser residues of the C/EBP proteins phosphorylated by PKC (*), the basic DNA binding regions (BR-A, BR-B), the leucine zipper residues (,), and conserved phosphorylation site Ser residues (stippling) are as indicated (Refs: 31, 47-49, 17, 50, 46, 51, 18, 16, 52, and 20, respectively).

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