

Protein kinase C regulates the phosphorylation and oligomerization of ERM binding phosphoprotein 50

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Abstract

Ezrin–Radixin–Moesin (ERM) binding phosphoprotein 50 (EBP50, a.k.a. NHERF-1) is a scaffold protein essential for the localization and coordinated activity of apical transporters, enzymes and receptors in epithelial cells. EBP50 acts via multiple protein binding interactions, including oligomerization through interactions of its PSD95–Dlg–ZO1 (PDZ) domains. EBP50 can be phosphorylated on multiple sites and phosphorylation of specific sites modulates the extent of oligomerization. The aim of the present study was to test the capacity of protein kinase C (PKC) to phosphorylate EBP50 and to regulate its oligomerization. In vitro experiments showed that the catalytic subunit of PKC directly phosphorylates EBP50. In HEK-293 cells transfected with rat EBP50 cDNA, a treatment with 12 myristate 13-acetate (PMA) induced a translocation of PKC α and β isoforms to the membrane and increased ³²P incorporation into EBP50. In co-transfection/co-precipitation studies, PMA treatment stimulated EBP50 oligomerization. Mass spectrometry analysis of full-length EBP50 and phosphorylation analyses of specific domains, and of mutated or truncated forms of EBP50, indicated that PKC-induced phosphorylation of EBP50 occurred on the Ser³³⁷/Ser³³⁸ residue within the carboxyl-tail domain of the protein. Truncation of Ser³³⁷/Ser³³⁸ also diminished PKC-induced oligomerization of EBP50. These results suggest the PKC signaling pathway can impact EBP50-dependent cellular functions by regulating EBP50 oligomerization.

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Introduction

Ezrin–Radixin–Moesin (ERM) binding phosphoprotein 50 (EBP50; a.k.a. NHERF-1) is a membrane-cytoskeleton linking protein localized in the apical region of epithelial cells [1–3]. Through protein–protein interactions in the apical region, EBP50 coordinates the activities of transporters, enzymes and receptors [3–7]. EBP50 is tethered to

the actin cytoskeleton via a binding interaction of its carboxyl end with ezrin [8], while its amino portion consists of two PSD95–Dlg–ZO1 (PDZ) domains. PDZ domains consist of conserved sequences of ~90 amino acids. They bind the COOH-tail of proteins that terminate with a PDZ ligand motif (–[T/S]–X–[Hydrophobic]) [9,10]. Proteins with PDZ domains typically serve to sequester and coordinate the activities of integrated proteins within membrane microdomains [11]. To diversify and amplify their binding interactions, PDZ proteins either contain multiple PDZ domains, contain additional protein binding motifs or oligomerize. Oligomerization of these proteins may be mediated by PDZ domain-independent [12] or PDZ domain-dependent mechanisms [13,14]. EBP50 oligomeri-

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zation is mediated by PDZ–PDZ interactions [14] and while being oligomerized, EBP50 retains the ability to concurrently bind integral membrane proteins to its PDZ domains [15,16].

EBP50 oligomerization is both positively and negatively regulated through site-specific phosphorylation [16–18]. Ser²⁸⁹ in rabbit EBP50 is a putative site of G protein-coupled receptor kinase 6a (GRK6a)-dependent phosphorylation [19]. A S289D mutation, which mimics constitutive phosphorylation at this site, enhances EBP50 oligomerization [16]. Rabbit EBP50 may also be phosphorylated on Ser²⁷⁷ and Ser³⁰¹ by the cyclin-dependent kinase cdc2 during mitosis [18] and, in contrast with phosphorylation at Ser²⁸⁹, phosphorylation at these sites inhibits EBP50 oligomerization. The aim of the present study was to investigate the capacity of protein kinase C (PKC) to phosphorylate EBP50 and to regulate EBP50 oligomerization. Several lines of evidence suggest that PKC might contribute to the regulation of EBP50 functions. During activation, PKC isoforms translocate from the cytosol to the plasma membrane, a site where EBP50 resides to interpose between membrane proteins and the underlying cytoskeleton. Protein–protein interactions of other PDZ domain-containing proteins are regulated by PKC phosphorylation. For example, PKC isoforms associate with EBP50 via the protein receptor, *receptor for activated C kinase 1* (RACK1) and PKC activity regulates the net activity of NHE3 and CFTR, two EBP50-bound proteins [20–24]. Furthermore, it was shown in a recent study that PKC phosphorylates the PDZ2 domain of human EBP50 with a consequent modulation of associated CFTR activity [25]. The results in the present study demonstrate that PKC directly phosphorylates EBP50 on a previously uncharacterized phosphorylation site, Ser³³⁷/Ser³³⁸ within the C-tail domain, and that PKC-mediated phosphorylation of EBP50 enhances EBP50 oligomerization.

Methods

Preparation of EBP50 mutants

EBP50 point mutants were generated by overlap polymerase chain reaction from wild type Flag-EBP50 [14] using mismatched complementary primers to incorporate the appropriate mutation and wild-type primers encompassing the start codon and the termination codon (Table 1). Truncated EBP50, Δ 336 and Δ 346 were generated from wild type Flag-EBP50 using specific primers (Table 1). Products were digested with *NotI*, an internal restriction site within EBP50, and *BamHI*, and subsequently ligated into the p3XFlag-CMV7 (Sigma, St Quentin Fallavier, France) vector digested with the same enzymes. *HindIII* and *BamHI* sites were positioned before and after the start and stop codons, respectively. All constructs were confirmed by automated sequencing (UCHSC Cancer Center DNA sequencing core, UCHSC). The four Flag-tagged constructs of distinct EBP50 domains and HA-EBP50 vector have been described previously [14].

Cell culture and transfections

HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml). Grown to ~80% confluence, HEK-293 cells were transfected with Lipofectamine transfection reagent (Invitrogen SARL; Cergy Pontoise, France) according to the manufacturer's instructions. Transfection constructs included 2 μ g of the following cDNAs; (1) Flag-EBP50 with or without HA-EBP50; (2) Flag-EBP50 and HA vector; (3) HA-EBP50 and Flag vector; (4) HA vector and Flag vector [14].

Table 1
Nucleotide sequence of EBP50 primers

A. EBP50 mutagenesis		
EBP50A277	Sense	GCCAGCTTCAGAAG CCCCC AGGCCAGC
	Antisense	GCTGGCCTGGGG G CTTCTGAAGCTGGC
EBP50A287	Sense	GGCAAGATCTGCC CC AGCGATAACAGTG
	Antisense	CACTGGTATCGCTGG CGG CAGATCTTGCC
EBP50A288	Sense	GCAAGATCTGCCTCC GGC GATAACAGTGAGG
	Antisense	CCTCACTGGTATCG GG CGGAGGCAGATCTTGC
EBP50A287/A288	Sense	GCAAGATCTGCC CCCGCC GATAACAGTGAGG
	Antisense	CCTCACTGGTATCG GGCGG CAGATCTTGC
EBP50A299	Sense	GCTAAATGCCCAAGATG CT CCCAAGAGACACG
	Antisense	CGTGTCTCTTGGG AG CATCTTGGGCATTTAGC
HINDIII EBP50	Sense	GGTAAGCTTAAGATGAGCGCGGACGCAGCG
BAMHI EBP50	Antisense	GGTGGATCTGCTCAGAGGTTGCTGAAGAG
B. EBP50 truncations		
EBP50 Δ 336	Sense	GGTAAGCTTAAGATGAGCGCGGACGCAGCG
	Antisense	TTATTAGGATCCCTAGCGCTTCTGGTGGGCCCT
EBP50 Δ 346	Sense	GGTAAGCTTAAGATGAGCGCGGACGCAGCG
	Antisense	TTATTAGGATCCCTACCAGTCCATCTGCGGGGG

Bold letters indicate mutant codons.

In studies requiring PKC activation, HEK-293 cells were serum-deprived for 20 h and then treated with 1 μ M phorbol-12-myristate-13-acetate (PMA; Calbiochem; VWR International SA, France) or vehicle for 20 min.

Immunoprecipitation of HA-EBP50 or Flag-EBP50

For immunoprecipitation of HA-EBP50 or Flag-EBP50, transfected HEK-293 cells were washed twice with ice-cold PBS and then scrapped and collected in 500 μ l of ice-cold Lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail tablet (Complete; Amersham; Saclay, France) and a phosphatase inhibitor cocktail set II tablet (Calbiochem; VWR International SA, France). The lysate was rocked (30 min to 2 h, 4°C) and the insoluble material was removed by centrifugation (20,000 \times g, 30 min, 4°C). The protein concentration was determined by BCA protein assay (Pierce; Brebières, France) and 500 μ g of total protein in a final volume of 500 μ l was incubated with 50 μ l of anti-HA (Roche; Meylan, France) or anti-Flag (Sigma; St. Quentin Fallavier, France) affinity matrix overnight at 4°C. The matrix was washed four times with ice-cold PBS buffer and the bound proteins eluted with 100 μ l of Laemmli sample buffer (Bio-Rad; Marne La Coquette, France) or 5 \times PAGE buffer (5% SDS, 25% sucrose, 50 mM Tris; 5 mM EGTA; pH 8.0).

Gel electrophoresis, protein staining and Western blotting

Proteins were denatured and reduced in 5 \times PAGE buffer (5% sodium dodecyl sulfate, 25% sucrose, 50 mM Tris, 5 mM EDTA, 0.2 M dithiothreitol; pH 8.0; 95°C for 10 min) and then separated on 12% or 4–14% gradient separating gels. Gels were either silver stained or stained with Coomassie for total protein detection or transferred onto nitrocellulose membranes for Western blot analysis. For silver staining, gels were fixed in 50% methanol/5% acetic acid, washed in 50% methanol and then in water, sensitized in 0.02% sodium thiosulfate and incubated in 0.1% silver nitrate (20 min; 4°C). After a final wash in water, development was achieved by incubation in 2% sodium carbonate with 0.04% paraformaldehyde and arrested in 5% acetic acid [26]. For Coomassie blue staining, gels were incubated in Coomassie stain (0.1% Coomassie Blue, 50% MeOH, 10% acetic acid) overnight and subsequently destained (50% MeOH, 10% acetic acid). For Western blotting, proteins were transferred onto nitrocellulose. EBP50 proteins were detected by incubation with antibodies against Flag (M2-anti-Flag; Sigma; St. Quentin Fallavier, France) or HA (12CA5-anti-HA; Roche; Meylan, France) followed by incubation with peroxidase-conjugated secondary antibodies (1:60,000; Jackson ImmunoResearch; West Grove, PA) and detection by enhanced chemiluminescence (Pierce; Brebières, France).

EBP50 phosphorylation assays

EBP50 phosphorylation was assayed by the relative incorporation of 32 P into EBP50 in vitro and in intact HEK-293 cells. For in vitro phosphorylation assays, Flag-EBP50 was immunoprecipitated from HEK-293 transfected cells, bead-bound Flag-EBP50 was washed in MES Buffer (50 mM MES, 1.2 mM EGTA, 12 mM MgCl₂; pH 6.0) and then incubated (12 min; 30°C) in MES buffer containing 0.1 mM ATP and 0.1 μ Ci/ μ l 32 P-ATP with or without PKC catalytic subunits from rat brain (35 mU PKC activity, Calbiochem; La Jolla, CA). The beads were washed three times in PBS and Flag-EBP50 proteins were solubilized by the addition of 5 \times PAGE. For metabolic labeling assays, HEK-293 cells were transfected with Flag-EBP50 cDNA, incubated for 48 h, washed in phosphate-free DMEM and incubated for 10 min in phosphate-free DMEM. Cells were then incubated for 20 min in phosphate-free DMEM containing 100 μ Ci 32 Pi (Amersham; Piscataway, NJ), washed three times with PBS and subjected to Flag-EBP50 immunoprecipitation. In both assays, Flag-EBP50 proteins were subjected to EBP50 immunoblotting or protein staining and to 32 P detection by autoradiography.

PKC activation assay

The activation of PKC isoforms in HEK-293 cells was assessed by measuring the translocation of PKC α and PKC β from cytosolic to membrane fractions. HEK-293 cells were plated at 2 \times 10⁶ cells/dish, serum-deprived for 20 h and treated with 1 μ M PMA or vehicle for 20 min at 37°C. Cells were washed twice in ice-cold PBS, scrapped and collected in 500 μ l of ice-cold Tris–sucrose buffer (10 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 0.2 mM CaCl₂, protease inhibitor (Complete tablets; Amersham; Saclay, France) and phosphatase inhibitors (Phosphatase inhibitor cocktail set II; Calbiochem; VWR International SA, France). Cell lysates were incubated (10 min at 4°C), sonicated, and EDTA was added to a final concentration of 1 mM. Nuclei and unbroken cells were removed by centrifugation (1000 \times g; 10 min; 4°C). The resulting supernatant was centrifuged (100,000 \times g; 60 min; 4°C). The supernatant from this spin (cytosol fraction) was removed and saved. The pellet (crude membranes) was resuspended in 500 μ l of ice-cold Tris–sucrose–Triton buffer (20 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM 2-mercaptoethanol, protease and phosphatase inhibitors cocktail), sonicated and centrifuged (100,000 \times g; 60 min; 4°C). Insoluble material was discarded and the supernatant was collected as the Triton soluble membrane fraction. Protein concentration was determined by the Bradford method (Bio-Rad; Marne La Coquette, France). Immunoblotting was performed using monoclonal antibodies raised against PKC α and PKC β isoforms (Transduction Laboratories; Le Pont de Claix, France).

Mass spectrometry analysis of EBP50 phosphorylation sites

To identify sites of phosphorylation on EBP50, Flag-EBP50 was expressed in HEK-293 cells, immunoprecipitated and separated by gel electrophoresis. The Flag-EBP50 band was visualized by silver staining, excised, minced and washed [27]. The samples were divided into three equal portions and subjected separately to in-gel digestion by the addition of 0.2 μg trypsin (Roche; Indianapolis, IN), 0.2 μg subtilisin (Boehringer Mannheim; Ridgefield, CT) or 0.2 μg elastase (Boehringer-Mannheim), followed by overnight incubation at 37°C [28]. The proteolyzed suspensions were combined, lyophilized and resuspended in 5% formic acid. This suspension was separated by sequential cation exchange and reverse phase chromatography and subjected to electron spray ionization tandem mass spectrometry (Thermo-Finnigan LCQ Deca Mass Spectrometry) [29]. The resultant spectra were identified using SEQUEST analysis program and sites of phosphorylation were determined with DTASelect analysis program [28].

EBP50 oligomerization assay

Oligomerization of EBP50 was evaluated in HA-EBP50/Flag-EBP50 co-transfected HEK-293 cells by measuring the amount of Flag-EBP50 that co-precipitated with HA-EBP50 as previously described [14]. Forty eight hours following co-transfection, the co-transfected cells were PMA-treated as described above, HA-EBP50 was immunoprecipitated and the relative abundance of co-precipitated Flag-EBP50 was measured by Flag Western blotting. Western blots of HA-EBP50 and Flag-EBP50 in initial lysates and HA-EBP50 in the precipitated samples were run as controls.

Statistical analysis

A paired *t* test for two groups or an analysis of variance for groups of three was performed on densitometric studies of phosphorylation and co-precipitated Flag-EBP50. Statistical significance was determined at $P < 0.05$ by the Students–Newman–Keuls test.

Results

In vitro phosphorylation of EBP50 by PKC catalytic subunit

The capacity of PKC to directly phosphorylate EBP50 was assessed by incubating the catalytic subunit of PKC with isolated Flag-EBP50 in the presence of ^{32}P -ATP (Fig. 1). In the absence of the PKC catalytic subunit, no phosphorylation of EBP50 was observed. The addition of the PKC catalytic subunit resulted in marked EBP50 phosphorylation. Subsequent incubation in alkaline phosphatase reduced the levels of incorporated ^{32}P , confirming that the ^{32}P signal arose from phosphorylation of the protein. These in vitro studies

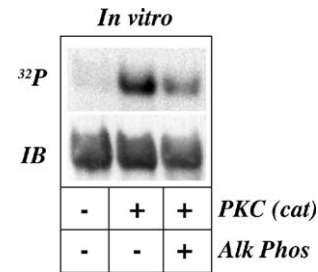


Fig. 1. Phosphorylation of EBP50 by the PKC catalytic subunit. Flag-EBP50 was immunoprecipitated from HEK-293 transfected cells and incubated in vitro with or without the catalytic subunit of PKC (cat), in the presence of ^{32}P -ATP. The amounts of immunoprecipitated Flag-EBP50 are shown on the immunoblot (IB). The autoradiogram (^{32}P) shows that ^{32}P incorporation was markedly increased by incubation of EBP50 with the PKC catalytic subunit and that incorporated ^{32}P was sensitive to subsequent alkaline phosphatase treatment (Alk Phos). Results are representative of three separate experiments.

demonstrated that the catalytic subunit of PKC is able to recognize and phosphorylate EBP50.

Intracellular phosphorylation of EBP50 by the PKC pathway

To determine if activation of the PKC pathway stimulates EBP50 phosphorylation within intact cells, Flag-tagged EBP50 was transfected into HEK-293 cells and the degree of phosphorylation was evaluated in basal serum-free conditions, and in response to PKC activation. PKC α , - β and - δ , which were previously reported as being the major endogenous PKC isoforms in HEK-293 cells [30], were readily detected in HEK-293 cell lysates (data not shown). The phorbol ester PMA was used to induce PKC activation in these cells. After 20 h in serum-free conditions, essentially all of PKC α and PKC β were distributed in the cytosolic fraction (Fig. 2A). After 20 min of PMA treatment, approximately half of both PKC α and PKC β had translocated to the membrane fraction (Fig. 2A). PKC δ showed a mixed distribution under basal conditions but also showed a translocation to the membrane fraction in response to PMA (data not shown).

Flag-EBP50 transfected cells were treated with vehicle or PMA for 20 min in $^{32}\text{P}_i$ containing media and assayed for incorporation of ^{32}P into EBP50 (Fig. 2B). In intact cells, phosphorylation of EBP50 was consistently detected under basal conditions and PMA treatment caused a further increase in EBP50 phosphorylation (2.2 ± 0.2 times control; $n = 7$), indicating that EBP50 is an intracellular substrate of the PKC signaling pathway.

EBP50 oligomerization in response to PKC activation

Previous studies have described disparate effects of phosphorylation on EBP50 oligomerization [16–18]. These opposing effects are likely due to differences in the site(s) of phosphorylation by distinct kinases. The effect of PKC

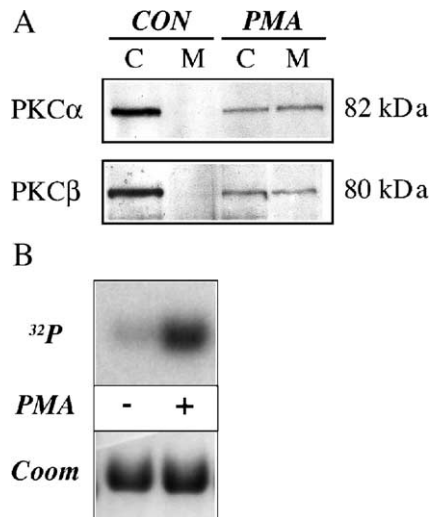


Fig. 2. PKC-dependent intracellular phosphorylation of EBP50. (A) PKC activation was induced by PMA treatment, as ascertained by the translocation of PKC isoforms to the cell membrane, in HEK-293 cells. PKC α and PKC β immunoblots were performed on cytosolic (C) and membrane (M) fractions of HEK-293 cells. Under serum-free conditions (CON), little PKC α or PKC β was detected in the membrane fraction. In cells treated for 20 min with 1 μ M PMA, approximately half of PKC α and PKC β had translocated from the cytosol to the membrane fraction. (B) EBP50 phosphorylation was increased in response to PKC activation. HEK-293 cells transfected with Flag-EBP50, were incubated without or with 1 μ M PMA for 20 min in the presence of 32 Pi. Flag-EBP50 was immunoprecipitated and assayed for 32 P incorporation. The amounts of immunoprecipitated Flag-EBP50 are shown by Coomassie staining (Coom). The autoradiogram (32 P) showed that EBP50 phosphorylation was markedly increased in PMA-treated cells (2.2 ± 0.2 times control; $n = 7$). Results are representative of three separate experiments.

phosphorylation on EBP50 oligomerization was evaluated in HA-EBP50/Flag-EBP50 co-transfection/co-precipitation studies. PMA treatment had no discernible effect on the total amounts of HA-EBP50 and Flag-EBP50 and did not modify the amount of HA-EBP50 that was immunoprecipitated from co-transfected HEK-293 cells. PMA treatment did, however, induce a significant increase in the amount of Flag-EBP50 (3.7 ± 0.8 times control; $n = 9$; $P < 0.05$) that co-precipitated with HA-EBP50 (Fig. 3). This indicates that PKC-dependent phosphorylation of EBP50 enhances oligomerization of the protein.

Analysis of EBP50 domain(s) phosphorylated by the PKC pathway

The specific site(s) that are phosphorylated by the PKC pathway were sought. Initially, the sites of EBP50 phosphorylation that existed under broad activation of intracellular signaling pathways were determined in Flag-EBP50 from transfected cells maintained in presence of serum. Sites of EBP50 phosphorylation were then identified by mass spectrometry and subsequent phospho-analysis of the resultant spectra (Fig. 4). This methodology does not provide quantitative information regarding the percentage of the total protein that is phosphorylated on any particular

site and may not detect every site that is phosphorylated on a protein. The methodology does directly identify specific sites that are phosphorylated within the protein population tested. In the present study, repeated analysis allowed 88% of the entire EBP50 protein sequence to be evaluated at least one time for phosphorylation. Not previously described, two residues in the PDZ1 domain (Thr⁷¹ and Ser⁷⁷) were identified as being phosphorylated on some of the peptides analyzed. This is of particular interest given that the PDZ1 domain is the region where many of the high affinity binding interactions of EBP50 occur [31]. Furthermore, four distinct amino acid residues in the C-tail domain were detected in a phosphorylated state (Fig. 4). These residues include Ser²⁷⁷, Ser²⁸⁷, Ser²⁸⁸ and Ser²⁹⁹. Three of these sites, Ser²⁷⁷, Ser²⁸⁷ and Ser²⁹⁹, correlate with residues in rabbit EBP50 that have been shown in mutational studies to influence EBP50 oligomerization through phosphorylation [16–18]. Interestingly, in all peptide strings assessed, Ser²⁸⁷ and Ser²⁸⁸ were invariably either both phosphorylated or both dephosphorylated.

Efforts were then made to determine specific site(s) of PKC-dependent phosphorylation in EBP50. Flag-tagged constructs of EBP50 domains (PDZ1, PDZ2, PDZ1/2, C-Tail) were generated (Fig. 5A), expressed in HEK-293 cells and evaluated for basal and PKC-dependent phosphorylation (Fig. 5B). PDZ1, PDZ2 and PDZ1, 2 domains failed to show detectable levels of phosphorylation upon either basal conditions or PKC activation. In contrast, the C-tail domain was phosphorylated under basal conditions and the level of phosphorylation was increased by PMA treatment (Fig. 5B).

To evaluate if any of the four amino acid residues in the C-tail domain that were detected in a phosphorylated state by mass spectrometry analysis contributed to either the basal or PKC-stimulated phosphorylation, these serine residues were individually mutated to alanine, a non-phosphorylatable amino acid. In addition, because residues Ser²⁸⁷ and Ser²⁸⁸ were consistently found as being concurrently phosphorylated in mass spectrometry phospho-analyses, a double S287A/S288A mutant was also generated. Whereas

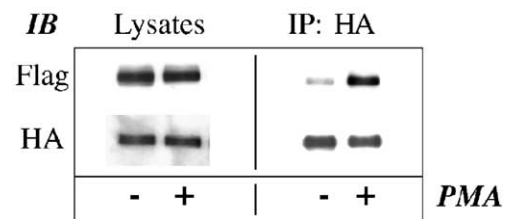


Fig. 3. PKC-dependent regulation of EBP50 oligomerization. HEK-293 cells were co-transfected with HA-EBP50 and Flag-EBP50 and incubated without (–) or with (+) 1 μ M PMA for 20 min. Total lysates from (–) and (+) cells contained similar amounts of HA- and Flag-EBP50 proteins. PMA treatment had no effect on the amount of HA-EBP50 that was HA-immunoprecipitated from HEK cell lysates but caused a significant increase in the amount (3.7 ± 0.8 times control; $n = 9$) of Flag-EBP50 that co-precipitated (i.e. oligomerized) with HA-EBP50. Results are representative of three separate experiments.

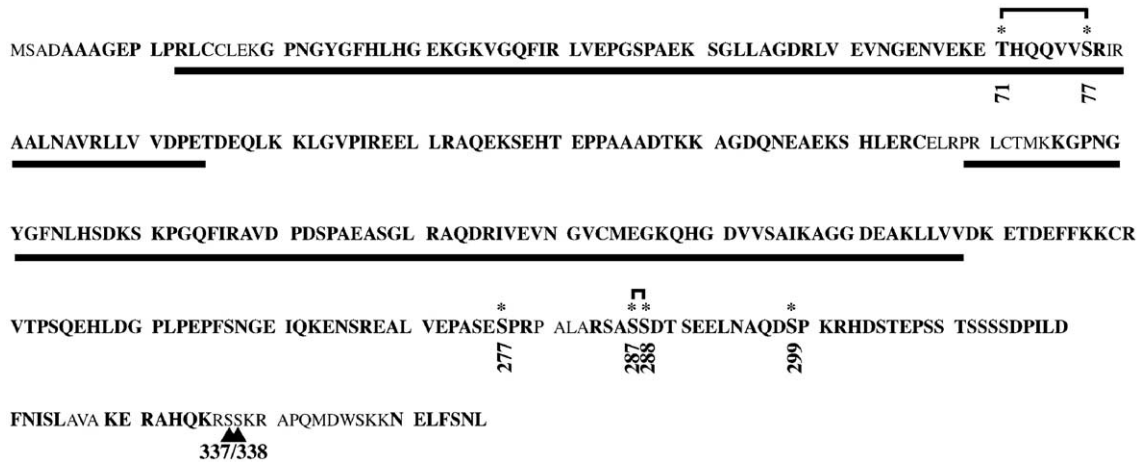


Fig. 4. Screening of EBP50 phosphorylation sites by mass spectrometry. HEK-293 cells were transfected with Flag-EBP50 and incubated in the presence of serum for 48 h. Flag-EBP50 was immunoprecipitated and subjected to mass spectrometry analysis to identify potential sites of phosphorylation. The PDZ1 and PDZ2 domains of EBP50 are highlighted by heavy underlines. Analysis covered 88.5% of the EBP50 sequence (bold letters). Comparative mass analysis positively identified six sites of phosphorylation (with asterisks). Two sites reside within the PDZ1 domain and four sites in the C-terminal domain. In all spectra, the amino acid pairs Thr⁷¹/Ser⁷⁷ and Ser²⁸⁷/Ser²⁸⁸ were always concurrently either phosphorylated or dephosphorylated (looped asterisks). Two sites not covered in the mass spectrometry analysis, Ser³³⁷ and Ser³³⁸ (arrowheads), were later found to be the primary sites for PKC-dependent phosphorylation.

the S277A, S288A and S299A mutations did not affect the level of phosphorylation under basal conditions (Fig. 6A), the S287A mutation, either alone or in combination with S288A (as S287A/S288A) showed no detectable phosphorylation under basal conditions (Fig. 6A). Consistent with previous observations [19], this confirms that Ser²⁸⁷ accounts for most of the phosphorylation of EBP50 under basal conditions. However, each of these alanine mutants

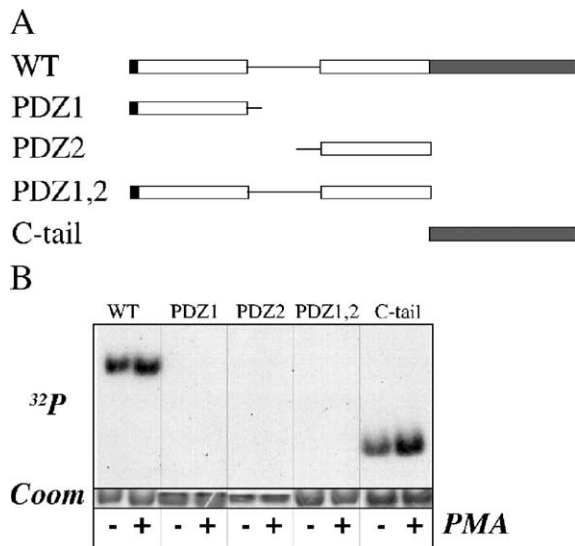


Fig. 5. Analysis of basal and PKC-induced phosphorylation of EBP50 domains. (A) Four Flag-tagged constructs of distinct EBP50 domains (PDZ1, PDZ2, PDZ1/2 and C-tail) were generated. (B) HEK-293 cells were transfected with the constructs shown in panel A and were incubated with or without 1 μM PMA for 20 min in the presence of ³²Pi. Flag-EBP50 was immunoprecipitated and assayed for ³²P-incorporation. The amounts of immunoprecipitated recombinant proteins are visualized by Coomassie staining (Coom). The autoradiogram (³²P) shows that both basal and PKC-induced phosphorylation of EBP50 occur on the C-tail domain. Results are representative of three separate experiments.

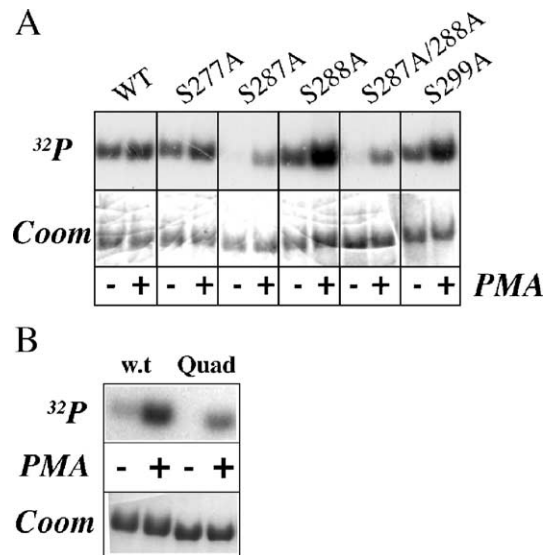


Fig. 6. Analysis of potential C-tail sites of basal and PKC-induced phosphorylation. (A) EBP50 proteins with serine to alanine mutations at Ser²⁷⁷, Ser²⁸⁷, Ser²⁸⁸ and Ser²⁹⁹ were evaluated for their degree of phosphorylation under basal and PMA-treated conditions. A double S287A/S288A mutant was also generated and tested. HEK-293 cells were transfected with Flag-tagged EBP50 mutants and were incubated with or without 1 μM PMA for 20 min in the presence of ³²Pi. The amounts of immunoprecipitated recombinant proteins are visualized by Coomassie staining (Coom). The autoradiogram (³²P) showed little or no basal phosphorylation in the S287A and S287A/S288A mutants. PMA induced an increase in phosphorylation in all mutants, indicating that the primary site of PKC phosphorylation was not one of these individual residues. Results are representative of three separate experiments. (B) To determine if the detected PMA-induced phosphorylation resulted from the cumulative effect of phosphorylating all four known C-tail phosphorylation sites, all four residues were mutated to alanine within the same construct (Quad). Despite the multiple mutations, PMA continued to induce phosphorylation of the EBP50 protein.

demonstrated increased phosphorylation over basal levels following PMA treatment (Fig. 6A), indicating that none of these sites, at least on its own, accounted for the PKC-dependent phosphorylation of EBP50.

To determine if two or more of these four sites could account for the PKC-dependent phosphorylation of EBP50, all four serine residues were mutated to alanine (Quad mutant) and PMA-dependent phosphorylation was evaluated. As anticipated, basal phosphorylation of the Quad mutant was almost completely suppressed. In paired experiments, densitometric analysis showed that the Quad mutant was significantly less phosphorylated ($9 \pm 3\%$ of w.t.) than wild type EBP50 under basal conditions. PMA-dependent phosphorylation of the Quad mutant still occurred (Fig. 6B). When corrected for their different basal levels of phosphorylation, there was no difference in the PMA-induced phosphorylation between w.t. EBP50 (2.2 ± 0.2 times basal level; $n = 4$) and Quad mutant EBP50 (equivalent to 2.1 ± 0.3 times w.t. basal level).

These results indicated that the PMA-dependent phosphorylation did not occur on the four sites within the C-tail domain that were identified by mass spectrometry and likely occurred on previously uncharacterized residues within the C-tail domain of EBP50. Three serine residues in the terminus of the C-tail, Ser³³⁷, Ser³³⁸ and Ser³⁴⁷, were embedded within consensus sequences for PKC phosphorylation. Truncation mutants were generated to remove the Ser³⁴⁷ residue ($\Delta 346$) or the Ser³³⁷/Ser³³⁸ and Ser³⁴⁶ residues ($\Delta 336$) and evaluated for PKC-dependent phosphorylation. PMA treatment of cells expressing the $\Delta 336$ mutant had markedly blunted phosphorylation in response to PMA treatment (Fig. 7). Paired studies showed that PMA treatment increased phosphorylation of wild type EBP50 (2.1 ± 0.4 times w.t. basal levels; $n = 5$) but not $\Delta 336$ EBP50. Phosphorylation levels of $\Delta 336$ EBP50 were unchanged under basal conditions (0.8 ± 0.1 times w.t. basal levels; $n = 5$) or following PMA treatment (1.0 ± 0.1 times w.t. basal levels; $n = 5$). PMA treatment of cells

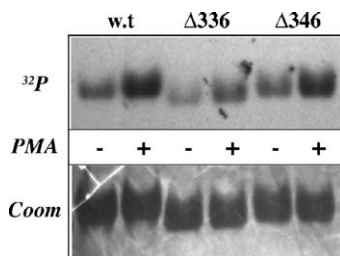


Fig. 7. Analysis of terminal C-tail sites of PKC-induced phosphorylation. Sequence analysis suggested Ser³³⁷/Ser³³⁸ and Ser³⁴⁷ were potential PKC phosphorylation sites. To determine if this was the case, EBP50 was truncated at Ser³³⁶ and Ser³⁴⁶ and PMA-induced phosphorylation of each truncation mutant was assessed. Compared with wild type EBP50 (w.t.), truncated EBP50 (Ser³³⁶) showed a markedly reduced phosphorylation response to PMA (³²P). In contrast, phosphorylation of truncated EBP50 (Ser³⁴⁶) following PMA treatment was retained. Coomassie staining (Coom) showed relatively equal protein loading.

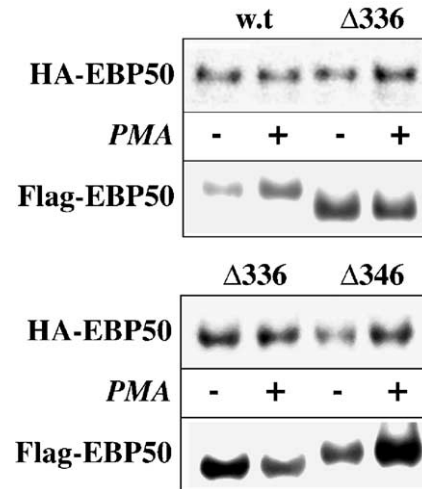


Fig. 8. Analysis of PMA-induced oligomerization of truncated EBP50 ($\Delta 336$). The effect of the loss of Ser³³⁷/Ser³³⁸, the primary site of PKC phosphorylation, on EBP50 oligomerization was evaluated. (Upper panel) PMA-induced oligomerization of the EBP50 ($\Delta 336$) mutant was initially compared to the wild type response. HA-immunoprecipitates showed similar levels of precipitated HA-EBP50 in all fractions and, as shown above, greater levels of wild type Flag-EBP50 co-precipitated with HA-EBP50 in cells treated with PMA. In contrast, PMA treatment failed to induce an increase in co-precipitation of Flag-EBP50 $\Delta 336$. (Lower panel) When PMA-treatment effect on oligomerization was compared between $\Delta 336$ and the $\Delta 346$ mutants, PMA again failed to increase the levels of co-precipitated Flag-EBP50 ($\Delta 336$) but PMA-induced oligomerization was retained in the Flag-EBP50 ($\Delta 346$) mutant.

expressing the $\Delta 346$ mutant, which contain the Ser³³⁷/Ser³³⁸ residues, showed increased degree of phosphorylation on the protein to levels observed in wild type protein. These studies suggest that the Ser³³⁷/Ser³³⁸ residues account to a major extent for PMA-induced phosphorylation of EBP50.

The ability of PMA treatment to increase EBP50 oligomerization was evaluated in the $\Delta 336$ mutant. In paired studies, oligomerization of the $\Delta 336$ mutant was significantly reduced as compared to wild type controls (Fig. 8; upper panel). While oligomerization in PMA treated cells increased 2.1 ± 0.2 times basal levels in cells expressing wild type Flag-EBP50 ($n = 4$), oligomerization was not increased in PMA treated cells expressing the Flag-EBP50- $\Delta 336$ mutant (0.7 ± 0.1 times basal levels in $\Delta 336$ cells, $n = 4$; Fig. 8). This effect was appeared specific to the Ser³³⁷/Ser³³⁸ residues since PMA treatment of cells expressing EBP50 that was truncated beyond these residues ($\Delta 346$) increased its oligomerization (1.5 ± 0.4 times untreated $\Delta 346$ cells; $n = 4$) in response to PMA treatment (Fig. 8; lower panel).

Discussion

EBP50 (a.k.a. NHERF-1) was first identified as a phosphorylated factor required for cAMP-inhibition of NHE3 activity in epithelial cells of the proximal tubule

[1,2]. It was later determined that the regulation of NHE3 by EBP50 did not require EBP50 phosphorylation [32]. Subsequently, EBP50 was shown to oligomerize through PDZ–PDZ interactions [14] and the phosphorylation status of EBP50 was found to moderate the oligomerization process [16,18,19]. Furthermore, there is both a positive and negative effect of phosphorylation on oligomerization depending upon the site of phosphorylation. Mutation studies showed that EBP50 phosphorylation on Ser²⁸⁹ (rabbit EBP50) by GRK6A increased oligomerization [16,19] while phosphorylation of Ser²⁷⁹ and Ser³⁰¹ by cdc2 kinase inhibited oligomerization [18]. Rather than within the PDZ domains, the site of protein–protein interaction, these three sites each reside in the C-tail domain of EBP50 and correlate with Ser²⁷⁷, Ser²⁸⁷ and Ser²⁹⁹ in rat EBP50. Results in the present study provide evidence that PKC can directly phosphorylate EBP50 (Fig. 1), that intracellular activation of PKC (Fig. 2A) induces EBP50 phosphorylation (Fig. 2B) and that PKC-mediated phosphorylation enhances EBP50 oligomerization (Fig. 3). The site of PKC-mediated phosphorylation, however, occurs on residues distinct from those previously described.

Mass spectrometry analysis of rat EBP50 isolated from HEK-293 cells maintained in serum identified six distinct sites of phosphorylation (Fig. 4). Two novel sites of phosphorylation were identified within the PDZ1 domain. While these sites were not responsible for an appreciable amount of either basal or PKC-induced phosphorylation, these sites are of significant interest since the PDZ1 domain is not only involved in oligomerization but interacts with a number of EBP50 binding partners [33]. The signaling pathway that caused their phosphorylation in serum-containing conditions remains to be identified. Four other sites, each residing within the C-tail domain, were also shown to be phosphorylated. These include homologous residues to the sites phosphorylated on rabbit EBP50 by either GRK6A (Ser²⁸⁷) or cdc2 kinase (Ser²⁷⁷ and Ser²⁹⁹). The fourth site, Ser²⁸⁸, was coordinately either phosphorylated or unphosphorylated with Ser²⁸⁷. Consistent with previous findings [19], Ser²⁸⁷ was identified by site direct mutagenesis studies as a major site of basal phosphorylation (Fig. 6A). While sequence analysis predicted that two of the sites identified by spectrometry analysis, Ser²⁷⁷ and Ser²⁹⁹, are potential PKC phosphorylation sites, their mutation did not individually preclude PKC-mediated phosphorylation. Furthermore, mutation of Ser²⁸⁷, Ser²⁸⁸ or a combination of all four of the identified phosphorylation sites within the C-tail domain also failed to ablate PMA-induced phosphorylation of EBP50 (Figs. 6A,B). This suggested that the PKC-dependent phosphorylation occurred on a novel phosphorylation site within the C-tail domain. While mass spectrometry analysis covered 88% of the EBP50 protein, several other potential phosphorylation sites within the C-tail domain remained unevaluated. This included three serine residues (Ser³³⁷, Ser³³⁸, Ser³⁴⁷) that were embedded within potential PKC consensus sites. Truncation of the Ser³³⁷/

Ser³³⁸ residues markedly reduced PMA-induced phosphorylation (Fig. 7) and abolished oligomerization (Fig. 8) of EBP50.

Several characteristics of PKC are consistent with its role in regulating EBP50 functions. First, specific PKC isoforms regulate molecular events that occur at the cytoplasmic surface of the plasma membrane, the site where EBP50 functions to cluster and sequester interactive proteins. Among these specific isoforms, PKC α , - β and - δ are the most abundantly expressed isoforms in HEK-293 cells [30] and each translocated to the membrane following PMA activation. Second, PKC isoforms interact with EBP50 both directly and indirectly.

PKC α and PKC β are both capable of interacting with the Receptor for Activated C Kinase-1 (RACK1), which in turn binds EBP50 [20–22]. PKC α has a PDZ binding motif in its C-terminus and can interact directly with EBP50 [34]. In addition, PKC α binds to the PDZ domain protein PICK1 [35,36]. Though not yet demonstrated, PICK1 could interact with EBP50 through heterologous PDZ–PDZ interactions. These interactions would position the PKC isoforms to phosphorylate and regulate proteins within the EBP50 complex including EBP50 itself. One well-documented interaction is that occurring between EBP50 and the cAMP-regulated Cl⁻ channel CFTR. The EBP50–CFTR interaction moderates the distribution and activity of CFTR in the apical membrane of numerous epithelial cell types. Inhibition of PKC activity inhibits cAMP-mediated CFTR function suggesting that constitutive PKC activity modifies PKA activation of CFTR [24]. Furthermore, potentiation of cAMP responses has also been observed in phorbol-stimulated epithelial cells expressing CFTR [37–39]. In contrast, recent co-transfection studies in Chinese hamster ovary cells have shown that PKC phosphorylated the Ser¹⁶² residue of the human EBP50 PDZ2 domain and inhibited the stimulatory effect of EBP50 on CFTR by disrupting EBP50–CFTR interaction [25]. The effects attributed to the Ser¹⁶² residue in human EBP50 (accession # O14745) are likely not universal since this residue is not conserved across species and is an asparagine residue in rabbit (accession # Q28619), rat (accession # Q9JJ19) and mouse (accession # P70441). The use of rat EBP50 in the above studies, with an unphosphorylatable residue at Ser¹⁶², may account for the discrepancy in sites of PKC phosphorylation between the two studies. Reflecting the complexity of EBP50 phosphoregulation, the present study also identified novel sites of phosphorylation in the PDZ1 (Ser⁷¹/Thr⁷⁷) and C-tail (Ser³³⁷/Ser³³⁸) domains of EBP50. Results also demonstrate that activated PKC phosphorylates EBP50 at Ser³³⁷/Ser³³⁸ (Fig. 7), sites that are conserved between human, rat, mouse and rabbit forms of EBP50, to promote its oligomerization (Fig. 8). Importantly, PKC-mediated EBP50 oligomerization is positioned to play a pivotal role in influencing the activity of its various binding partners.

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