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Phosphorylation of murine homeodomain protein Dlx3 by protein kinase C

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Abstract The Dlx3 homeodomain gene is expressed in terminally differentiated murine epidermal cells. As demonstrated for differentiation-specific granular markers, Dlx3 is activated in primary mouse keratinocytes cultured in vitro by increasing the level of the extracellular Ca^{2+}. This activation is mediated through a protein kinase C-dependent (PKC) pathway. In this study, we investigated whether PKC can modulate the activity of murine Dlx3 protein. Using in vitro kinase assays, we show that PKC enzymes phosphorylate the Dlx3 protein. Using keratinocyte nuclear extracts for the kinase reaction, we determined that Dlx3 protein is phosphorylated, and the phosphorylation is inhibited by the PKC-specific inhibitor GF109203X, suggesting that Dlx3 is phosphorylated by PKC in vivo. Of the PKC isoforms present in the epidermis, we tested α, δ, ε and ζ. Dlx3 is primarily phosphorylated by PKCα. By deletion and mutational analysis, we show that the serine residue S^{138}, located in the homeodomain of Dlx3 protein, was specifically phosphorylated by PKC. The phosphorylation of purified Dlx3 proteins by PKC partially inhibited formation of complexes between Dlx3 protein and DNA. These results suggest that Dlx3 protein can be directly phosphorylated by PKC and this affects the DNA binding activity of Dlx3. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Dlx3; Homeodomain; Protein kinase C; Phosphorylation; Nuclear localization signal; Epidermis

1. Introduction

The stratified epidermis is characterized by outward migration of keratinocytes from the proliferative basal compartment to the upper, terminally differentiated cornified layer of the skin. The differentiation process is associated with the sequential induction and repression of structural and enzymatic stratification-specific markers [1]. In primary basal mouse keratinocytes cultivated in vitro, this process can be induced by increasing by Ca^{2+} concentration in the culture medium [2]. Activation of protein kinase C (PKC) has been shown to be essential for expression of the late differentiation markers loricrin and profilaggrin [3].

Dlx3, a murine ortholog of the *Drosophila Distal-less* homeodomain protein [4], is a member of the Dlx vertebrate family. Dlx3 is a transcriptional activator with an AT-rich DNA binding site [5], which is primarily expressed in the differentiated granular layer of the epidermis and in the hair matrix cells of the hair follicle [6]. Transgenic temporal and spatial mis-expression of Dlx3 in the basal layer caused an abnormal skin phenotype, characterized by cessation of proliferation and premature differentiation of the basal cells judged by the upregulation of expression of late differentiation markers [6]. Disruption of the DLX3 coding sequence has been associated with the autosomal dominant human trichodento-osseous syndrome, characterized by defects in ectodermal derivatives such as hair and teeth, and craniofacial bone abnormalities [7]. The expression of Dlx3, as for the differentiation markers, is activated by Ca^{2+} in keratinocytes induced to differentiate in culture [8]. These data support a role for Dlx3 as a determinant factor in the activation of expression of granular markers during the terminal differentiation of keratinocytes.

PKC consists of a multigene family of closely related serine-threonine kinase isozymes that have been implicated in numerous biological processes including differentiation [9]. The α, δ, ε, η, and ζ isoforms of PKC are expressed in murine keratinocytes [10,11]. Ca^{2+} changes the subcellular distribution of PKC in vitro [11,12], and the calcium-induced expression of genes for the epidermal differentiation markers transglutaminase, loricrin, and profilaggrin is dependent upon PKC activation [3]. The importance of PKC in keratinocyte differentiation is substantiated by the fact that PKC activators such as 12-*O*-tetradecanoylphorbol-13-acetate also induce late differentiation marker expression in vitro in the absence of changes in extracellular Ca^{2+} levels [13]. The AP-1 transcription factor is known to be a nuclear target of PKC signal transduction [14]. The transcription of involucrin, one of the keratinocyte terminal differentiation marker, is activated by the PKC signaling pathway through AP-1 signal transduction [15].

We have been studying the regulation of Dlx3 transcription during keratinocyte differentiation [16]. Since epidermal differentiation is dependent on PKC activation, and Dlx3 is thought to be involved in this process, we examined whether PKC phosphorylates Dlx3 protein and if these posttranslational modifications modulate its function.

2. Materials and methods

2.1. Plasmid construction

To express the murine Dlx3 protein in bacteria, the cDNA of the Dlx3 gene was inserted in the pET28a *Escherichia coli* expression vector (Invitrogen), at *Bgl*II and *Xho*I sites. Each deletion mutant DNA fragment of Dlx3 was constructed by PCR and inserted into the *Bgl*II and *Xho*I site of pET28a. The following primers were used:

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pET124–287 (5′-CGCAGATCTGGCAAGCCCAAAAAGGTCCG-AAAGCCGCG-3′ and 5′-CCGCTCGAGTCAGTACACAGCCCC-AGGGTTAGG-3′), pET 130–188 (5′-GGAAGATCTCGAAAG-CCGCGAACGATCTACT-3′ and 5′-CCGCTCGAGATAGAGCT-TTTTGAACTTGGAGCGG-3′), pET 195–287 (5′-GGAAGATCT-CTGGAACACAGCCCCAACAACAGT-3′ and 5′-CCGCTCGA-GTCAGTACACAGCCCCAGGGTTAGG-3′). Amino acid mutations in the Dlx3 protein were created by replacing the codons for Ser^{137}, Ser^{138}, Ser^{182} and Thr^{134} with codons for Ala by site-directed mutagenesis (QuickChange, Stratagene). All mutations were confirmed by DNA sequencing.

2.2. Phosphorylation assays

In vitro phosphorylation reactions were performed as follows: approximately 100 ng of each purified bacterial protein was incubated with 25 ng (0.04 U) of PKC (Promega) at 37°C for 30 min in assay buffer (40 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, bovine serum albumin at 100 μg/ml, 0.4 mM $CaCl_2$, 100 μg/ml phosphatidylserine) and 0.1 μl of [γ-^{32}p]ATP (4500 Ci/mmol; NEN Dupont). Reactions were terminated by adding SDS loading buffer and boiling for 3 min. Proteins were subjected to SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane. The transferred membrane was analyzed by Western blotting with T7 antigen Ab (Invitrogen) and exposed to X-ray film overnight.

For the kinase assays with nuclear extract and PKC-specific inhibitor, 100 ng of purified Dlx3 protein was incubated with 5 μg of primary keratinocyte nuclear extract and the PKC inhibitor GF109203X (GF, bisindolylmaleimide I, Alexis) or PKA inhibitor H-89 (Alexis), in the same assay buffer described above and immunoprecipitated with anti-His antibody (Santa Cruz). The samples were then analyzed by Western blotting and exposed to X-ray film.

2.3. Preparation of bacterial fusion proteins

His-tagged fusion proteins were prepared as described by Novagen. Briefly, 100 ml of cultured *E. coli* BL21 (DE3) (Novagen) was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. His-tagged proteins were obtained using His-Bind resin according to Novagen. GST-tagged fusion proteins were prepared according to the manufacturer's guide using glutathione-Sepharose 4B (Pharmacia).

2.4. Preparation of nuclear extracts and gel retardation assays

Primary mouse keratinocytes were isolated from BALB/c newborn mouse skins and grown in Eagle's minimal essential medium lacking Ca^{2+}, with 8% Chelex-treated fetal bovine serum. Nuclear extracts were prepared from primary mouse keratinocyte cultures as described by Andreas and Faller [17]. Gel retardation analysis was carried out according to Park and Morasso [16], using ^{32}P-end-labeled Dlx3 binding consensus sequence (5′-CGGATCCATAATTGCTGGAATTCC-3′) oligonucleotide [5].

2.5. Mass spectrometry

Stained bands after SDS–PAGE of phosphorylated and non-phosphorylated homeodomain of Dlx3 were excised and broken into pieces with a microhomogenizer in 0.2-ml PCR plastic tubes. Gel pieces were sequentially extracted with 2×100 μl 50% acetonitrile in 25 mM NH_4CO_3 pH 8.1, 100% acetonitrile and dried under vacuum. The gels were allowed to swell in 20 μg/ml sequencing grade modified trypsin (Promega) in 25 mM NH_4CO_3 pH 8.1 and digested overnight at 37°C. Protein fragments were extracted with 100 μl 60% acetonitrile, 0.5% trifluoroacetic acid (TFA), the extracts were concentrated 6–8-fold in vacuum and were freed from salts on a C_{18} zip-tip (Millipore). The peptide mixture was mixed 1:1 with a freshly prepared matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.3% TFA) and spotted on sample plates.

Mass spectra were collected on a Voyager DE-PRO MALDI-TOF mass spectrometer in the delayed extraction mode. Sixty-four to 128 scans from a nitrogen laser (337 nm) were averaged in each recorded spectrum.

3. Results

Expressed Dlx3 protein was immunoprecipitated with anti-His antibody and protein A agarose, and incubated with a nuclear extract of mouse keratinocytes in assay buffer, with or without 1 μM or 10 μM of PKC-specific inhibitor GF or 10 μM of PKA-specific inhibitor H-89, as a control. As shown in Fig. 1A, Dlx3 protein was specifically phosphorylated in nuclear extract, and this phosphorylation was inhibited by GF, but not by H-89. This result suggested that the Dlx3 protein could be phosphorylated by PKC in vivo. Furthermore, we show that purified Dlx3 protein is phosphorylated

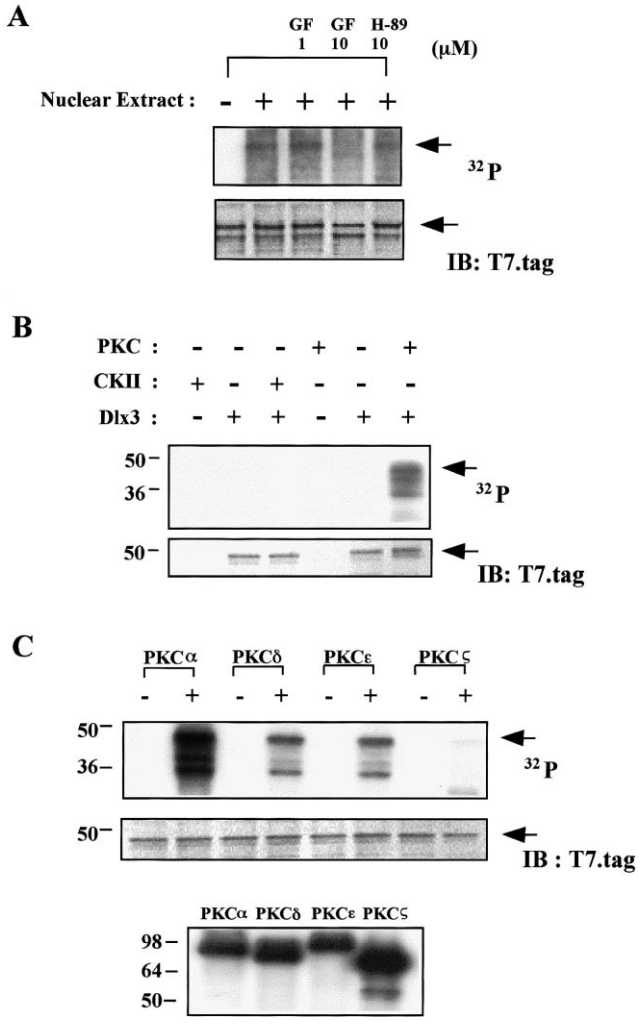

Fig. 1. Dlx3 is phosphorylated by PKC. A: Bacterially expressed Dlx was immunoprecipitated with anti-His antibody and incubated with 5 μg of nuclear extract of mouse keratinocytes. The PKC inhibitor GF was added in the reaction at 1 μM (lane 3) and 10 μM concentrations (lane 4). The PKA inhibitor H-89 was also added in the reaction (lane 5). Samples were subjected to SDS–PAGE, and transferred to a PVDF membrane. The amount of Dlx3 protein in each reaction was determined by Western blotting analysis (lower panel) using the T7 tag antibody (Novagen). The membrane was exposed to X-ray film for autoradiography. Arrows indicate the Dlx3 protein. B: Dlx3 protein (100 ng) was incubated for 30 min at 37°C in the presence or absence of PKC and γ-ATP. Dlx3 protein was also incubated with casein kinase II (CKII). Samples were then separated by electrophoresis on SDS–PAGE. The amount of Dlx3 protein used in each reaction was determined by Western blot analysis (lower panel). C: Dlx3 protein was incubated in the presence or absence of PKC isoforms α, δ, ε, ζ (Calbiochem) in the presence of γ-ATP. The amount of Dlx3 protein used in each reaction is shown in the Western blot in the lower panel using the T7 tag antibody (Novagen). The bottom panel shows the autophosphorylation of each PKC isoform, demonstrating the specific activity of each PKC isoform.

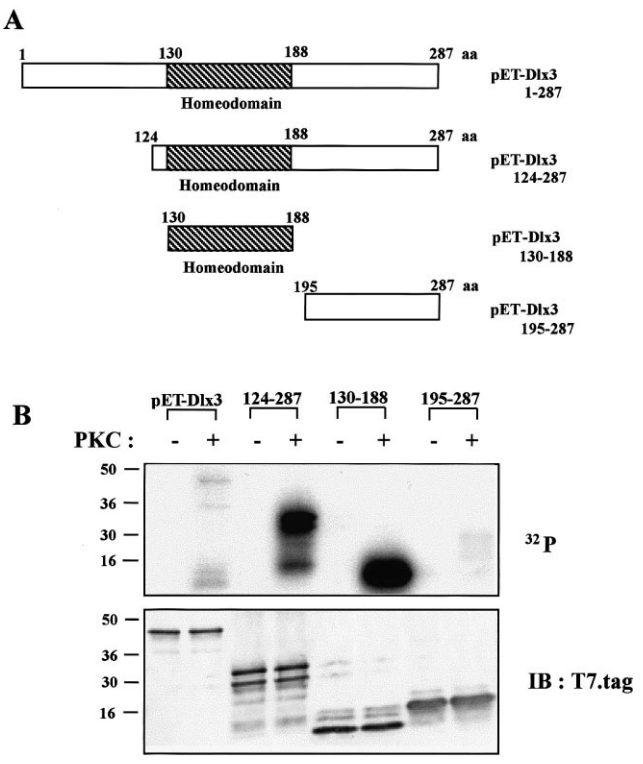

Fig. 2. Phosphorylation by PKC within the homeodomain of Dlx3. A: Schematic representation of Dlx3 deletions. B: In vitro PKC phosphorylation of Dlx3 proteins described in A. Each mutant protein was incubated with γ-ATP in the presence or absence of PKC. Each sample was subjected to SDS–PAGE and transferred to a PVDF membrane. The upper panel shows the results of autoradiography. The amount of each protein is shown in the bottom panel by Western blot.

by PKC in vitro, by performing kinase reaction with purified PKC (Promega). PKC from Promega consists primarily of the α and β isozymes, and is at least 90% pure PKCα by SDS–PAGE. Casein kinase II (CKII) was also tested for the ability to phosphorylate Dlx3. As shown in Fig. 1B, Dlx3 was specifically phosphorylated by PKC and no detectable phosphorylation was observed in assays with CKII.

Several subtypes of the PKC family (α, δ, ε, η, and ζ isoforms) are expressed in keratinocytes. To examine which PKC subtype was able to phosphorylate Dlx3, we performed in vitro kinase assays with the commercially available PKCα, PKCδ, PKCε, and PKCζ (Calbiochem). Dlx3 protein was strongly phosphorylated by PKCα, to a much lesser extent by PKCδ and PKCε and not by PKCζ (Fig. 1C). The specific activity of each PKC isoform was not much different from each other, as is shown by autophosphorylation of each PKC isoform in Fig. 1C, bottom panel. These results showed that in vitro, the Ca^{2+}-dependent PKCα was the most effective kinase of Dlx3. Since Dlx3 localizes to the nucleus in differentiated keratinocytes [18], we corroborated the presence of the PKCα isoform in the nucleus, as has been shown in NIH3T3 upon treatment with phorbol ester [19], by Western blot analysis of nuclear extracts of suprabasal epidermal cells (data not shown).

To determine the phosphorylation site(s) within the Dlx3 protein, we incubated PKC with: the full-length Dlx3 protein (pET 1–287), amino-terminal deletion mutant (pET 124–287), homeodomain (pET 130–188), and carboxy-terminal (pET 194–287) (Fig. 2A). As shown in Fig. 2B, PKC phosphorylated the amino-terminal deletion mutant (124–287) and homeodomain of Dlx3 protein (130–188). PKC weakly phosphorylated the full-length Dlx3, but not the carboxy-terminal. The result delineated the phosphorylation site(s) to the homeodomain region of Dlx3. Interestingly, the amino-terminal deletion mutant Dlx3 (124–287) and homeodomain (130–188) were more strongly phosphorylated by PKC than full-length Dlx3. Analysis of the amino acid sequence in the homeodomain region indicated three serine residues (Ser^{137}, Ser^{138}, and Ser^{182}) and three threonine residues as potential candidates for phosphorylation sites (Fig. 3A). Before the determination of the specific phosphorylation site(s), we performed mass spectrometry for analysis of the molecular weight of proteolytic peptides comparing the wild type and phosphorylated homeodomains of Dlx3 (Fig. 3B). We found that for the peptide containing amino acids of the phosphorylated form valued by 80 (approx. MW of phosphate) when compared to the non-phosphorylated corresponding peptide (134–146: TIYSSYQLAALQR; MW 1513) (Fig. 3B, right panel, arrow indicated). Peak heights shown in Fig. 3B do not reflect relative amounts. As mass spectra are recorded in the positive mode, the introduction of a negative group by phosphorylation is expected greatly to reduce the peak height at *m/z* 1593. This result suggested that one of the serine residues (S^{137}, S^{138}) or the threonine residue (T^{134}) was phosphorylated in this region. Since trypsin cuts the peptide bond after an Arg or Lys residue, the potential phosphorylation of residue S^{182} could not be assessed by mass spectrometry. Therefore, we tested all three serine residues (S^{137}, S^{138}, S^{182}) with in vitro mutagenesis analysis. In vitro mutagenesis was performed, in which each potential phosphorylation site including S^{182} residue was replaced with an alanine. Of the three serine residue mutant Dlx3 proteins, $Dlx3^{S137A}$ was phosphorylated, whereas $Dlx3^{S138A}$ and $Dlx3^{S182A}$ were phosphorylated weakly or not at all (Fig. 3C, left panel). We also tested one threonine residue (T^{134}) and the double serine residue mutant $Dlx3^{S137,138A}$. $Dlx3^{T134A}$ was phosphorylated while $Dlx3^{S137,138A}$ was not phosphorylated (Fig. 3C, right panel). These results support that the S^{138} residue in the Dlx3 protein homeobox region was the primary site of phosphorylation by PKC.

To determine whether phosphorylation of Dlx3 protein could affect the functions of Dlx3 as a transcriptional activator, we tested the DNA binding affinities of the phosphorylated and non-phosphorylated forms of Dlx3 by gel retardation assay using the consensus Dlx3 binding site (ATAATTGC) [5]. We prepared GST-Dlx3 fusion protein and incubated it in the presence or absence of PKCα. Gel retardation assays were performed with phosphorylated and non-phosphorylated GST-Dlx3 fusion proteins. The amounts of protein used in this assay were approximately equivalent to 50, 100, and 200 ng in lanes 1, 2, and 3 for the non-phosphorylated form and lanes 5, 6, and 7 for the phosphorylated form. As shown in Fig. 4A, the intensity of the complexes formed with phosphorylated Dlx3 was decreased when compared to that of the non-phosphorylated form. The shifted bands were scanned and plotted against protein concentration (Fig. 4B). Phosphorylation of Dlx3 shifted the plotted line downward. These results suggest that the phosphorylated Dlx3 had lower DNA binding affinity than the non-phosphorylated protein.

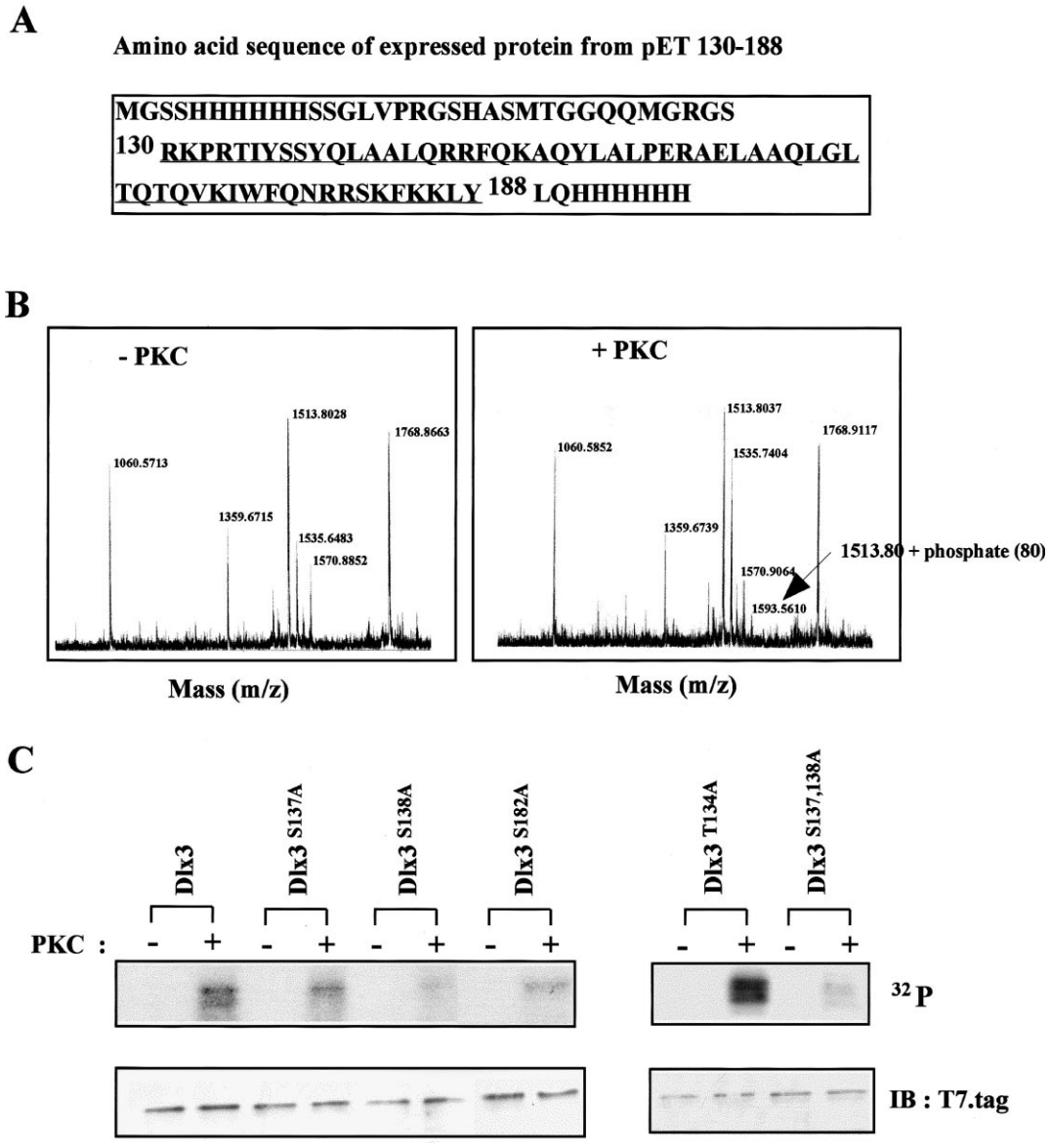

Fig. 3. Determination of the PKC phosphorylation site in the homeodomain of Dlx3. A: Schematic representation of the amino acids in the homeodomain region (underlined). Three serine residues and three threonine residues are in this homeodomain region. B: Spectra from mass spectrometry analysis of non-phosphopeptide (left panel) and phosphopeptide (right panel) of the Dlx3 homeodomain region (phosphorylated by PKC in vitro). The numbers on each peak represent the molecular weight of each proteolytic peptide. The sequence of each peptide was identified based on the molecular weight. The arrow indicates the new peak that by MW corresponds to the 1513.80 peak plus the phosphate group. C: Each mutant Dlx3 protein was incubated with γ-ATP in the presence or absence of PKC. Each sample was subjected to SDS–PAGE and transferred to a PVDF membrane. The upper panel presents the results of autoradiography. The amount of protein in each reaction was analyzed by Western blotting (lower panel).

4. Discussion

The Dlx3 gene is expressed in the suprabasal layer of skin. Dlx3 expression is dramatically increased in cultured primary mouse keratinocytes induced to differentiate by extracellular calcium. Since PKC activation is required for keratinocyte differentiation, we hypothesized that there is a relationship between regulation of Dlx3 protein and the PKC signaling pathway in this process. In this study, we present evidence that the Dlx3 protein is a substrate of PKC in vitro, and that phosphorylation of Dlx3 modulates its DNA binding. Dlx3 protein is phosphorylated with nuclear extracts of mouse keratinocytes and this phosphorylation is inhibited by the PKC inhibitor GF109203X, which suggests that Dlx3 protein is phosphorylated by PKC in vivo. Delineation of the phosphorylation site(s) of Dlx3 was examined by in vitro phosphorylation using truncated Dlx3 mutant proteins. We have also shown that Dlx3 protein is phosphorylated in vitro primarily by purified PKCα. Nishikawa et al. [20] determined the optimal peptide substrate sequence for nine of the PKC isoforms. PKCα selected peptides with basic amino acids at positions −7, −6, −4, and −2. At position −5, PKCα strongly selected peptides with Arg at this position and preferred peptides with Ala or Lys at the +5 position. The sequence around S^{138} has an Arg at −5 and Ala at +5 and basic amino acids at the amino-terminal of S^{138}, while S^{137} and S^{182} have a Phe at

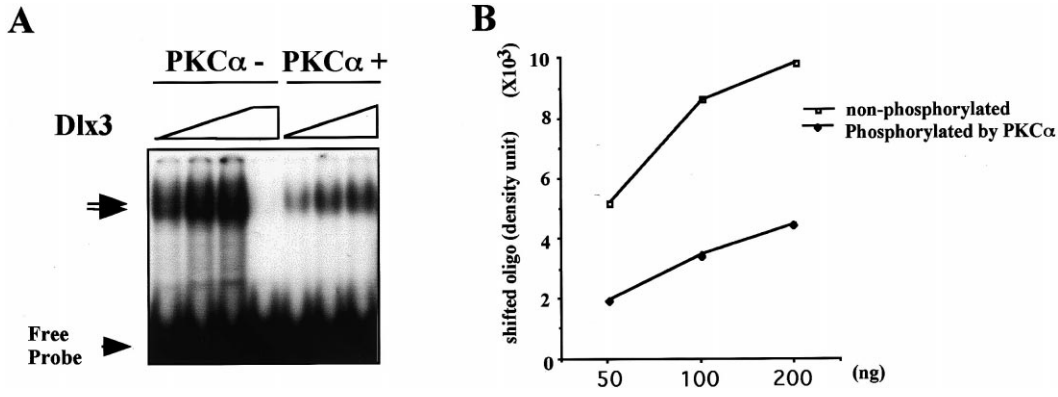

Fig. 4. PKC phosphorylation modulates Dlx3 function. A: Increasing amounts of GST-Dlx3 fusion protein from 50 ng to 200 ng and unlabeled ATP were incubated for 30 min at 37°C in the presence or absence of PKCα (Calbiochem). Samples were incubated with radiolabeled oligonucleotides encoding the consensus Dlx3 binding site for gel retardation assay. Arrowhead indicates the complex formed by GST-Dlx3 and probe. B: The shifted bands were scanned and plotted against protein concentration.

position −5. Based on the report by Nishikawa et al., the S^{138} residue is a relatively good substrate for PKCα by sequence comparison. In addition to PKCα, PKCη has been strongly associated with keratinocyte differentiation [21]. However, we were not able to evaluate the ability of PKCη to phosphorylate Dlx3. Thus, we cannot rule out the possibility that PKCη can phosphorylate Dlx3 and may also regulate its function. Future studies will address this possibility.

As has recently been shown for other homeodomain proteins [22,23], Dlx3 protein is phosphorylated within the homeodomain region. Interestingly, the full-length Dlx3 protein was phosphorylated to a much lesser extent than the N-terminal deletion. Our data indicate that the N-terminal region of Dlx3 in some way interferes with the phosphorylation in the homeodomain, possibly due to three-dimensional conformation. However, when the full-length Dlx3 protein was phosphorylated by PKC, a decreased DNA binding activity was observed.

The process of cellular differentiation partly results from differential gene expression regulated through specific functions of transcription factors, and the function is dependent on nuclear localization, DNA binding and transactivating properties. Recently, we have reported that the Dlx3 protein has a nuclear localization signal (NLS) located in the N-terminal region of the homeodomain [18]. The delineated Dlx3 bipartite NLS is necessary not only for intracellular localization but also for transcriptional activity, protein:protein interaction and DNA binding. Homeodomain proteins direct DNA binding through the homeodomain region, where the N-terminal arm of the homeodomain is oriented such that it inserts into the minor groove of the DNA. The Dlx3 bipartite NLS and the specific phosphorylation site for PKC determined in this study lie within the N-terminal arm. Altogether, these results suggest that modifications such as phosphorylation within the NLS might affect the ability of Dlx3 to bind DNA (TAATT consensus motif) by interfering with minor groove DNA recognition and binding. A recent report has shown that phosphorylation of the homeodomain protein Csx/Nkx2.5 by CKII increased the DNA binding affinity [22]. Therefore, by phosphorylation, homeodomain protein function can be modulated positively or negatively. Our findings suggest that the function of Dlx3 protein is modulated in vitro by PKC phosphorylation.

From the PKC isoforms expressed in epidermis (α, δ, ε, η, ζ), the isoforms δ, ε, and η are involved in the regulation of human involucrin gene expression [15]. PKCα is an important component of the signaling pathway regulating terminal differentiation of keratinocytes [24]. In our results, Dlx3 protein was phosphorylated primarily by PKCα and this modification reduced the DNA binding. We have evidence that PKC inhibitors repress the transcription of the Dlx3 gene upon differentiation [25]. Therefore, Dlx3 gene expression and function in the keratinocytes is hypothetically under both positive and negative regulation by PKC.

Since DNA binding may reveal only a part of Dlx3 protein function, we cannot rule out the influence of phosphorylation on other biological functions. Thus, the characterization of the full biological function of PKC phosphorylation of Dlx3 protein will require further studies.

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