Protein kinase C (PKC) family requires phosphorylation of itself to become competent for responding to second messengers. Much attention has been focused on elucidating the role of phosphorylation in PKC activity; however, it remains unknown where this modification takes place in the cells. This study examines whether anchoring protein is involved in the regulation of PKC phosphorylation. A certain population of PKCε in rat brain extracts as well as that expressed in COS7 cells was associated with an endogenous anchoring protein CG-NAP (centrosome and Golgi localized PKN-associated protein). Pulse chase experiments revealed that the associated PKCε was an immature species at the hypophosphorylated state. In vitro binding studies confirmed that non-hypophosphorylated PKCε directly bound to CG-NAP via its catalytic domain, whereas sufficiently phosphorylated PKCε did not. PKCε mutant at a potential phosphorylation site of Thr-566 or Ser-729 to Ala, possessing almost no catalytic activity, was associated and co-localized with CG-NAP at Golgi/centrosome area. On the other hand, wild type and a phosphorylation-mimicking mutant at Thr-566 were mainly distributed in cytosol and represented second messenger-dependent catalytic activity. These results suggest that CG-NAP anchors hypophosphorylated PKCε at the Golgi/centrosome area during maturation and serves as a scaffold for the phosphorylation reaction.

PKC family is a key player in the cellular responses mediated by the second messenger diacylglycerol and the phorbol ester tumor promoters (1). Although the allosteric control is an essential feature of its action as a signal transducer, it has been revealed that an additional two types of control are also important as follows: one is phosphorylation of kinases themselves (2, 3), and the other is specific subcellular localization mediated by anchoring proteins (4, 5). The PKC family has three conserved in vivo phosphorylation sites as follows: the activation loop site and two sites located C-terminal to the kinase domain (6, 7), with the exception of the atypical PKC isozymes having a Glu residue at the most carboxyl position. Modification of these sites by combination of trans- and autophosphorylation is required to gain catalytically competent conformation in order to respond to second messengers (8–11). It has been found that the activation loop site of PKCε as well as other kinases such as PKB and p70S6K is phosphorylated by phosphoinositide-dependent protein kinase 1 (PDK1) (12–15). Because PDK1 kinase activity is constitutive (16), activation loop phosphorylation may be controlled by localization and conformation of the substrates. For instance, phosphorylation of PKB is triggered by translocation to the membrane through binding its pleckstrin homology domain with phosphatidylinositol(3,4,5)-trisphosphate, causing a conformational change that exposes the activation loop phosphorylation site (17). It is likely that the PKC family is also recruited to the particular place through binding with lipid or with anchoring proteins to receive phosphorylation.

We have previously reported that CG-NAP (centrosome and Golgi localized PKN-associated protein) serves as an anchoring protein for protein kinases (PKN and PKA) and protein phosphatases (PP1 and PP2A) at centrosome and the Golgi apparatus (18). Among PKC isozymes, PKCε was demonstrated to be localized to the Golgi apparatus when expressed in NIH3T3 cells (19). Therefore, we examined whether PKCε associates with CG-NAP.

In this study, we have found that immature non- or hypophosphorylated PKCε associates with CG-NAP at Golgi/centrosome area, and sufficiently phosphorylated PKCε dissociates from CG-NAP as a “mature” PKCε responsive to the incoming second messenger signals, suggesting that CG-NAP serves as a protein scaffold for the phosphorylation of PKCε.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—COS7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin. Rat PKCε cDNA (20) and human CG-NAP cDNA (18) were described previously. Mammalian expression plasmids for HA- and FLAG-tagged proteins were constructed by inserting corresponding cDNA fragments into pT7B01-HA and pT7B01-FLAG (21), respectively. Expression plasmids were introduced into COS7 cells by electroporation.

**In Vitro Mutagenesis**—Expression plasmids for PKCε mutants were generated by QuickChange Site-directed Mutagenesis Kit (Stratagene) using appropriate oligonucleotide primers and pT7B01-HA/PKCE.

**Immunoprecipitation and Immunoblotting**—Cells were lysed with Lysis Buffer (50 mM Tris-HCl at pH 7.5, 1% Nonidet P-40, 0.15 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 10 μg/ml leupeptin). After centrifugation, cleared lysates were incubated with an appropriate antibody at 4 °C for 2 h, and then protein A-Sepharose (Amersham Pharmacia Biotech) was added and incubated for a further 30 min. The resin was washed with the same buffer, and then the bound proteins were processed for immunoblotting (22) or kinase assay.

**Received for publication, June 16, 2000, and in revised form, July 27, 2000**

**Published, JBC Papers in Press, August 16, 2000, DOI 10.1074/jbc.M005285200**

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Printed in U.S.A.

**THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 44, Issue of November 3, pp. 34592–34596, 2000**

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**Associa of Immature Hypophosphorylated Protein Kinase Cε with an Anchoring Protein CG-NAP**

Association of Immature Hypophosphorylated Protein Kinase Cε with an Anchoring Protein CG-NAP*

*This work was supported in part by research grants from the Ministry of Education, Science, Sports and Culture of Japan and the “Research for the Future” program of the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PKC, protein kinase C; CG-NAP, centrosome and Golgi localized PKN-associated protein; PKA, protein kinase A; GST, glutathione S-transferase; HA, hemagglutinin; APase, alkaline phosphatase; PS, phosphatidylinerine; FBS, fetal bovine serum; DO, diolein.
Preparation of Rat Brain Extracts—Rat brain was homogenized by Dounce homogenizer in the Lysis Buffer and then centrifuged at 100,000 x g for 20 min. Cleared lysates were used as brain extracts.

Pulse-Chase Experiment—COS7 cells transiently expressing HA-PKC\(\epsilon\) were incubated in methionine/cysteine-free medium containing 10% dialyzed FBS and then pulse-labeled for 5 min with \([35S]\)methionine/cysteine (10.4 MBq/ml). The cells were further incubated in methionine/cysteine (210 \(\mu\)g/ml) for 0, 15, 30, 60, and 120 min. After washing with ice-cold PBS, the cells were lysed with the Lysis Buffer and processed for immunoprecipitation.

Alkaline Phosphatase (APase) Treatment of PKC\(\epsilon\)—PKC\(\epsilon\) in the immunoprecipitates or purified preparation was treated with calf intestine alkaline phosphatase (New England Biolabs) at a final concentration of 1000 units/ml in a buffer containing 50 mM Tris-HCl at pH 8.0, and 1 mM MgCl\(_2\) at 37 °C for 1 h.

Preparation of Recombinant Proteins in E. coli—Bacterial expression vectors to generate recombinant virus was constructed by inserting PKC\(\epsilon\) cDNA into pBlueBacHis/GST (23). His\(_6\)/GST-tagged recombinant PKC\(\epsilon\) was expressed and purified by using glutathione-Sepharose 4B as described previously (23).

Binding Assay of Dephosphorylated PKC\(\epsilon\) with CG-NAP—FLAG-tagged deletion mutant of CG-NAP was expressed in COS7 cells together with FLAG-tagged CG-NAP. Cell extracts were immunoprecipitated with anti-FLAG M2 (aFL) followed by immunoblot with anti-HA 3F10 (aHA). Lanes 6 and 7 were done with the second antibody preadsorbed with murine immunoglobulin. Schematic representation of PKC\(\epsilon\) is shown on the right with positions of Lys-437 in ATP-binding site and three potential phosphorylation sites. Names and positions of the deletion mutants are shown below. Mutant representing minimum binding region to CG-NAP is shaded. Mutant P6-(364–725) was used in Fig. 4B.

Preparation of Recombinant PKC\(\epsilon\) in Insect Cells—Transfer vector to generate recombinant virus was constructed by inserting PKC\(\epsilon\) cDNA into pBlueBacHis/GST (23). His\(_6\)/GST-tagged recombinant PKC\(\epsilon\) was expressed and purified by using glutathione-Sepharose 4B as described previously (23).

Binding Assay of Dephosphorylated PKC\(\epsilon\) with CG-NAP—FLAG-tagged deletion mutant of CG-NAP was expressed in COS7 cells and immunoprecipitated with anti-FLAG Affi-Gel-agarose (Eastman Kodak Co.). Then the immunoprecipitates were mixed with insect cell-expressed PKC\(\epsilon\) pretreated with or without APase and incubated at 4 °C for 1 h in Binding Buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 2 mM MgCl\(_2\), 1 mM EDTA, and 1 mM diithiothreitol) containing 8 mM NaPO\(_4\) at pH 7.2 to inhibit APase activity. After washing the resin with the Binding Buffer, the bound proteins were analyzed by immunoblotting.

Preparation of Recombinant Proteins in E. coli—Bacterial expression plasmids for glutathione S-transferase (GST)- and His\(_6\) epitope-tagged proteins were constructed by using pGEX4T (Amersham Pharmacia Biotech) and pRSET (Invitrogen), respectively. Tagged recombinant proteins were purified as described previously (18).
Association of Immature PKCε with CG-NAP

**GST-Pulldown Assay of In Vitro Phosphorylated PKCε**—Bacterially expressed His-tagged PKCε fragment was phosphorylated by incubation with insect cell-expressed PKCε at 30 °C for 2 h in a buffer containing 20 mM Tris-HCl at pH 7.5, 4 mM MgCl₂, 100 μM ATP, and 40 μM arachidonic acid as an activator (24). Then the reaction mixture was 5-fold diluted with the Binding Buffer and incubated with bacterially expressed GST-tagged CG-NAP fragment at 4 °C for 1 h. Then glutathione-Sepharose 4B was added and incubated for further 30 min. After washing the resin with the same buffer, the bound proteins were analyzed by immunoblotting.

**Antibodies**—Polyclonal antibodies against CG-NAP (αEE and αBH) were described previously (18). The following antibodies were purchased: anti-PKCε and anti-PKA-R1α (Transduction Laboratories); anti-phospho-PKCε (Upstate Biotechnology); mouse anti-HA 12CA5, and rat anti-HA 3F10 (Roche Molecular Biochemicals); anti-FLAG M2 (Kodak); anti-His₆ antibody (Qiagen); rhodamine-conjugated anti-rabbit IgG, and dichlorotriazinylaminofluorescein-conjugated anti-mouse IgG (Chemicon International); peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology).

**Kinase Assay**—Immunoprecipitates of PKCε were resuspended with a buffer containing 20 mM Tris-HCl at pH 7.5, and 4 mM MgCl₂. Then aliquots were incubated at 30 °C for 5 min in a reaction mixture (25 μl) containing 20 mM Tris-HCl at pH 7.5, 4 mM MgCl₂, 40 μM ATP, 18.5 kBq of [γ-³²P]ATP, and 100 μM PKCε peptide (25) in the presence or absence of activator 8 μg/ml phosphatidylserine (PS) and 0.8 μg/ml diolein (DO). The incorporation of [³²P] into PKCε peptide from [γ-³²P]ATP was monitored as described previously (25).

**Immunofluorescence Microscopy**—Cells grown on cover glasses were fixed with 3.7% formaldehyde in 0.2 M NaPO₄ at pH 7.2 and permeabilized using 0.3% Triton X-100. Cells were blocked with 5% normal goat serum in PBST (20 mM NaPO₄ at pH 7.5, 150 mM NaCl, and 0.03% Triton X-100) and then incubated with the relevant antibody for 1 h at room temperature. The primary antibody was visualized by subsequent incubation with the appropriate secondary antibody conjugated with either rhodamine or dichlorotriazinylaminofluorescein. The fluorescence was observed under a confocal laser scanning fluorescent microscope (Zeiss) as described (18).

**RESULTS AND DISCUSSION**

Association of PKCε with CG-NAP—Exogenously expressed PKCε was co-immunoprecipitated with endogenous CG-NAP by anti-CG-NAP (Fig. 1A, lane 3). The co-precipitated PKCε consisted of fast and slow migrating species, whereas PKCεs in cell extracts appeared as a single band with slow mobility (Fig. 1A, lane 1). A kinase-negative mutant, PKCε/K437M (Fig. 1A,
Association of Immature PKCe with CG-NAP

Fig. 5. Co-localization of CG-NAP with PKCe mutated at potential phosphorylation sites. A, Ser-729 phosphorylation of PKCe mutants. HA-tagged PKCe mutants expressed in COS7 cells were immunoprecipitated (IP) with anti-HA 12CA5 (aHA) followed by immunoblot with aPKCe or aPS729. B, kinase activity of PKCe mutants. The immunoprecipitates prepared in A were examined for kinase activity using SPKCe peptide as a substrate (25) in the presence or absence of activator PS/DO as described under “Experimental Procedures.” The values shown are means ± S.E. of triplicates. C, association of PKCe mutants with CG-NAP. Extracts of COS7 cells expressing HA-tagged PKCe mutants were double-stained with anti-HA and aCG-NAP. Schematic representation of PKCe with positions of mutated amino acid residues is shown at the bottom. The results shown are representative of three separate experiments.

Lane 2) mutated at ATP-binding site, was also co-immunoprecipitated with CG-NAP efficiently (Fig. 1A, lane 4). Association between PKCe and CG-NAP was observed with endogenous proteins in rat brain extracts (Fig. 1B, lane 3), suggesting that this association is physiological. Binding regions of PKCe and CG-NAP were examined by immunoprecipitation using deletion mutants (Fig. 2, A and B, respectively). Names of the deletion mutants of PKCe and CG-NAP were designated P1–P6 (Fig. 2A, right) and C1–C5 (Fig. 2B, right), respectively, with amino acid numbers. Binding regions of PKCe and CG-NAP were located in P2-(540–725) within the catalytic domain and C4-(3451–3899) at the C terminus, respectively.

Preferential Association of Immature Hypophosphorylated PKCe with CG-NAP—To examine whether the fast migrating PKCe appears during biosynthesis, pulse-chase labeling of newly synthesized PKCe was performed. As shown in Fig. 3A, wild type PKCe was synthesized as a fast migrating species and then converted to a slow migrating species within 120 min. Phosphatase treatment of the latter species resulted in shift to the fast mobility (Fig. 3B, lane 3), indicating that the migration shift during biosynthesis is caused by phosphorylation. We next examined whether association with CG-NAP changes during biosynthesis of PKCe. 32P-Labeled fast migrating PKCe at 0 and 30 min of chase was co-immunoprecipitated with CG-NAP (Fig. 3C, lanes 1 and 2), whereas the slow migrating species at 30 and 120 min of chase was rarely detected in the precipitates (Fig. 3C, lanes 2 and 3). These results indicate that CG-NAP preferentially associates with immature PKCe at non- or hypophosphorylated states.

Direct Binding of Hypophosphorylated PKCe with CG-NAP—To assess the possibility that the association with CG-NAP depends on phosphorylation states of PKCe, in vitro binding studies were performed. PKCe has three potential phosphorylation sites of Thr-566 in the activation loop and Thr-710 and Ser-729 in the C-terminal region (Fig. 2A, right). An antibody recognizing PKCe phosphorylated at Ser-729 (aSer(P)-729) was used to monitor phosphorylation/dephosphorylation reactions. As shown in Fig. 4A, insect cell-expressed phosphorylated PKCe specifically bound to FLAG-tagged CG-NAP/C4-(3451–3899), and the binding was increased when PKCe was dephosphorylated by APase (lane 2). Conversely, the effect of phosphorylation was examined by using bacterially expressed PKCe/P6-(364–725) or PKCe/P1-(364–737) (see Fig. 2A) as a binding fragment and insect cell-expressed PKCe as a kinase. Either fragment bound to GST-tagged CG-NAP/C4-(3451–3899) (Fig. 4B, lanes 1 or 5). The binding was diminished by phosphorylation of PKCe/P6-(364–725) (Fig. 4B, lane 2) containing potential phosphorylation sites of Thr-566 and Thr-710. Furthermore, phosphorylation of PKCe/P1-(364–737) completely abolished the binding (Fig. 4B, lane 6). PKCe/P1-(364–737) contains an additional phosphorylation site of Ser-729 located outside of the binding region (amino acids 540–725, see Fig. 2A). Phosphorylation of this site may affect conformation of the binding region; thus, we examined the effect of its mutation. PKCe/P3-(540–737), in which Ser-729 was not phosphorylated (data not shown), was efficiently co-immunoprecipitated with CG-NAP/C3-(2876–3899) (Fig. 4C, lane 1). Mutation of Ser-729 to phosphorylation-deficient Ala did not affect the association (lane 2), whereas mutation to phosphorylation-mimicking Asp resulted in dissociation as expected (lane 3). These results confirmed that CG-NAP directly binds to PKCe at non- or hypophosphorylated states.

Co-localization of CG-NAP with PKCe Mutated at Potential Phosphorylation Sites—PKCe co-immunoprecipitated with CG-NAP contained a slow migrating species as well as the fast migrating species (Fig. 1A, lane 3). This slow migrating species...
appeared to be different from the mature PKCe, as it was not detected by α(Ser\(^{\text{P}}\))-729 (data not shown). To investigate subcellular localization and catalytic activity of these immature PKCe associated with CG-NAP, mutants of the potential phosphorylation sites were examined. Phosphorylation-mimicking mutant of activation loop Thr-566 (PKCe\(^{\text{T566E}}\)) showed similar properties to wild type PKCe (whose majority was mature form) as follows: slow migration (Fig. 5A, left); efficient phosphorylation at Ser-729 (Fig. 5A, right); second messenger-dependent catalytic activation (Fig. 5B); lower affinity to CG-NAP (Fig. 5C); and cytosolic localization (Fig. 5D). On the other hand, phosphorylation-deficient mutants of Thr-566 (PKCe\(^{\text{T566A}}\)) and Ser-729 (PKCe\(^{\text{S729A}}\)) as well as kinase-negative mutant (PKCe\(^{\text{K437M}}\)) represented different properties as follows: fast migration (Fig. 5A, left); no phosphorylation at Ser-729 (Fig. 5A, right); and almost no basal and second messenger-dependent catalytic activity, although PKCe\(^{\text{S729A}}\) showed slight activation (Fig. 5B). These phosphorylation-deficient mutants were efficiently associated and co-localized with endogenous CG-NAP at Golgi/centrosome area (Fig. 5, C and D, respectively). These results suggest that immature hypophosphorylated PKCe associated with CG-NAP is catalytically incompetent and co-localized at Golgi/centrosome area.

In addition, Ser-729 was phosphorylated in PKCe\(^{\text{T566E}}\) but not in PKCe\(^{\text{T566A}}\) (Fig. 5A), suggesting that Thr-566 phosphorylation within the activation loop is a prerequisite for Ser-729 phosphorylation. Ser-729 of a kinase-negative mutant PKCe\(^{\text{K437M}}\) was not phosphorylated, either. Furthermore, overexpression of wild type PKDK1 resulted in increase in Ser-729 phosphorylation of wild type PKCe but not of PKCe\(^{\text{K437M}}\) (data not shown). These results suggest that Ser-729 phosphorylation depends on the catalytic competency of PKCe following the Thr-566 phosphorylation by PKDK1.

Our results led us to hypothesize the maturation process of PKCe as follows: newly synthesized non-phosphorylated PKCe associates with CG-NAP at Golgi/centrosome area; Thr-566 is phosphorylated by PKDK1 followed by phosphorylation of the C-terminal sites Thr-710 and Ser-729 probably by auto-phosphorylation; sufficiently phosphorylated PKCe dissociates from CG-NAP and distributes to cytosol as mature PKCe responsive to the incoming second messenger signals. In addition, dephosphorylation by some phosphatase may re-associate with CG-NAP. PKCe and PKCBII lacking phosphates at Ser-657 and Ser-660, respectively (equivalent to Ser-729 of PKCe), are found to be structurally unstable as follows: thermo-labile, phosphatase-sensitive, and protease-sensitive (10, 11). Therefore, CG-NAP may mediate the maturation of PKCe either directly by providing a scaffold for the phosphorylation reactions or indirectly by protecting unstable immature PKCe species. Although our findings suggest that Ser-729 of PKCe is auto-phosphorylated, we cannot exclude the possibility that a heterologous kinase might be involved in this reaction. PKD1 has been demonstrated to phosphorylate Ser-473 of PKB (equivalent to Ser-729 of PKCe), when it is bound to the C-terminal peptide of PRK2 (26), an isozyme of PKN. Recently, the C-terminal part of PKN has been revealed to associate with PKD1 (27). As CG-NAP anchors PKN (18), PKN might modify PKD1 activity, for instance, to phosphorylate Ser-729 of PKCe on CG-NAP. Further study will clarify the mechanism of PKCe phosphorylation taking place on CG-NAP. Now the growing number of kinases has been demonstrated to be regulated by phosphorylation (2). The role of the anchoring protein CG-NAP for the phosphorylation of PKCe described here may provide insight into the regulation of other members of AGC kinase superfamily.

Acknowledgment—We thank Y. Nishizuka for encouragement.

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