Nucleolin Is a Protein Kinase C-ζ Substrate

CONNECTION BETWEEN CELL SURFACE SIGNALING AND NUCLEUS IN PC12 CELLS*

Guisheng Zhou, M. Lamar Seibenhener, and Marie W. Wooten‡

From the Department of Zoology, Auburn University, Auburn, Alabama 36849-5414

We have previously shown that protein kinase C (PKC)-ζ is activated and required for nerve growth factor (NGF)-induced differentiation of rat pheochromocytoma PC12 cells (Wooten, M. W., Zhou, G., Seibenhener, M. L., and Coleman, E. S. (1994) Cell Growth & Diff. 5, 395–403; Coleman, E. S., and Wooten, M. W. (1994) J. Mol. Neurosci. 5, 39–57). Here we report the characterization and identification of a 106-kDa nuclear protein as a specific substrate of PKC-ζ. NGF treatment of PC12 cells resulted in translocation of PKC-ζ and coincident phosphorylation of a protein that was localized within the nucleoplasm of nuclei isolated from PC12 cells. Addition of PKC-ζ pseudosubstrate peptide in vitro or myristoylated peptide in vivo diminished phosphorylation of pp106 in a dose-dependent fashion. Likewise, addition of purified PKC-ζ, but neither PKC-α nor δ, to nuclear extracts resulted in an incremental increase in the phosphorylation of pp106. Expression of dominant-negative PKC-ζ inhibited NGF-induced phosphorylation of pp106, by comparison overexpression of PKC-ζ enhanced basal phosphorylation without a noticeable effect upon NGF-induced effects. Amino acid sequence analysis of four peptides derived from purified pp106 revealed that this protein was homologous to nucleolin. Using an in vitro reconstitution system, purified nucleolin was likewise shown to be phosphorylated by purified PKC-ζ. The staining intensity of both enzyme and substrate in the nucleus increased upon treatment with NGF. In vivo labeling with 32P i and stimulation of PC12 cells with NGF followed by immunoprecipitation with anti-nucleolin antibody corroborated the in vitro approach documenting enhanced phosphorylation of nucleolin by NGF treatment. Taken together, the findings presented herein demonstrate that nucleolin is a target of PKC-ζ that serves to relay NGF signals from cell surface to nucleus in PC12 cells.

Nerve growth factor (NGF) is required for the survival, differentiation, and guidance of neurons to their targets (1). Rat pheochromocytoma PC12 cells, derived from an adrenal tumor of adrenergic neural crest origin, respond to NGF by cessation of division, neurite outgrowth, development of excitatory membranes, and differentiation into a sympathetic neuronal phenotype (2). Several signaling molecules and second messenger systems have been identified that participate in relaying signals from the NGF receptor to the nucleus, one of which is protein kinase C (PKC).

PKC is a ubiquitously expressed serine/threonine kinase which has been implicated in a wide variety of cellular processes (3–5). PKC is a multigene family consisting of 12 structurally related isoforms which have different tissue distribution, as well as cofactor and substrate specificities (4, 5). Based upon structural features the isoforms of the PKC family can be grouped into three related groups: classical/conventional, cPKCs (α, β1,11, and γ) that are sensitive to calcium/diacylglycerol and tumor promoting phorbol esters; novel/atypical (δ, η, and θ) that are sensitive to diacylglycerol and phorbol esters but insensitive to calcium; and atypical aPKCs (ζ and ι/λ) that are insensitive to all three regulators, diacylglycerol, calcium, and phorbol esters (5). The precise role and placement of PKC within the NGF signaling cascade has been unclear and controversial. Upon treatment of PC12 cells with NGF, diacylglycerol, an endogenous PKC activator, is generated (6) followed by PKC activation (7, 8). PKC activators, such as phorbol esters, mimic certain biological activities of NGF in PC12 cells (9, 10). In addition, NGF has been shown to stimulate translocation of PKC activity and activation of specific isoforms (11, 12). Likewise, certain NGF-specific transcripts are induced in response to PKC (13). A requirement for PKC as part of the induction pathway leading to NGF-stimulated neurite outgrowth has also been documented (14, 15). The PKC inhibitor sphingosine blocks NGF-induced neurite outgrowth in PC12 cells (14) and microinjection of PKC antibodies inhibits NGF-induced neurite outgrowth and c-fos expression (15). In contrast, however, down-regulation or removal of cellular PKC pools by chronic treatment with phorbol esters has no effect on neurite outgrowth (16) or NGF-induced early and secondary responsive gene expression (17, 18). Collectively these findings document that NGF-dependent responses in PC12 cells occur through a pathway that is sphingosine-sensitive and phorbol ester-insensitive. These observations prompted us to characterize the expression of PKC isoforms in PC12 cells (12) and to further investigate the activation of these isoforms in response to NGF (19). We have shown that NGF leads to changes in all PKC isoforms (19, 20). To begin to unravel the role of this multigene family in neuronal differentiation and to provide clear insight into the role of the isoforms in neurite outgrowth, we have employed a reductionist view to their study. Since removal of both cPKC and nPKC does not abrogate NGF responses, we postulated that the aPKC pathway was dominant to those regulated by either the cPKC or nPKC isoforms for differentiation. This would be consistent with the inability of phorbol esters to inhibit NGF-induced neurite outgrowth. Thus, we elected to focus on whether NGF activated the phorbol ester insensitive/atypical PKC-ζ isoform. We documented activation
of atypical/PKC-ζ in response to NGF and parallel inhibition of this isoform by sphingosine (19). Whereas, removal of PKC-ζ attenuates NGF responsiveness (20). Taken together, these findings reveal that NGF-dependent responses leading to neurite outgrowth in PC12 cells are characterized by a pathway that is sphingosine sensitive, phosphor-ester-insensitive involving atypical PKC (14, 16, 19–21).

As an extension of our previous studies, we sought to identify a substrate of PKC-ζ that was integral to NGF responses of PC12 cells, this led to the identification of a nuclear phosphoprotein, pp106. In this report, we characterize pp106 as a specific substrate for PKC-ζ. Purification and amino acid sequence analysis reveal that pp106 is the nuclear protein, nucleolin. These findings provide new clues for the role(s) PKC-ζ plays in NGF signaling and further support a growing body of evidence documenting a role for this kinase in RNA processing.

**EXPERIMENTAL PROCEDURES**

**Reagents—**PC12 cells were obtained from the American Type Culture Collection (Rockville, MD). SB cells and recombinant baculovirus containing the coding regions of PKC-ζ were from the Laboratory of Biochemistry, University of Freiburg, Germany. Plasmids pRCEMVzeta and pRCE-MVzeta were gifts from D. J. Moscat (Consorzio Superiore de Investigaciones Cientificas-Universidad Autonoma de Madrid, Spain). Polyclonal antinucleolin antibody was a gift from Dr. Renato J. Aguilera (University of California at Los Angeles, Department of Biology).

**Materials—**2.5 S NGF and epidermal growth factor were purchased from Bioproducts for Science (Indianapolis, IN). Monoclonal antibody against PKC-ζ was obtained from Transduction Laboratories (Lexington, KY). Monoclonal antibodies against retinoblastoma gene product (p107) and a polyclonal antibodies against both phosphatidylinositol 3-kinase p110 and PKC-ζ were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against topoisomerase I were both from Dr. W. C. Earnshaw (The Johns Hopkins University, MD) and ImmuNoVision (Springdale, AR). Antibodies against PKC-α and PKC-δ were obtained from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology (Lake Placid, NY), respectively. Secondary sheep anti-mouse horseradish peroxidase-labeled antisera, ECL reagents, and Hyperfilm were purchased from Amersham. Goat anti-rabbit/rat Red. E. coli nucleolin was of 0.14 Mr units, phosphorylated with 15 µCi of [γ-32P]ATP (a 1:4 mixture of 200 µCi of γ-32P-ATP and 750 µCi ATP) for 10 min at 30 °C (24). Essentially the same method was used to examine phosphorylation of purified nucleolin by purified PKC-ζ. The reaction was terminated by addition of 100 µl of SDS sample buffer. Thereafter, the sample was boiled for 5 min and separated on 7.5% SDS-polyacrylamide gel electrophoresis. The gel was then stained, destained, dried, and exposed to x-ray film at −80 °C. Changes in the phosphorylation state of pp106/nucleolin were determined by densitometry.

**Expression and Purification of PKC-α, -ζ, -ι—**Spodoptera frugiperda (SB) cells (5 × 10⁶) were seeded onto 100-mm dishes and incubated in IPL-41 insect medium for 1 h at 27 °C. After removing the medium, recombinant baculoviruses containing coding regions of PKC-α, -ζ, and -ι were added at a multiplicity of infection = 10 plaque forming units/cell and incubated with the cells for 1 h. Afterward, the inoculum was removed and 10 ml of fresh medium added. After a 4-day incubation, the medium was harvested at 37 °C in PKC buffer containing 10 mM Tris, 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA, 100 µM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 10 µM aprotinin, 100 µM sodium fluoride) containing 2 mM MgCl₂. Nuclei were isolated following a previously established procedure (21, 23). In brief, cells were incubated at room temperature for 2 min and cooled in ice water for 5 min. Nonidet P-40 was added to a final concentration of 1%. After one dissection through a 20-gauge needle, the nuclear solution was adjusted to 5 mM MgCl₂. The supernatants were taken to be a mixture of cytoplasm and plasma membrane. The nuclear pellet was washed once more with PKC sonication buffer containing 5 mM MgCl₂, resuspended in 300 µl of PKC sonication buffer containing 0.1% Triton X-100, and sonicated for 10 s. Centrifugation of proteins (different fractions were detected using the Bio-Rad dye binding method using bovine serum albumin as a standard. Nuclei obtained in this manner possess intact nuclear membranes and were free from significant cytoplasmic contamination (21).

**Western Blotting—**Western blot analysis of PKC-ζ was carried out using 50 µg of nuclear protein denatured in SDS sample buffer (125 mM Tris, pH 6.8, 20% glycerol, 1.5 mM 2-mercaptoethanol, 15 mM SDS, 0.2 mg/ml bromphenol blue) and separated using a 10% SDS-polyacrylamide gel (19). The separated proteins were transferred to nitrocellulose and processed for immunoblotting with isoform-specific antisera as previously described (19, 20). The relative changes in intensities were determined by densitometry (Molecular Dynamics Personal Densitometer SI, Sunnyvale, CA).

**Expression and Purification of PKC-ζ—**To a reaction (100 µl, total volume), 25 µg of nuclear protein was mixed with 65 µl of pre-mix containing 17.75 mM PIPES, pH 6.5, 10 mM MgCl₂, and 20 µg/ml phosphatidylserine. In the presence or absence of 150 µM PKC-ζ pseudosubstrate peptide, the assay was initiated by adding 5 µl of [γ-32P]ATP (a 1:4 mixture of 200 µCi of γ-32P-ATP and 750 µCi ATP) for 10 min at 30 °C (24). Essentially the same method was used to examine phosphorylation of purified nucleolin by purified PKC-ζ. The reaction was terminated by addition of 100 µl of SDS sample buffer. Thereafter, the sample was boiled for 5 min and separated on 7.5% SDS-polyacrylamide gel electrophoresis. The gel was then stained, destained, dried, and exposed to x-ray film at −80 °C. Changes in the phosphorylation state of pp106/nucleolin were determined by densitometry.

**Cell Culture—**PC12 cells, PC12PKC-ζ+, and PC12PKC-ζ− were seeded onto 100-mm plates coated with rat tail collagen, grown in RPMI 1640 medium, and maintained in a 92% air, 8% CO₂ atmosphere.
loaded onto a heparin-agarose column, washed with column buffer containing 200 mM NaCl. Bound proteins were eluted with column buffer containing 600 mM NaCl. Fractions were collected and an aliquot was examined by SDS-polyacrylamide gel electrophoresis/autoradiography. The fractions that contained pp106 were pooled and glycerol was added to a final concentration of 1%. The pooled fractions were loaded onto a Sephacryl S-200-HR and eluted with S-200 column buffer (20 mM Tris, pH 7.5, 2 mM EGTA, 2 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 2.5 μg/ml P-nitrophenyl phosphate, and 100 μM sodium fluoride). The column was developed with S-200 column buffer. The homogeneity of the purified 106-kDa protein was confirmed by the presence of a single radioactive 106-kDa protein band upon long-term exposure of the autoradiogram and a single protein band upon staining of the gel.

Microsequencing of pp106 Peptides—Purified 106-kDa nuclear protein was isolated, separated by electrophoresis on a 7.5% SDS gel, and transferred to polyvinylidene difluoride. The 106-kDa protein band was excised and subjected to in-gel tryptic digestion. The eluate was dried, redissolved in 0.1% trifluoroacetic acid, and processed for microsequencing. The tryptic peptides were loaded onto an N-terminal protein sequencer (Model 470A, Applied Biosystems, Foster City, CA) for automated Edman degradation. The sequences derived from this analysis were entered into a data bank and compared with entries in the nonredundant protein sequence database.

Immunofluorescence Microscopy—PC12 cells were treated with NGF (50 ng/ml) for 0–30 min (acute). Thereafter, the cells were placed on ice and immediately fixed with cold PBS, followed by isolation of nuclei as described previously (20, 23). The isolated nuclei were resuspended in PBS and allowed to sediment onto polylysine-coated glass slides for 15 min and fixed in 4% (v/v) paraformaldehyde for 20 min. The nuclei were permeabilized by incubation in 80% methanol in PBS for 60 min at −20 °C. Alternatively, localization was monitored in cells that were plated directly onto coverslips that had been coated with a mixture of collagen/ polylysine (4:1, v/v) and treated with NGF (50 ng/ml for 3 days). The coverslips were rinsed with PBS and incubated for 3 min in 2% (v/v) paraformaldehyde in PBS and then incubated for another 3 min in 4% (v/v) paraformaldehyde in PBS. Fixed nuclei or cells were blocked in PBS containing 1% bovine serum albumin and 0.1% (v/v) saponin for 2 h at 27 °C. Thereafter, polyclonal primary antibody either anti-PKC-ζ or anti-nucleolin (1:250) was added in blocking buffer overnight and incubated at 4 °C. The coverslips were rinsed three times, 5 min each, followed by addition of goat-antirabbit IgG-fluorescein isothiocyanate conjugated antibody (12 μg/ml) in blocking buffer for 2 h in the dark at 27 °C. Thereafter, the coverslips were mounted in glycerol/PBS and observed using a Nikon Optiphot epifluorescence microscope. As control, samples were processed without primary antibodies, or in the case of the PKC-ζ antibody that had been previously preincubated with peptide antigen. In either case, no background fluorescence could be detected.

In Vivo Labeling and Immunoprecipitation of Nucleolin/pp106—In brief, PC12 cells were labeled overnight in growth media which had been diluted by half with serum-free/phosphate-free RPMI 1640 containing 10% FCS. The fractions were collected and an aliquot was examined by SDS-polyacrylamide gel electrophoresis/autoradiography. The fractions that contained pp106 were pooled and glycerol was added to a final concentration of 1%. The pooled fractions were loaded onto a Sephacryl S-200-HR and eluted with S-200 column buffer (20 mM Tris, pH 7.5, 2 mM EGTA, 2 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 2.5 μg/ml P-nitrophenyl phosphate, and 100 μM sodium fluoride). The column was developed with S-200 column buffer. The homogeneity of the purified 106-kDa protein band was confirmed by the presence of a single radioactive 106-kDa protein band upon long-term exposure of the autoradiogram and a single protein band upon staining of the gel.

NGF-Induced Phosphorylation of a 106-kDa Nuclear Protein and Translocation of PKC-ζ—We initiated our search for substrates of PKC-ζ that might play a role in NGF signaling by examining nuclear lysates prepared from PC12 cells following exposure to NGF. We observed that phosphorylation of pp106 was enhanced when cells were cultured at low cell density, and to lesser extent, in subconfluent cultures and almost absent in cells cultured at high cell density. In parallel, samples were Western blotted with PKC-ζ antibodies. An NGF-dependent increase in immunoreactivity of PKC-ζ (Fig. 1B) was likewise observed in nuclei. Activity changes in nuclear PKC-ζ were monitored by phosphorylation of e-peptide, ERMRPRKRGSSVRRV, a synthetic peptide corresponding to amino acids 149–164 of PKC-ζ pseudosubstrate motif substituting Ser for Ala in mice (19). NGF treatment resulted in a 35% increase in nuclear PKC-ζ activity, as well as, enzyme translocation from the cytoplasm to the nucleus. As transport of enzyme into the nucleus as observed by immunoelectron microscopy (12). To examine whether NGF could directly promote phosphorylation of pp106 at the nuclear level, isolated nuclei were directly stimulated with NGF: no change in the phosphorylation of the 106-kDa protein was observed (data not shown). To examine the localization of pp106 and PKC-ζ within the nucleus, nuclei were fractionated into envelope and nucleoplasmic fractions. Endogenous phosphorylation analysis revealed that phosphorylated

FIG. 1. NGF induces an increase in the phosphorylation of a 106-kDa nuclear protein paralleled by translocation of nuclear PKC-ζ. PC12 cells were stimulated with NGF (100 ng/ml) for various times (0–30 min) as indicated. A, densitometric scan of an autoradiogram from an endogenous protein phosphorylation assays. Plotted are the relative changes in the phosphorylation state of pp106 as quantitated by densitometry of the autoradiogram. The data are representative of five separate experiments. Inset, autoradiogram showing enhanced phosphorylation of pp106. The positions of the molecular mass standards, 116 and 97 kDa, are shown. B, PC12 cells were stimulated with NGF (100 ng/ml) for various times as indicated. The nuclear fractions were subjected to Western blotting and probed with isofom-specific antisera to PKC-ζ (1:2000). Immunoreactivity was quantitated by densitometry. Inset, a representative autoradiogram of a PKC-ζ immunoblot. The positions of the molecular mass standards, 97 and 66 kDa, are shown.
pp106 was restricted to nucleus and localized within the nucleoplasm (Fig. 2A). PKC-ζ itself was likewise enriched in the nucleoplasm post-NGF treatment (Fig. 2B). Thus, we hypothesized that pp106 might be a direct substrate of PKC-ζ, since it was phosphorylated in a manner that was concomitant with the translocation kinetics exhibited by PKC-ζ (Fig. 1, A and B) and hence was chosen as a candidate protein for further study.

NGF-induced Phosphorylation of pp106 Is Mediated by PKC-ζ—We evaluated the specificity of pp106 to serve as a substrate of PKC-ζ by examining the effect which inclusion of PKC-ζ pseudosubstrate peptide had upon NGF-induced phosphorylation of pp106 both in vivo and in vitro. PKC-ζ pseudosubstrate peptide is a synthetic peptide corresponding to amino acids 113–129 of PKC-ζ regulatory subunit (SIYRGRGARRWRK-PLL), which is homologous between all members of the atypical PKC family. The pseudosubstrate motif suppresses PKC activity by interacting with the substrate-binding pocket in the catalytic domain (28) and can be used as a specific PKC inhibitor (8). Addition of PKC-ζ pseudosubstrate peptide into the endogenous protein phosphorylation assay diminished phosphorylation of pp106 in a dose-dependent manner in the range of 5–200 μM (Fig. 3A). By comparison, addition of pseudosubstrate peptide 19–36, which inhibits classical PKC isoforms (α, β, γ) did not significantly diminish NGF-stimulated phosphorylation of pp106. Likewise, pretreatment of the cells with myristoylated PKC-ζ pseudosubstrate peptide, which can be efficiently transported across the cell membrane (29), inhibited phosphorylation of pp106. In contrast, cPKC 19–36 myristoylated pseudosubstrate peptide, corresponding to the conserved pseudosubstrate motif of PKC-α, -β, or -γ had no inhibitory effect on phosphorylation of pp106 either in vitro or in vivo (data not shown). We next examined whether addition of purified PKC-ζ directly to nuclear extracts would support phosphorylation of pp106. Addition of PKC-ζ likewise resulted in a dose-dependent increase in the phosphorylation of pp106 (Fig. 3B), by comparison no significant change in the phosphorylation state of pp106 was observed following addition of either purified PKC-α or PKC-δ isoforms. Thus, the 106-kDa nuclear protein appears to be a preferred substrate of atypical PKC-ζ compared with either classical or nonclassical PKC.

Overexpression of a mutant PKC-ζ, which rendered the dominant-negative phenotype, was used as another approach to confirm the functional involvement of PKC-ζ in NGF-induced pp106 phosphorylation. pRcCMVζmut construct contains a point mutation in codon 275 resulting in substitution of lysine by tryptophan rendering the enzyme inactive (22). By competing with native PKC-ζ, dominant-negative PKC-ζ inhibited
NGF-induced phosphorylation of the 106-kDa nuclear protein. By comparison, overexpression of PKC-ζ significantly enhanced basal phosphorylation of pp106 which was further enhanced upon addition of NGF (Fig. 4). Similar patterns of phosphorylation were observed independent of atypical PKC gene constructs employed (X. laevis-PKC-ζ, mouse/rat PKC-ζ, or human PKC-ζ). Taken together, these experiments establish pp106 as a likely nuclear substrate of atypical PKC.

pp106 Is Homologous with Nucleolin—A wide spectrum of intracellular proteins have been shown to undergo phosphorylation and dephosphorylation following NGF treatment in PC12 cells (30–32). Several proteins with an approximate mass of 100 kDa likewise exhibit altered phosphorylation states in response to NGF. Proteins such as Nsp100 (33), calmodulin-100 kDa likewise exhibit altered phosphorylation states in cells (30–32). Several proteins with an approximate mass of intracellular proteins have been shown to undergo phosphorylation (35), however, we excluded these proteins since pp106 was not immunoprecipitated by antibodies against either pp106 or nucleolin by phosphoamino acid analysis (Fig. 3). Phosphoamino acid analysis revealed Ser phosphorylation of either pp106 or nucleolin by purified PKC-ζ (27). The purified protein was blotted to polyvinylidifluoride membrane, followed by Lys-C digestion. Four peptides obtained from reverse phase HPLC separation of the digested products were sequenced by automatic Edman degradation (Fig. 5). A SwissProt data base search for similarity revealed the amino acid sequence of each peptide displayed complete homology with that of rat nucleolin, a major nuclear phosphoprotein with apparent mass of 105–110 kDa.

We next set up an in vitro reconstitution assay to examine whether purified nucleolin could be phosphorylated directly by purified PKC-ζ. Increasing concentrations of PKC-ζ lead to increased phosphorylation of nucleolin (Fig. 6A). Phosphorylation of nucleolin was not observed with preparations of either PKC-α or PKC-δ isozymes (data not shown), which was consistent with previous findings (Fig. 3). Phosphoamino acid analysis revealed Ser phosphorylation of either pp106 or nucleolin by PKC-ζ (Fig. 6B). Immunofluorescence was used to examine the localization of both kinase and substrate. To enhance detection, we examined the translocation utilizing intact nuclei rather than whole cells. Nuclei isolated employing this method are concentrated in the nuclear membrane. During longer term treatment (3 days) both nucleolin and PKC-ζ colocalized to the perinuclear region of PC12 cells (Fig. 8, A and B).

To provide further support that nucleolin/pp106 was a PKC-ζ substrate whose phosphorylation is mediated by NGF, the findings were corroborated by an alternate set of experiments. PC12 cells were labeled overnight with orthophosphosphate followed by NGF stimulation and immunoprecipitation with antinucleolin antibody (26). We observed enhanced phosphorylation of pp106/nucleolin in vivo reaching a maximum at 5 min post-NGF treatment (Fig. 9A). The phosphorylation of nucleolin was likewise dependent upon the dose of NGF (Fig. 9B).
In summary, we documented the following: 1) nuclear translocation of PKC-ζ concomitant with phosphorylation of the 106-kDa nuclear protein; 2) direct phosphorylation of pp106 by PKC-ζ and not other PKC isoforms; 3) inhibition of pp106 phosphorylation by atypical pseudosubstrate both in vitro and in vivo; 4) enhanced basal phosphorylation of pp106 by overexpression of PKC-ζ; 5) phosphorylation of purified nucleolin/pp106 by purified PKC-ζ; 6) copurification, as well as, localization of both nucleolin and PKC-ζ to similar sites within the cell; and last, 7) NGF-induced phosphorylation of pp106 in vivo by immunoprecipitation with anti-nucleolin antibody paralleled the findings in vitro. Collectively, these findings demonstrate that pp106 is the nuclear protein nucleolin which serves as a substrate for PKC-ζ and connects NGF cell surface signaling with the nucleus.

DISCUSSION
Atypical PKC-ζ has been implicated in a variety of cellular functions such as maturation of X. laevis oocytes (36), proliferation of mouse fibroblasts (37), maintenance of long term potentiation (38), brain development (39), insulin-induced glucose transport (40), platelet-derived growth factor-stimulated stromelysin gene expression (41), gene transcription through the activation of NFκB (42), a component of the angiotensin II signaling pathway in vascular smooth muscle cells (43), regu-

**FIG. 6.** Purified pp106/nucleolin is phosphorylated by purified PKC-ζ in vitro. A, purified pp106 (0.4 μg) was mixed with various concentrations of purified PKC-ζ in an in vitro reaction. Inset, representative autoradiogram of the reaction. The positions of the molecular mass standards, 116 and 97 kDa, are shown. B, phosphoamino acids analysis of residues phosphorylated by PKC-ζ in either pp106 (lane 1) or nucleolin (lane 2). The amino acid standards are shown.

**FIG. 7.** Temporal localization of PKC-ζ and in PC12 nuclei. PC12 cells were treated with 50 ng/ml NGF (0 min, A/E; 5 min, B/F; 15 min, C/G; and 30 min, D/H), nuclei isolated, followed by staining with antibody to PKC-ζ (A–D) or nucleolin (E–H). Arrow indicates the concentration of PKC-ζ (D) and nucleolin (H) in the nuclear membrane.

**Fig. 8.** Detection of PKC-ζ and nucleolin in differentiating PC12 cells. PC12 cells were treated with 50 ng/ml NGF for 3 days prior to fixation and staining with antibodies against either PKC-ζ (A) or nucleolin (B). Arrow points to colocalization/accumulation of both substrate and kinase in the perinuclear region of PC12 cells.

**FIG. 9.** Treatment of PC12 cells with NGF results in phosphorylation of nucleolin/pp106 in vivo. PC12 cells were labeled with orthophosphate, stimulated with NGF followed by immunoprecipitation of nucleolin and SDS-polyacrylamide gel electrophoresis/autoradiography. A, PC12 cells were treated with 50 ng/ml NGF for various times as indicated. B, autoradiogram showing dose-dependent increases in the phosphorylation of nucleolin by NGF treatment. The positions of the molecular mass standards, 116, 97, and 66 kDa are shown; arrow indicates the position of pp106/nucleolin.
lation of COX transcription (44), NGF responses (21), and more recently PKC-ζ has been shown to play a role in cell death (45).

Nucleolin is a major constituent of nucleoli in exponentially growing cells (46) and functions in the organization of nucleolar chromatin (47), packaging of pre-rRNA (48), rDNA transcription (49), and ribosome assembly by shuttling between the nucleus and the cytoplasm (50). In addition, nucleolin has been reported to serve as substrate for casein kinase II during interphase of the cell cycle (51, 52) and for the cell cycle-regulatory cdc2 kinase during mitosis (53, 54). Nucleolin shuttles from nucleus to cytoplasm, however, we observed that only the protein localized within the nucleus is phosphorylated by PKC-ζ. It is possible that the protein assumes an alternate conformation upon exit from the nucleus which masks the phosphorylation site. Interestingly, phosphorylation of nucleolin has been shown to regulate its helicase activity (55) and thus, phosphorylation by PKC-ζ, as well as other kinases may likely regulate its functional abilities in chromatin organization, rRNA packaging, rDNA transcription, or ribosome assembly (56). The translocation of PKC-ζ itself may be directed by a bipartite nuclear targeting motif within the enzyme (20) or proteins which facilitate import into the nucleus. Although, fibroblast growth factor-2 has been shown to directly stimulate phosphorylation of nucleolin through a casein kinase II-mediated pathway directly at the nuclear membrane (56), we failed to observe enhanced phosphorylation of nucleolin by NGF under similar conditions. Thus, this observation further underscores the differences in the two growth factors and suggests that NGF mediates movement of PKC-ζ into the nucleus by a pathway that originates at the plasma membrane. Alternatively, the local concentration of second messenger within the vicinity of the nucleus may specifically activate PKC-ζ within that particular microenvironment. In this regard, PKC-ζ is activated by phosphatidic acid, which is generated by the activation of phospholipase D that is also localized to the nuclear membrane (57, 58) and may play a role as a second messenger.

Interestingly, overexpression of X. laevis PKC-ζ resulted in a somewhat unexpected finding. Although enhanced phosphorylation of pp106 was observed by introduction of PKC-ζ construct, overexpression itself did not result in a dramatic increase in NGF-stimulated phosphorylation levels. To rule out any possible differences in gene constructs we have employed a critical study of various genes of the atypical family.6 The X. laevis gene originally cloned by Moscat and colleagues (36) displays 90% homology with the human PKC-ζ cloned by Selbie et al. (59). PKC-ζ is likewise homologous to the mouse PKC-λ. Using various constructs of atypical PKCs (X. laevis PKC-ζ (36), mouse PKC-ζ (60), and human PKC-λ (59)) we have found no differences in NGF-mediated responses in the phosphorylation pattern to nucleolin.6 Both genes, s/λ, and ζ, are highly homologous encoding proteins with similar functional properties. A growing body of data suggests that signaling pathways likely maintain their differential specificity depending upon the cellular background in which they are expressed. Thus, it is possible in cells other than PC12 that atypical PKCs-s/λ and ζ may possess differential modes of regulation or that nucleolin may be phosphorylated in a differential manner. Our overexpression studies therefore suggest that atypical PKC may interact with other upstream signaling pathways since the basal phosphorylation state of the downstream target, nucleolin, was enhanced. The findings presented herein, along with other studies (12, 61–63), further implicate a role for this PKC-ζ in mediating nuclear responses. Interestingly, PKC-ζ accumulates within the perinuclear membrane during longer term treatment with NGF and thus, may play a role in signals necessary for longer term morphological differentiation.

Nucleolin has been shown to preferentially interact with some specific regions of DNA (64, 65) with the nonphosphorylated form serving as a negative regulator of transcription (46, 48), perhaps by altering the topography of DNA (55). Nucleolin also binds to the 3′-untranslated region of amyloid protein precursor (APP) mRNA (66). Thus, a possible role for PKC-ζ is to modulate nucleolin-RNA interactions. In this regard, hnRNP A1 has been shown to serve as a substrate of PKC-ζ (67); phosphorylation impairs hnRNP A1 RNA binding and its ability to promote strand annealing. Both hnRNP A1 and s/λ share similar properties in their ability to bind reiterated AUUUA sequences (68). We speculate that PKC-ζ may regulate the formation of a complex between hnRNP A1 and nucleolin and thus affects its ability to bind certain mRNAs, such as APP. Additionally, the APP gene itself may be directly regulated by PKC-ζ. Previous findings have shown that s/λ promoter activation to be dependent upon PKC-ζ (42). APP has both a s/λ regulatory site (69) and is also regulated by NGF (70). Thus, APP may serve as an example of a gene whose expression is regulated by the “double-control” model (67) subject to coordinate regulation both at the transcriptional and translational levels by PKC-ζ.

Numerous questions remain to be answered such as the signaling route taken to mediate this response; the physiological second messenger signal cascade that drives this response; the relationship of NGF-receptor components in this process; the effect of PKC-ζ mediated phosphorylation on shuttling of nucleolin; and effects of differential phosphorylation by cdc2, casein kinase II, and PKC-ζ on RNA binding and helicase activity. Obviously there is a great deal more work that is needed to address these questions.

Acknowledgments—We thank members of our laboratory, as well as, Drs. Anthony Moss, James Sartin, John Weete, and Catherine Wernecke for reading, assistance, and discussion during the course of this study. We thank Dr. Carol M. Beach (University of Kentucky, Lexington, KY) for assistance with microsequence analysis.

REFERENCES

1. Levi-Montalcini, R. (1987) EMBO J. 6, 1145–1154
2. Greene, L. A., and Tischler, A. S. (1982) Adv. Cell Neurobiol. 3, 373–414
3. Parker, P. J., Kour, G., Marais, R., Mitchell, F., Pearse, C., Schap, D., Stabel, S., and Webster, C. (1989) Mol. Cell. Endocrinol. 65, 1–11
4. Nishizuka, Y. (1992) Science 258, 607–614
5. Hug, H., and Sarre, T. F. (1985) Biochem. J. 291, 329–343
6. Chan, B. L., Chao, M. V., and Saltiel, A. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1756–1760
7. Hama, T., Huang, K.-P., and Guroff, G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2353–2357
8. Hesaley, L. E., and Johnson, G. L. (1989) Mol. Pharmacol. 35, 331–338
9. McTigue, M., Cremins, J., and Halegoua, S. (1985) J. Biol. Chem. 260, 9041–9056
10. Greenberg, M. E., Greene, L. A., and Ziff, E. B. (1985) J. Biol. Chem. 260, 14101–14110
11. Kondratyev, A. D., Popova, O. N., Severin, S. E., Choladze, M. A., Shmyrev, I. I., Tubasheva, I. A., Zaitseva, E. E., Posypanova, G. A., and Severin E. S. (1990) FEBS Lett. 264, 75–77
12. Wooten, M. W., Zhou, G., and Wooten, M. W., Coleman, E. S. (1994) Cell Growth & Differ. 5, 395–403
13. Altmann, D. S., and Neet, K. E. (1989) J. Biol. Chem. 264, 3538–3544
14. Machida, C. M., Scott, J. D., and Ciment, G. (1991) J. Cell Biol. 114, 1037–1048
15. Szeberenyi, J., Erhardt, P., Cai, H., and Cooper, G. M. (1992) Oncogene 7, 2105–2113
16. Wooten, M. W., Zhou, G., Seibenhener, M. L., and Coleman, E. S. (1994) Cell Growth & Differ. 5, 395–403
17. Coleman, E. S., and Wooten, M. W. (1994) J. Mol. Neurosci. 5, 39–57
18. Wooten, M. W., Zhou, G., and Seibenhener, M. L. (1997) J. Neurosci. Res. 49, 1–11
19. Berra, E., Diaz-Meco, M. T., Dominguez, I., Municio, M. M. Sastre, L., Lozano, J., Chapkin, R. S., and Moscat, J. (1993) Cell 74, 555–563

3 M. L. Seibenhener, Y. M. Wang, J. Heikila, G. Zhou, J. O. Weete, and M. W. Wooten, manuscript in preparation.
