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Nerve Growth Factor Stimulates the Interaction of ZIP/p62 With Atypical Protein Kinase C and Targets Endosomal Localization: Evidence for Regulation of Nerve Growth Factor-Induced Differentiation

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Abstract Atypical protein kinase Cs zeta and lambda/iota play a functional role in the regulation of NGF-induced differentiation and survival of pheochromocytoma, PC12 cells [Coleman and Wooten, 1994; Wooten et al., 1999]. Here we demonstrate an NGF-dependent interaction of aPKC with its binding protein, ZIP/p62. Although, ZIP/p62 was not a PKC-ι substrate, the formation of a ZIP/p62-aPKC complex in PC12 cells by NGF occurred post activation of PKC-ι and was regulated by the tyrosine phosphorylation state of aPKC. Furthermore, NGF-dependent localization of ZIP/p62 was observed within vesicular structures, identified as late endosomes by colocalization with a Rab7 antibody. Both ZIP/p62 as well as PKC-ι colocalized with Rab7 upon NGF stimulation. Inhibition of the tyrosine phosphorylation state of PKC-ι did not prevent movement of ZIP/p62 to the endosomal compartment. These observations indicate that the subcellular localization of ZIP/p62 does not depend entirely upon activation of aPKC itself. Of functional importance, transfection of an antisense p62 construct into PC12 cells significantly diminished NGF-induced neurite outgrowth. Collectively, these findings demonstrate that ZIP/p62 acts as a shuttling protein involved in routing activated aPKC to an endosomal compartment and is required for mediating NGF's biological properties. J. Cell. Biochem. 82: 452–466, 2001.

Key words: PC12; atypical protein kinase C; scaffold; nerve growth factor; tyrosine phosphorylation; neurotrophin

Protein kinase C (PKC) represents a family of structurally and functionally related serine/threonine kinases. Conventional or classical PKCs (cPKCs: α, βI, βII, γ) require Ca^{2+} and diacylglycerol (DAG) for activity; novel PKCs (nPKCs: δ, ε, η, σ) require only DAG; atypical PKCs (aPKCs: ζ and λ,/ι) require neither Ca^{2+} nor DAG. The cPKCs and nPKCs likewise bind phorbol ester and are subject to down regulation [Nishizuka, 1992; Toker, 1998]. Atypical PKCs ζ and λ/ι are highly homologous isoforms and respond to phosphatidylinositol 3,4,5-triphosphate rather than DAG or Ca^{2+} for activation [Nakanishi et al., 1993; Selbie et al., 1993; Toker et al., 1994].

Notably, the aPKC isoforms are positioned upstream of NF-κB providing a regulatory role and protection against apoptosis [Diaz-Meco et al., 1993; Murray and Fields, 1997; Jamieson et al., 1999; Wooten et al., 1999]. In pheochromocytoma (PC12) cells, nerve growth factor (NGF) activates the aPKCs [Wooten et al., 1992, 1994, 1997]. Moreover, NGF's biological responses are dependent upon aPKC; the removal of aPKC inhibits cell differentiation as well as NGF-induced NF-κB activation [Coleman and Wooten, 1994; Wooten et al., 1999]. Conversely, overexpression of aPKC enhances PC12 cell survival and NGF responsiveness [Wooten et al., 1999].

Abbreviations used: NGF, nerve growth factor; ZIP/p62, Zeta-interacting protein; aPKC, atypical protein kinase C; PC12, pheochromocytoma.

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Migration of PKC, post activation, occurs in part through interaction with intramolecular receptors or binding proteins [Mochly-Rosen, 1995]. These proteins are believed to play a role in shuttling PKC to specific subcellular sites, or acting as substrates, are downstream effectors for activated PKC [Jaken, 1996; Mochly-Rosen and Gordon, 1998]. Binding proteins may also play a separate role serving as scaffolds to anchor the activated enzyme at a distinct subcellular site [Faux and Scott, 1996]. Thus signal specificity and localization of PKC is likely mediated by protein–protein interactions between the activated enzyme and the binding protein.

In searching for proteins that would bind aPKC, full length rat PKC-ζ DNA was used as bait for yeast two-hybrid cloning. A 65 kDa protein, named ZIP, zeta-PKC interacting protein [Puls et al., 1997] characterized by 1317 nucleotides coding for 439 amino acids was isolated from a rat brain cDNA library. A second independent screen of a human kidney cDNA library using the regulatory domain of zeta PKC as bait cloned a protein, p62, which was shown to specifically associate with both atypicals, PKC-ζ and PKC-λ/ι, but neither the classical or novel PKC isoforms [Sanchez et al., 1998]. P62 is the human homolog of rat ZIP, both proteins bind to the pseudosubstrate region in the regulatory domain of the aPKCs [Puls et al., 1997; Sanchez et al., 1998]. The structure of ZIP/p62 is defined by a ZZ zinc finger, two PEST sequences, and a unique putative protein-binding motif, $YXDEDX_5SDEE/D$[Puls et al., 1997]. ZIP/p62 is also homologous to a protein cloned as the phosphotyrosine-independent $p56^{lck}$ SH2-interacting protein, p62. In addition, ZIP/p62 shares 90% homology with A170, another SH2-binding protein [Ishii et al., 1997; Puls et al., 1997; Yanagawa et al., 1997]. Recent studies have shown ZIP/p62 to be evolutionarily conserved displaying ubiquitous tissue distribution [Gong et al., 1999].

Although aPKCs are required for NGF's effects [Coleman and Wooten, 1994], the contribution that ZIP/p62 plays in NGF signaling has not yet been examined. In this study, the role of NGF in mediating ZIP/p62-PKC-ι interactions, the factors that regulate the formation of the complex and the role of ZIP/p62 in subcellular routing of PKC-ι was examined. We demonstrate that the ZIP/p62-aPKC complex is localized to an endosomal compartment post-NGF treatment. In addition, we find that activation of PKC-ι via tyrosine phosphorylation enhances interaction between PKC-ι and ZIP/p62. Of functional relevance, removal of p62 diminished NGF-induced neurite outgrowth. Collectively, these data support the notion that ZIP/p62 is a shuttling protein that is involved in routing activated aPKC to an endosomal compartment.

MATERIALS AND METHODS

Materials

PC12 and HEK 293 cells were obtained from the American Type Culture Collection (Rockville, MD). Antibody to ZIP/p62 was a gift from Axel Puls (Kings College, London, England), while Suzanne Piefer (Stanford Medical School, Stanford, CA) provided affinity purified Rab7 antibody. M.T. Diaz-Meco and Jorge Moscat (Madrid, Spain) provided GFP-p62, antisense p62 and MBP-hnRNPA1. PKC-ι, α, δ, LAMP1, myc, and p62 monoclonal antibody was obtained from Transduction Labs (San Diego, CA). Antibodies to src and purified src enzyme were obtained from Upstate Biotechnology (Lake Placid, NY). Texas Red and Oregon Green conjugated antibodies were obtained from Molecular Probes (Eugene, OR). The ε-peptide (ERMRPRKRQGSVRRRV) was synthesized by the Macromolecular Structure Analysis Facility at the University of Kentucky (Lexington, KY). Goat anti-mouse and goat anti-rabbit horseradish peroxidase, ECL reagents and Hyperfilm were obtained from Amersham (Arlington Heights, IL). All other reagents and antibodies were obtained from Sigma (St Louis, MO).

Cell Culture and Treatment

PC12 cells were seeded onto 100 mm plates coated with rat tail collagen, grown in RPMI containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin), and maintained in a 92% air, 8% CO_2 atmosphere. HEK 293 cells were seeded similarly and grown in DMEM containing 10% heat-inactivated fetal calf serum, the same antibiotics as PC12 cells, and were maintained in a 95% air, 5% CO_2 atmosphere. Prior to experimentation, cells were incubated in reduced serum overnight at a ratio of 1 ml complete conditioned media to 5 ml serum free

media followed by stimulation with 100 ng/ml NGF at 37°C.

Immunoprecipitation

PC12 cells were harvested and pelleted by centrifugation, 8 min, 3,000 rpm, at 4°C. Cell pellets were then resuspended in cold PBS and spun down for 4 min at 4°C, 20,800*g*. PBS was removed and samples were sonicated for 8 s in 250 μl lysis buffer [Puls et al., 1997] (300 mM NaCl, 1.0% Triton X-100, 1.0% deoxycholic acid, 0.1% SDS, 50 mM NaF, 100 μM sodium orthovanadate, 40 mM $Na_4P_2O_7$, 5 mM EDTA, 20 mM EGTA, 25 mM HEPES, pH 7.6) at 4°C in the presence of protease inhibitors (20 μg/ml Aprotinin, 10 μg/ml Leupeptin, 1 mM PMSF). Lysates were incubated for 30 min at 4°C then diluted with 2.5 volumes of binding buffer (300 mM NaCl, 2.5 mM $MgCl_2$, 50 mM NaF, 100 μM sodium orthovanadate, 40 mM $Na_4P_2O_7$, 100 μM EDTA, 20 mM HEPES, pH 7.6, plus protease inhibitors). Samples were precleared with protein A-agarose, anti-rabbit agarose, or anti-mouse agarose for 30–45 min with end-over-end mixing followed by centrifugation at 4°C for 3 min (20,800*g*), and were normalized for total protein concentration by Bio-Rad dye assay with a BSA standard. Primary antibody was added and samples were incubated for 1.5 h at 4°C followed by addition of 30 μl of appropriate IgG agarose beads. Incubation was extended for an additional 1.5 h at 4°C. Immunoprecipitates were then washed four times in binding buffer, SDS sample buffer was added, and samples were boiled prior to SDS–PAGE (7.5%).

Immunecomplex Kinase Assay

PC12 cells were incubated for different times with NGF, extracted in lysis buffer (50 mM Tris [pH 7.5], 150 mm NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, protease inhibitors), and immunoprecipitated with affinity-purified rabbit polyclonal antibody. Immunoprecipitates were washed five times with lysis buffer containing 0.5 M NaCl. Samples were then resuspended in kinase buffer (35 mM Tris–HCl [pH 7.5], 10 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM $CaCl_2$, 1 mM *p*-nitrophenyl phosphate) containing 1 μg of myelin basic protein (MBP) and 5 μCi (100 mM) of [γ-^{32}P] ATP for 30 min at 30°C in a final volume of 40 μl. Reactions were terminated by addition of SDS sample buffer and separated by SDS–PAGE followed by exposure to X-ray film and quantitation with a computer-interfaced densitometer. MBP phosphorylation was normalized to the amount of PKC-ι in each sample and expressed as specific activity in arbitrary units [Wooten et al., 2000].

Western Blotting

Extracts were denatured in SDS sample buffer (125 mM Tris, pH 6.8, 20% glycerol, 1.5 M 2-mercaptoethanol, 15 mM SDS, 0.2 mg/ml bromophenol blue) and separated using SDS/PAGE. The separated proteins were transferred to nitrocellulose. Post-transfer, the blots were stained with Ponceau stain and marked, blocked by incubation in blocking buffer, 5% nonfat milk in PBS (ZIP/p62) or 7% nonfat milk in PBS (PKC-ζ, ι, δ, α) at 4°C for 2 h, and followed by incubation with antisera diluted in blocking buffer overnight at 4°C with rocking. Dilutions of antisera were: ZIP/p62 1:10,000, PKC-ι 1:2,000, PKC-δ1:1,000, or PKC-α 1:5,000. After incubation, the blots were washed for 4 × 15 min in PBS, pH 7.3, containing 0.1% Triton X-100 and 0.1% Tween 20, followed by incubation with 1:3000 dilution of goat anti-mouse horseradish peroxidase antisera in PBS, pH 7.3, containing 0.05% Triton X-100 and 0.05% Tween 20 for 2 h at room temperature with rocking. Blots were washed 8 × 15 min in PBS, pH 7.3, containing 0.1% Triton X-100 and 0.1% Tween 20, followed by antigen-antibody complex detection with ECL.

Expression and Purification of PKC-ι

In brief, *Spodoptera frugiperda* (SF9) cells (5×10^6) were seeded onto 100 mm dishes and incubated in IPL-41 insect medium for 1 h, 27°C. After medium was removed, recombinant baculoviruses containing coding regions of PKC-ι were added at a multiplicity of infection = 10 plaque forming units/cell and incubated with the cells for 1 h. The inoculum was then removed and 10 ml of fresh media was added. After a 4 day incubation, the cells were harvested, lysed at 4°C in PKC buffer (20 mM Tris, pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA, 100 μM PMSF, 1% Nonidet P-40) followed by centrifugation at 1000*g*, 15 min. The supernatant was collected and used to purify individually expressed PKC isoforms as described previously [Zhou et al., 1997]. The identity of purified PKC-ι was confirmed by gel staining and Western Blot analysis.

In Vitro Protein Phosphorylation

To a reaction, ZIP/p62, GST-ZIP/p62, MBP, MBP-hnRNPA1, or ε-peptide (ERMRPRKRQGSVRRRV) was incubated with PKC-ι (700 ng) in 17.75 mM PIPES, pH 6.5, 10 mM $MgCl_2$, 20 μg/ml phosphatidyl serine. The assay was initiated by adding 5 μl of [γ^{32}P] ATP (a 1:4 mixture of 200 μCi of [γ ^{32}P] ATP and 750 mM ATP) for 10 min, 30°C. The reaction was spotted onto Whatman P81 paper and Cerenkov counted. Alternatively, ZIP/p62 was phosphorylated by PKC-ι and terminated by addition of 100 μl of SDS sample buffer. The samples were subsequently boiled for 5 min and separated by SDS–PAGE (12%). The gel was stained, destained, dried, and exposed to X-ray film at −80°C. Relative changes in the phosphorylation of ZIP/p62 were determined by densitometry.

Preparation of GST-ZIP Fusion Proteins

A single colony of *E. coli* cells containing the recombinant GST-ZIP/p62 plasmid was inoculated into 10 ml of LB medium overnight at 37°C. The culture was diluted 1:100 into fresh, warm LB media with 50 μg/ml ampicillin at 37°C until the A600 reached 0.8–1.0. The culture was induced with 0.2 mM IPTG at 25°C overnight. The bacteria were collected by centrifugation, 4,000*g*, 20 min, 4°C and resuspended in 15 ml MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3) +1% Triton-X 100 + 500 mM PMSF, 5 μg/μl Leupeptin, 3.3 μg/ml Aprotinin. Lysozyme was added (1 mg/ml) and incubated at 4°C for 20 min. The cells were further lysed by sonication at 4°C. DNAse (4 μg/ml) and RNAse (2 μg/ml) were subsequently added. The lysates were then incubated for 20 min, 4°C followed by centrifugation, 15,000 × RPM, 45 min, at 4°C. To the supernatant, 50% glutathione-agarose beads in MTPBS were added and the mixture was incubated for 1 h, 4°C, with rotation. Beads were collected by brief centrifugation at 600*g*, 4°C, washed 3× in MTPBS, made 50% in MTPBS and 10% glycerol was added for storage. Protein concentration was determined using the Bio-Rad dye assay with a BSA standard.

Cobinding Assays

To an assay, 1.25 μg of PKC-ι and 12.5 μl of src buffer (100 mM Tris, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 2 mM DTT, 0.25 mM Na_3VO_4) was added. Assays were conducted in the absence or presence of increasing concentrations of src (1.5–6 Units) in a final volume of 45 μl. Reactions were initiated by adding 1 mM cold ATP and incubated for 10 min at 30°C. The reaction was terminated on ice and GST-ZIP/p62 equilibrated in MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3) was added at a ratio of 5:1 (ZIP:PKC-ι). Samples were rotated end-over-end for 3 h, 4°C, and then pelleted by centrifugation. The beads were washed 5× in MTPBS. SDS-sample buffer was added to each sample, followed by SDS–PAGE (10%), transfer to nitrocellulose and probed for PKC-ι-ZIP/p62 complexes by Western blot of PKC-ι. Blots were scanned and differences in PKC-ι bound to ZIP/p62 were evaluated by comparison of PKC-ι input into the cobinding assay vs. the amount recovered complexed to ZIP/p62. Equivalent amount of GST-ZIP/p62 added into the cobinding assay was verified by stripping blots (rinse 1 × PBS, wash 2 × 15 min each 0.1 M glycine, pH 2.5, wash 2 × 5 min each 10 M Tris, pH 8, wash 3 × 5 min each PBS + 0.1% Triton X-100 + 0.1% Tween 20) and reprobing for ZIP/p62 by Western blot.

Immunofluorescence Microscopy

PC12 cells were grown at 37°C on glass coverslips treated with polylysine in 24 well plates until confluency and treated with 100 ng/ml NGF, washed with PBS at 4°C and then incubated at 37°C for varying times. Cells were washed 2× with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature (RT), and rinsed again with PBS. Cells were permealized with 80% MeOH at −20°C for 10 min then washed with PBS+0.05% Triton X-100 and rinsed again with PBS. Aldehydes were quenched with 0.05 mM NH_4Cl for 1 min and then rinsed again with PBS followed by blocking with 1% BSA/PBS for 2 h at RT. Cells were treated with primary antibody: ZIP/p62 (1:250) (made in 1% BSA/PBS block) overnight at 4°C in humidified chambers and then washed 2× with PBS. Texas Red (8 μg/ml), was added in block for 2 h at RT in the dark. Coverslips were washed 5× in PBS + 0.2% Tween 20, rinsed in PBS, dH_2O, blotted dry and mounted and sealed onto slides. Double labeling experiments were conducted by transiently transfecting GFP-p62 followed by staining with either Rab7 (1:100), LAMP1 (1:100) or myc (1:250). Double label of endogenous proteins was employed for the localization of PKC-ι (1:100) and Rab7

(1:100), p62 (1:250) and Rab 7 (1:100) or PKC-ι (1:100) and p62 (1:250) along with goat anti-mouse Oregon Green and goat anti-rabbit Texas Red.

Subcellular Fractionation

In brief, to isolate various subcellular fractions (cytosol, endosomes, golgi, lysosomes, plasma membrane, nucleus) a previously established and well characterized protocol was employed [Sanchez et al., 1998]. PC12 cells were harvested and pelleted by centrifugation, 8 min, 3,000 rpm, at 4°C. Cell pellets were resuspended in cold PBS and spun down for an additional 8 min at 4°C, 3,000 rpm. Subcellular Fractionation Buffer (SCFB: 10 mM NaCl, 10 mM Tris, pH 7.5, 2 mM EGTA, 25 μg/ml Aprotinin, 25 μg/ml Leupeptin, 1 mM PMSF, 100 μM NaF) + 0.08% NP-40 was added to the pellet. Cells were then homogenized in a small Dounce homogenizer with a B pestle on ice for 50 strokes. After homogenization, the nuclear fraction was pelleted at 500*g* at 4°C and washed twice in SCF buffer + 10% sucrose + 1 mM $MgCl_2$ by centrifugation at 500*g* for 5 min at 4°C. Nuclear fractions were resuspended in SCFB + 250 mM sucrose + 5 mM $MgCl_2$, DNAse and RNAse were each added to 100 μg/ml, and fractions were rotated at 4°C for 1.5 h. Fractions were then centrifuged at 650*g* for 10 min and pellets were resuspended in 300 μl SCF Sonication buffer (20 mM Tris, pH 7.5, 1 mM Na_3VO_4, 100 μM NaF, 10 mM β-glyceroPO_4, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 1 mM PMSF, 10 μg/ml Leupeptin, 5 μg/ml Aprotinin). To the fraction, 1.2 ml of SCFB + 2.0 M NaCl was added dropwise and fractions were rotated for 30 min at 4°C. Postnuclear supernatant was collected, layered on top of a 62% sucrose solution and centrifuged at 27,000 rpm for 45 min in a Ti50.4 rotor. Interfaces were made to 45% sucrose and used in a sucrose step gradient: 1 ml 45% sucrose (interfaces), 750 μl 32% sucrose, 750 μl 18% sucrose. Gradients were centrifuged at 33,000 rpm for 60 min in a Ti50.4 rotor and fractions collected as follows: 18% sucrose, small membranes; 18/32% interface, endosomes; 32% sucrose, golgi; 32/45% interface, lysosomes; 45% sucrose, plasma membranes. Protein concentration was determined using the Bio-Rad dye assay with a BSA standard. Fractions obtained in this manner were used for immunoprecipitation or Western blot analysis.

Neurite Outgrowth

PC12 cells were transiently transfected using Lipofectamine 2000. Twenty-four hours later NGF (50 ng/ml) was added. The percentage of cells with neurite processes greater than two cell bodies in length was counted three days after addition of NGF. At least 300 cells were counted per treatment by an individual blind to the experimental design.

RESULTS

ZIP/p62 Associates With the aPKCs

To investigate the specificity of ZIP/p62's interaction with each of the PKC isoforms, PC12 cells were treated in a time frame where PKC-ι is maximally activated by NGF, 15 min [Wooten et al., 1992, 1994], followed by immunoprecipitation with anti-ZIP/p62 antibody. Both the protein bound by ZIP/p62 antibody and the unbound protein were immunoblotted with anti-PKC-α, PKC-δ, and PKC-ι antibodies (Fig. 1). Notably, only PKC-ι, but neither PKC-α nor PKC-δ, bound ZIP/p62. These findings thus validate and confirm the previous observation that ZIP/p62 selectively interacts with the atypical PKC, PKC-ι [Puls et al., 1997; Sanchez et al. [1998].

Studies were undertaken to determine if ZIP/p62 would serve as a substrate of PKC-ι. The phosphorylation of ZIP/p62 captured in an immune-complex, or as a GST-tagged protein was compared to other known substrates of PKC-ι (Fig. 2). Myelin basic protein (MyBP), and a peptide coding for amino acids 149–164 of the PKC-ε pseudosubstrate motif, which substitutes Ser for Ala^{159}, (ε peptide) have been identified as substrates for aPKCs [Kazanietz et al., 1993]. Substrate phosphorylation obtained with these substrates was compared to that obtained with hnRNPA1 [Municio et al., 1995]. ZIP/p62, compared to these well-defined aPKC substrates, is a relatively poor substrate (Fig. 2). These findings confirm the initial studies of Sanchez et al. [1998].

NGF Induces Formation of a ZIP/p62-aPKC Complex That is Modulated by Tyrosine Phosphorylation

Since NGF activates PKC-ι whereby it plays a role in mediating NGF responses [Wooten et al., 1992, 1994, 2000], we speculated that NGF may modulate formation of a ZIP/p62-PKC-ι

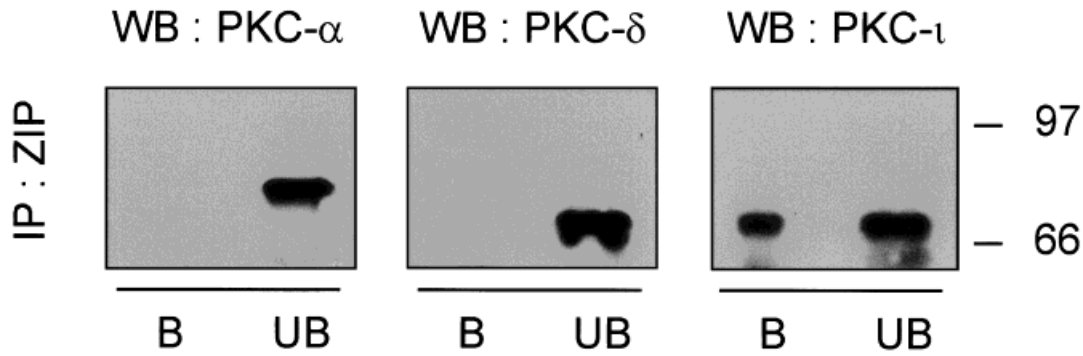

Fig. 1. The aPKCs coassociate with ZIP/p62. PC12 cell lysates treated with NGF 100 ng/ml for 15 min were immunoprecipitated with ZIP/p62 antibody. The immunoprecipitates (B) were immunoblotted and their unbound supernatants (UB) were Western blotted with anti-PKC-α, anti-PKC-δ and anti-PKC-ι antibodies. Similar results were obtained in three other experiments.

complex. To address this possibility, PC12 cells were stimulated by NGF and immunoprecipitated with anti-ZIP/p62 antibody followed by Western blot analysis with an anti-PKC-ι antibody. In resting unstimulated cells, a minor amount of ZIP constitutively associated with aPKC. However, treatment of PC12 cells with NGF induced a ~4-fold increase in the amount of PKC-ι bound to ZIP/p62 (Fig. 3A). NGF did not alter the levels of aPKC nor ZIP/p62 in cells. The interaction of PKC-ι with ZIP/p62 was transient. Also, changes in the concentration of NGF resulted in modulating the amount of PKC-ι bound to ZIP/p62 (Fig. 3B). Thus, the interaction between PKC-ι and p62 was regulated by NGF. To determine whether activation of PKC-ι preceded interaction between PKC-ι and p62, the activity of PKC-ι was determined by immunecomplex kinase assay. Maximum activation of PKC-ι by NGF took place between 5–15 min (Fig. 3C). By comparison, NGF-stimulated interaction between PKC-ι and ZIP/p62 was maximized within a 15–30 min time interval (Fig. 3A). Furthermore, decline in the amount of PKC-ι bound to ZIP/p62 (Fig. 3A), coincided with a parallel decline in the activity of PKC-ι (Fig. 3C). These findings suggest that upon activation of aPKC, the ZIP/p62 binding site is exposed. Thus, we postulated that activated enzyme induced a favorable conformation promoting association of PKC-ι with ZIP/p62, whereas inhibition of enzyme activity may result in diminished coassociation between the two proteins.

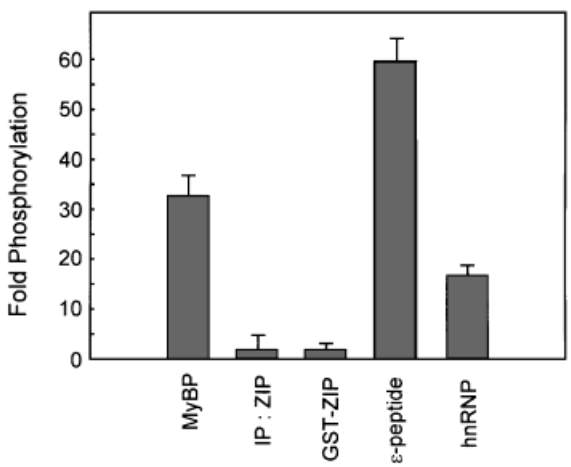

Fig. 2. ZIP/p62 is a poor substrate of PKC. The phosphorylation of known PKC-ι substrates, Myelin basic protein (MyBP), ε peptide, and MBP-hnRNPA1was compared to the phosphorylation of ZIP/p62 (immunoprecipitated ZIP/p62 from PC12 cells or a GST-ZIP/p62 fusion protein). The data were normalized on an equimolar basis for the amount of substrate input into the reaction and plotted as fold phosphorylation. Similar results were obtained in three other experiments.

We and others have previously shown that PKC-ι is phosphorylated and activated by the tyrosine kinase, src [Gschwendt et al., 1994; Konishi et al., 1997; Seibenhener et al., 1999; Wooten et al., 2001b]. Therefore, we sought to test whether tyrosine phosphorylation of PKC-ι by src might also regulate its interaction with ZIP/p62. Initially blots from Figure 3A and B were stripped and reprobed with anti-ptyr 4G10 antibody. However, due to low antibody specificity and the small amount of tyrosine-phosphorylated PKC-ι within the complex the tyrosine phosphorylation of PKC-ι bound to ZIP/p62 could not be assessed in this manner. Therefore, a GST-pull down assay was employed as an in vitro means to measure the influence that PKC-ι's tyrosine phosphorylation

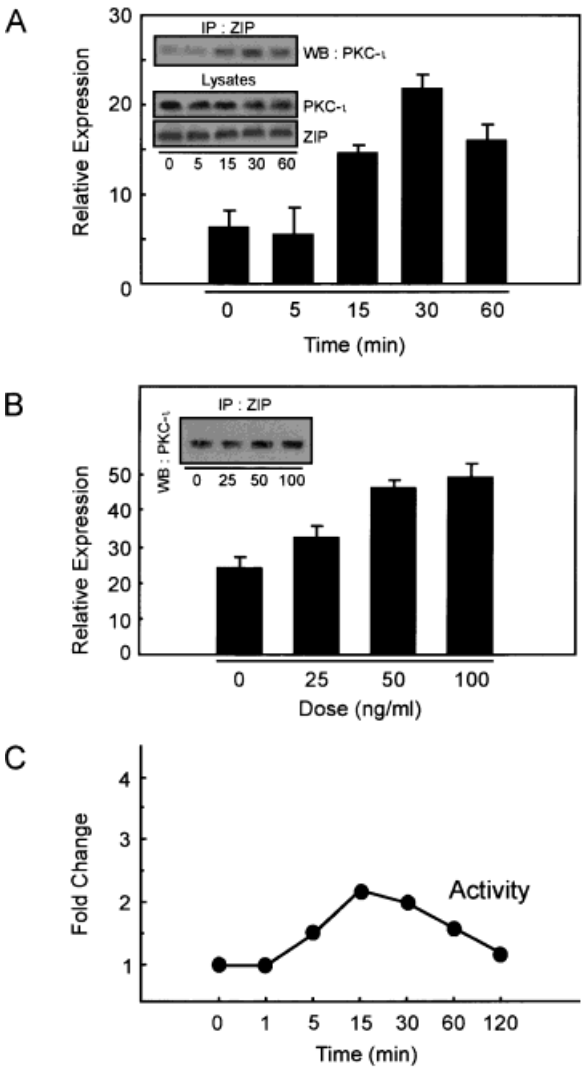

Fig. 3. NGF stimulates the formation of a ZIP/PKC-ι complex. **A:** PC12 cells were treated with NGF (100 ng/ml) for various times (0–60 min). The cell lysates were immunoprecipitated with anti-ZIP/p62 antibody and Western blotted with anti-PKC-ι antibody. Cell lysates (60 μg) were probed with antibody to both PKC-ι and ZIP/p62. **B:** PC12 cells were treated with increasing doses of NGF 0, 25, 50, 100 ng/ml, followed by immunoprecipitation with anti-ZIP/p62 antibody and Western blotted with anti-PKC-ι antibody. **C:** PC12 cells were treated with 100 ng/ml NGF followed by immunecomplex kinase assay of aPKC activity using PKC-ι antibody and ε-peptide as substrate. Similar results were obtained in three other independent experiments.

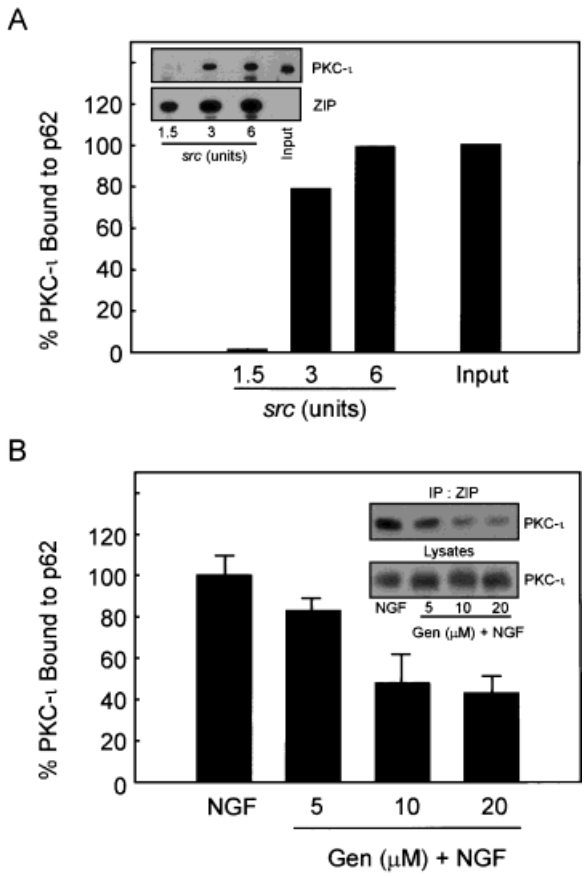

Fig. 4. Src phosphorylation of PKC-ι modulates interaction with ZIP/p62. **A:** Cobinding assays were prepared as described in Materials and Methods. Src (0–6.0 units) was added to stimulate phosphorylation of PKC-ι followed by addition of GST-ZIP/p62. PKC-ι input into the assay was included as a reference for determination of percent PKC-ι bound to ZIP/p62. Similar results were obtained in two other experiments. **B:** PC12 cells were pretreated with increasing concentrations of genistein for 1 h prior to treatment with 100 ng/ml NGF for 30 min. ZIP/p62 was immunoprecipitated followed by Western blot analysis with PKC-ι antibody. As control, the expression of PKC-ι was examined by Western blot analysis of equivalent amounts of protein (60 μg). Shown is the % of PKC-ι bound to ZIP/p62 represented as an average of three separate experiments mean±SEM (n = 3).

state had on its ability to bind ZIP/p62. PKC-ι was tyrosine phosphorylated by src in vitro followed by addition of GST-ZIP/p62. The complex was washed followed by immunoblotting for both the ZIP/p62 and PKC-ι (Fig. 4A). As control, the amount of PKC-ι input into the binding reaction was included on the blot for comparison. Increases in tyrosine phosphorylated PKC-ι, induced by src input into the assay, enhanced the amount of PKC-ι coassociated with ZIP/p62. A complementary approach was undertaken to validate these observations. We reasoned that inhibition of endogenous tyrosine kinases by genistein prior to addition of NGF could be used as a means to examine whether tyrosine phosphorylation plays a modulatory role on PKC-ι's association with ZIP/p62 in vivo. PC12 cells were treated with genistein, a potent tyrosine kinase inhibitor, followed by stimulation with NGF for 15 min. The interaction between ZIP/p62 with PKC-ι was evaluated

by co-immunoprecipitation (Fig. 4B). As concentration of the tyrosine kinase inhibitor increased, the amount of PKC-ι cobound with ZIP/p62 decreased. By comparison, the amount of aPKC in the lysate remained constant. At maximum dose of genistein, a 50% reduction in the amount of PKC-ι complexed to ZIP/p62 was observed. Genistein treatment likewise reduced the activity as well as tyrosine phosphorylation state of PKC-ι [Wooten et al., 2001b]. Therefore, these findings demonstrate that activation of PKC-ι by src phosphorylation is required for interaction of ZIP/p62 with PKC-ι.

In addition, similar findings were observed employing a separate cotransfection approach. HEK 293 cells were cotransfected with HA tagged PKC-ι, src, and ZIP/p62. Likewise the association between PKC-ι and ZIP/p62 increased upon cotransfection of constitutively active src (data not shown).

NGF Stimulates Formation of ZIP/p62 Vesicles Along With Movement of aPKC

Since ZIP/p62 is not a substrate for PKC-ι we reasoned that it might serve a shuttling function, participating in the movement of PKC-ι to a particular subcellular site [Mochly-Rosen and Gordon, 1998]. Therefore, we sought to examine the localization of ZIP/p62 as well as PKC-ι post treatment of PC12 cells with NGF (Fig. 5). Because ZIP/p62 binds to the TrkA receptor [Wooten et al., 2001a] which is subsequently internalized to an endosomal vesicular compartment [Beattie et al., 1996], we next determined whether ZIP/p62 could also be detected in vesicular structures. To examine intracellular localization of ZIP/p62, PC12 cells were treated with NGF for 1 h at 4°C. Afterwards, unbound NGF was extensively washed and the cells were incubated for different times at 37°C to allow endocytosis. NGF treatment lead to formation of vesicles which displayed a punctate staining pattern with ZIP/p62 antibody (Fig. 5). Previous studies have localized p62 within an endosomal compartment [Sanchez et al., 1998]. To validate the colocalization of ZIP/p62 in an endosomal compartment, PC12 cells were transiently transfected with GFP-p62 followed by staining with an affinity purified Rab7 antibody, a well characterized marker

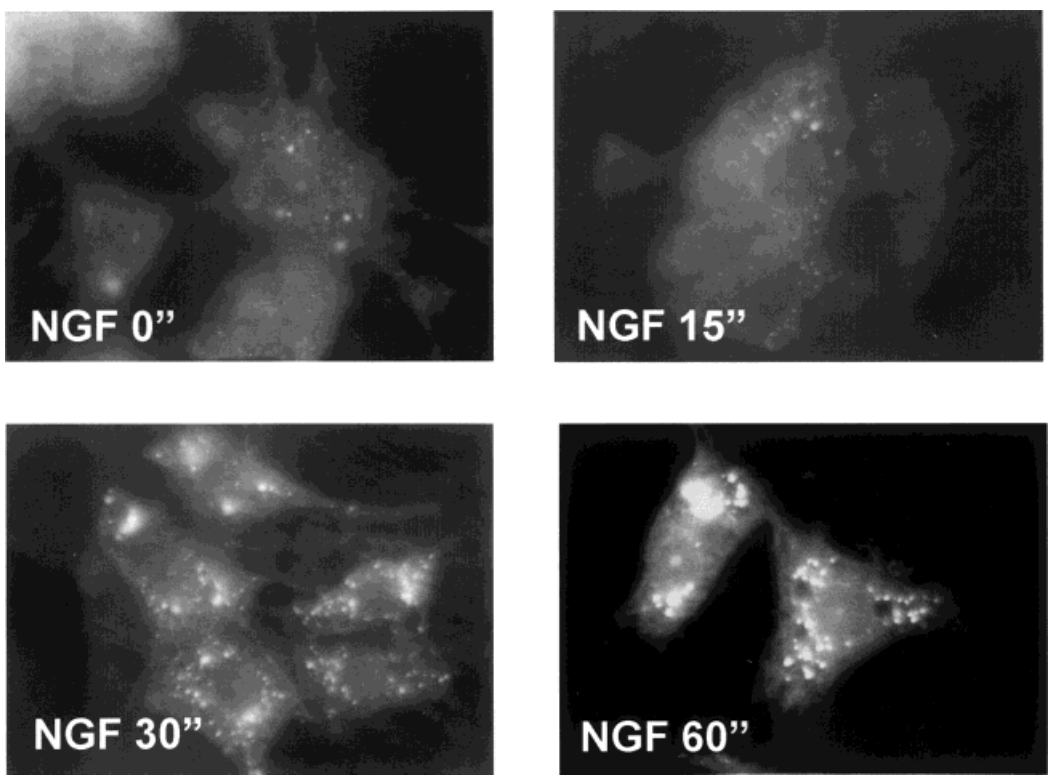

Fig. 5. NGF stimulates accumulation of ZIP/p62 vesicles. NGF (100 ng/ml) was bound to PC12 cells at 4°C for 1 h followed by washing of the cells and shift to 37°C for 0, 15, 30, 60 min, followed by fixation and staining with antibody to ZIP/p62. Similar results were obtained in three other experiments.

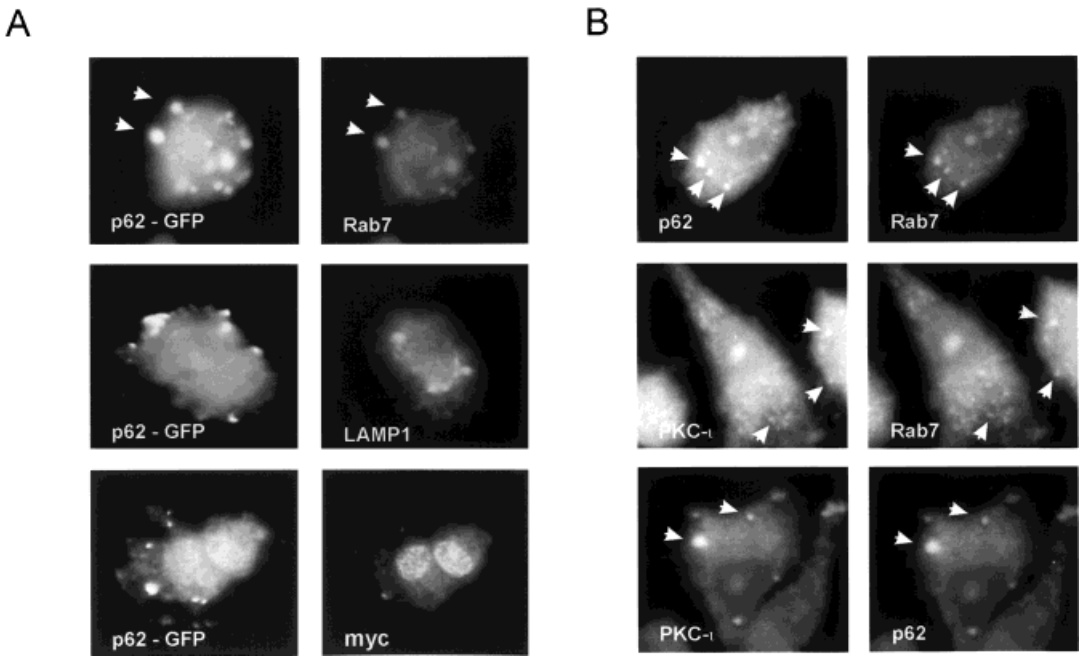

Fig. 6. Colocalization of p62-Rab7 and PKC-ι. **A:** PC12 cells were transfected with GFP-p62 followed by staining for either Rab 7, LAMP1 or myc. GFP-p62 staining colocalizes with Rab7. **B:** Alternatively, endogenous proteins were colocalized by treating cells with NGF (100 ng/ml) at 4°C for 1 h followed by washing of the cells and staining with either p62 and Rab7, PKC-ι and Rab7, or PKC-ι and p62. Vesicular structures identified as late endosomes by Rab7 staining colocalization with both PKC-ι and p62. Similar results were obtained in one other experiment.

of the late endosomal compartment [Soldati et al., 1995]. Both GFP-p62 and Rab7 staining overlapped, whereas GFP-p62 failed to colocalize with either LAMP1, a lysosomal marker, or myc, a nuclear marker (Fig. 6A). To study the colocalization of PKC-ι, Rab7, and ZIP/p62 double labeling was employed. Similar overlap in the staining patterns could be observed between PKC-ι and Rab7; ZIP/p62 and Rab7 or PKC-ι and ZIP/p62 (Fig. 6B). Collectively, these findings demonstrate that p62, PKC-ι, and Rab7 colocalize within the late endosomal compartment.

In order to address whether aPKC was routed along with ZIP/p62 to the late endosomes upon NGF stimulation, a subcellular fractionation approach was undertaken. To validate the separation of the endosomal fraction in our hands by use of this protocol, equivalent protein from the various fractions were blotted with affinity purified Rab7 antibody [Soldati et al., 1995]. Rab7 localized to 18–32% interface of the gradient (Fig. 7A). This fraction has previously been identified as being enriched in late endosomes employing this particular separation method [Sanchez et al., 1998]. Thus, this finding validates the application of this fractionation method for preparation of endosomes from PC12 cells. Experiments were undertaken to determine whether NGF stimulated an accumulation of ZIP/p62, as well as, aPKC within the endosomal compartment and also, to ascertain whether aPKC and ZIP/p62 moved simultaneously or independently of one another to this compartment. We observed an NGF-dependent increase in both PKC-ι and ZIP/p62 in the endosomal compartment (Fig. 7B and C). Endosomes prepared in this manner consistently resulted in an NGF-stimulated pool of ZIP/p62 within the endosomes at a time frame coincident with the time of accumulation observed employing immunofluorescent microscopy (30–60 min). Furthermore, activation of PKC-ι by NGF (15 min) preceded accumulation of either PKC-ι or ZIP/p62 within the endosomal compartment, suggesting that activation of aPKC may be required in order for transport of either PKC-ι or ZIP/p62 to take place.

Since the two proteins coassociate with each other as a function of the tyrosine phosphorylation state of PKC-ι (Fig. 4), studies were undertaken to determine if tyrosine phosphorylation

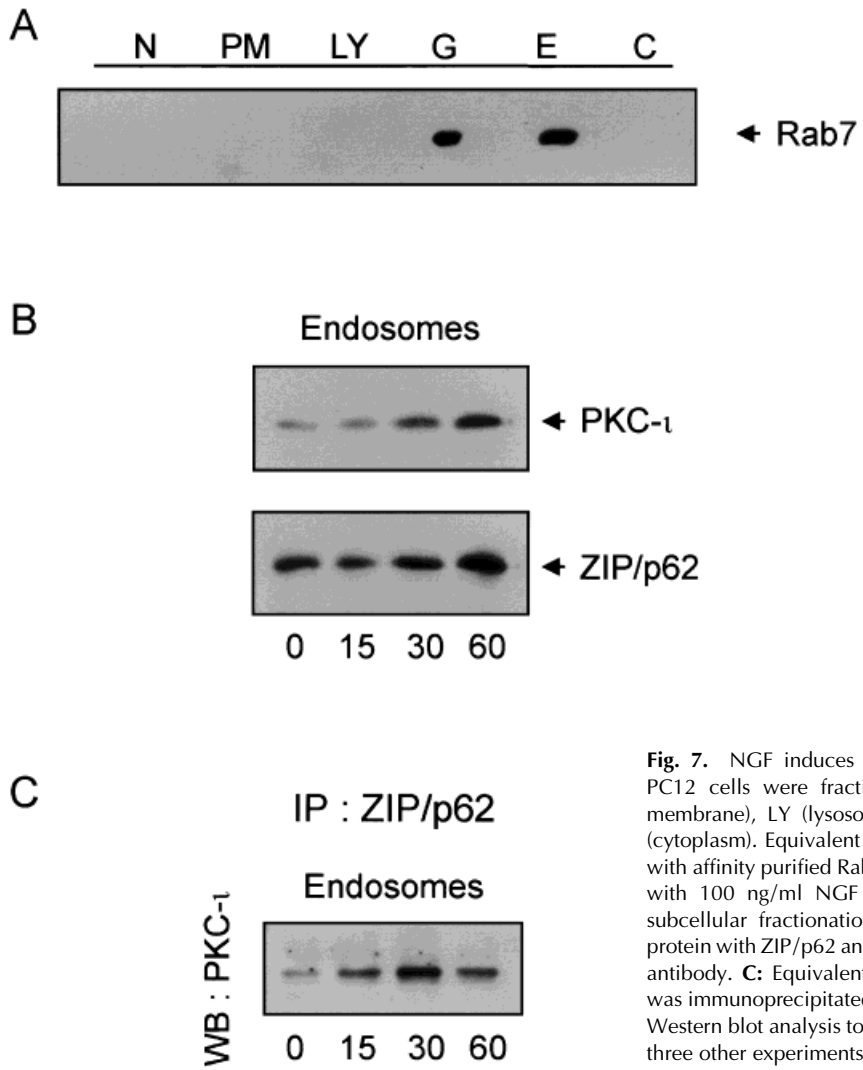

Fig. 7. NGF induces routing of ZIP/p62 to endosomes. **A:** PC12 cells were fractionated into N (nuclei), PM (plasma membrane), LY (lysosomes), G (golgi), E (endosomes), or C (cytoplasm). Equivalent protein from each fraction was blotted with affinity purified Rab7 antibody. **B:** PC12 cells were treated with 100 ng/ml NGF for 0, 15, 30, 60 min followed by subcellular fractionation, immunoprecipitation of equivalent protein with ZIP/p62 antibody, and Western blotting with PKC-ι antibody. **C:** Equivalent protein from the endosomal fraction was immunoprecipitated with antibody to ZIP/p62 followed by Western blot analysis to PKC-ι. Similar results were obtained in three other experiments.

was required for movement of aPKC to the late endosomal compartment. We reasoned that modulating the interaction between aPKC and ZIP/p62 may alter transport of aPKC to this fraction. Alternatively, it is also possible that ZIP/p62 may move independently of PKC activation or its coassociated cargo. To this end, we took advantage of our previous findings where inhibition of tyrosine phosphorylation was observed to block ZIP/p62-PKC-ι interaction (Fig. 4). PC12 cells were pretreated with genistein, followed by study of ZIP/p62 routing to the endosomal compartment (Fig. 8A and B). Inhibition of tyrosine protein kinase activity with genistein did not prevent targeting of ZIP/p62 to this component of the cell, but did reduce routing of PKC-ι with a parallel effect on the amount of aPKC cobound to ZIP/p62. These data suggest that movement of ZIP/p62 to this compartment could occur in the absence of aPKC activation. Furthermore, genistein did not effect appearance of NGF-stimulated vesicular structures stained by the ZIP/p62 antibody (data not shown), thus confirming our observations obtained by subcellular fractionation (Fig. 8A and B). Therefore, tyrosine phosphorylation regulates the activity and subsequently the ability of PKC-ι to bind ZIP/p62. However, the routing of ZIP/p62 itself to the endosome occurs independently of aPKC activation. Altogether, these findings suggest that delivery of PKC-ι is a two-step process. First, enzyme activation takes place resulting in binding of PKC-ι to ZIP/p62 and a second,

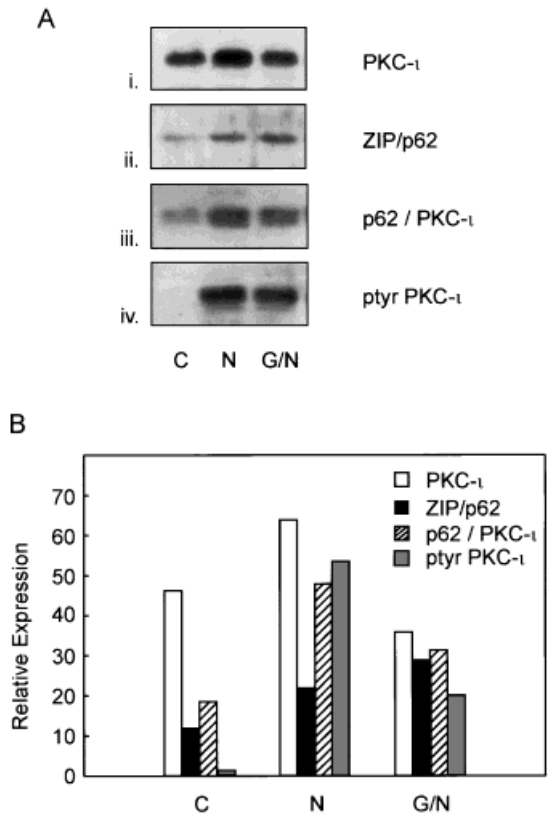

Fig. 8. Tyrosine phosphorylation modulates interaction of PKC-ι with ZIP/p62 and routing to the endosomes. **A:** PC12 cells were treated with genistein (10 μM) for 0 or 60 min followed by 100 ng/ml NGF for 0 or 30 min; C, no genistein nor NGF; N, NGF only; N/G, both genistein and NGF. The cells were then fractionated into components and equivalent protein from the late endosomes was either: i) blotted with antibody to PKC-ι, ii) blotted with antibody to ZIP/p62, iii) immunoprecipitated with anti-ZIP/p62 antibody coupled to agarose followed by Western blotting with antibody to PKC-ι, or iv) immunoprecipitated with antiphosphotyrosine antibody, 4G10, coupled to agarose followed by Western blotting to PKC-ι. **B:** The relative expression of PKC-ι, (i), ZIP/p62 (ii), PKC-ι cobound to ZIP/p62 (iii), or changes in the tyrosine phosphorylation-state of PKC-ι (iv) was determined by densitometric scan of the blots in (A). Essentially identical results were obtained in two other independent experiments.

independent signal is required to deliver ZIP/p62 to the endosome along with its cargo, PKC-ι.

ZIP/p62 Regulates NGF-Induced Neurite Outgrowth

Since PKC-ι is required for NGF-induced differentiation [Coleman and Wooten, 1994], we sought to determine whether p62 was likewise required to mediate NGF's biological activity. PC12 cells were transiently transfected with antisense p62 construct [Sanz et al., 2000]. Thereafter, the cells were stimulated with NGF for a period of 48 h followed by measure of the percentage neurite bearing cells (Table I). Transfection of antisense p62, but not p62 nor vector control, significantly reduced NGF-induced neurite outgrowth (Table I, Fig. 9A). Control cells and transfected cells were evaluated by Western blotting to determine the relative expression of p62 (Fig. 9B). Antisense p62 significantly diminished the expression of ZIP/p62. Thus, diminished NGF responsiveness was attributed to a decrease in the levels of ZIP/p62.

TABLE I. ZIP/p62 Effect on NGF–Induced Neurite Outgrowth

Treatment	Percentage cells with neurites
Control + NGF	65±12
Vector + NGF	58±16
P62 + NGF	62±9
Antisense p62 + NGF	28±11

PC12 cells were transiently transfected using Lipofectamine 2000. Twenty-four hours later NGF (50 ng/ml) was added. The percentage of cells with neurite processes greater than two cell bodies in length was counted three days after addition of NGF. At least 300 cells were counted per treatment. The experiment was replicated three independent times. (X±SEM, n = 3).

DISCUSSION

Scaffolding and shuttling proteins have gained wide attention as critical determinants in targeting activated PKC to particular subcellular sites [Mochly-Rosen, 1995; Faux and Scott, 1996; Mochly-Rosen and Gordon, 1998]. These proteins bind to the PKCs, relocate with them in response to stimuli, and then anchor PKC at a specific location within the cell. The docker protein, FRS2 has been recently identified as coimmunoprecipitating with activated aPKCs [Lim et al., 1999]. Like ZIP/p62, FRS2 does not exhibit an enzyme-substrate relationship with PKC. Similarly, RACKs (Receptors for Activated C Kinases) bind to inactive PKCs and shuttle them to specific locations within the cell [Ron et al., 1999]. Specifically, RACK1 binds inactive PKC-β_{II} at the cytoplasm of Chinese hamster ovary cells and translocates upon activation. It has been shown that RACK1 carries PKC-β_{II} to the Golgi; however, because RACK1 is a mobile protein and lacks a targeting consensus sequence motif, it does not anchor to a specific subcellular location. In fact, RACK1 shuttles PKC-β_{II} to various sites in different cell lines [Ron et al., 1999]. Proteins that interact

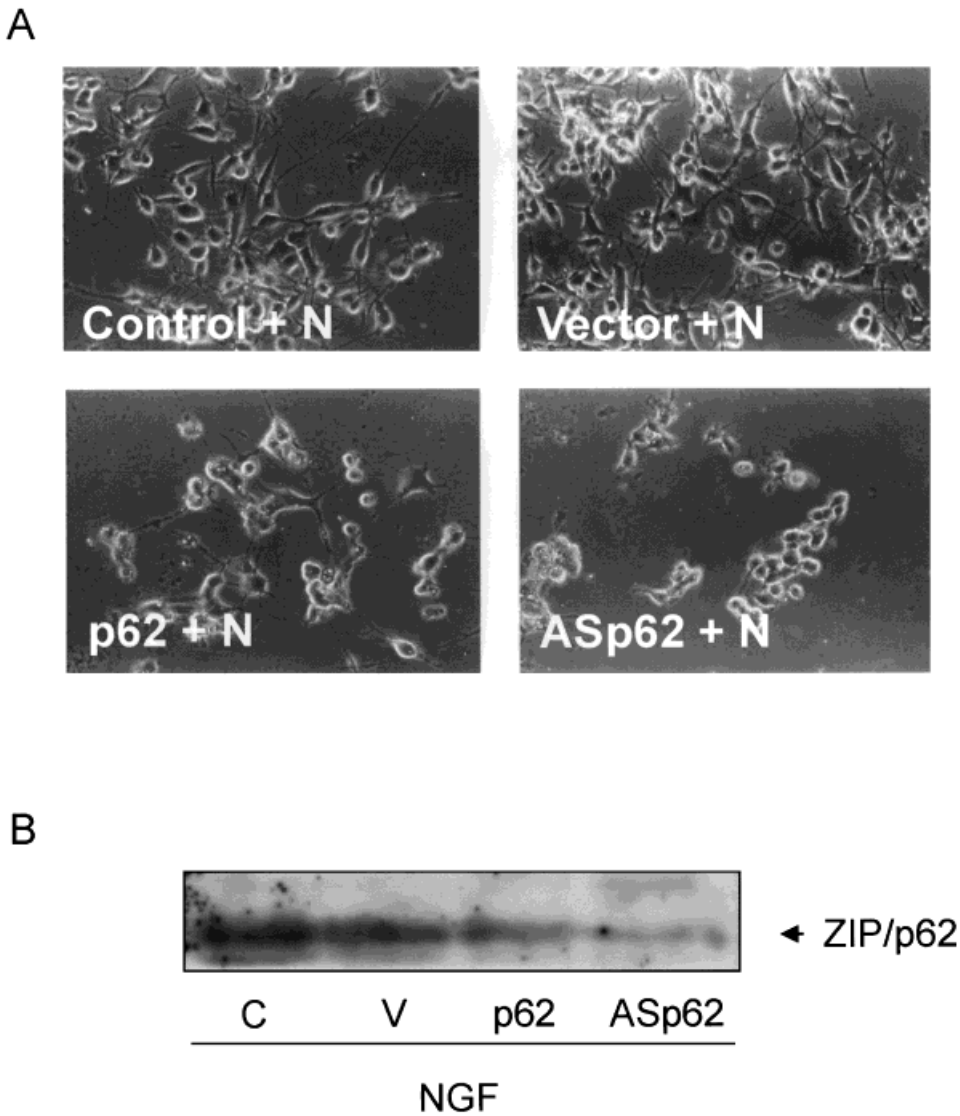

Fig. 9. Antisense ZIP/p62 blocks NGF-induced differentiation. **A:** PC12 cells were transiently transfected with nothing (control), vector pcDNA, full length ZIP/p62 or antisense (AS) ZIP/p62 as indicated. Twenty-four hours later NGF (50 ng/ml) was added followed by assessment of neurite outgrowth three days post addition of NGF (N). **B:** Control or transfected cells were collected and the expression of ZIP/p62 was examined by Western blot analysis. Transfection of the antisense (AS) construct significantly reduced the expression of ZIP/p62 by 85% of control levels.

with C kinases, PICKs, also exhibit an analogous behavior [Staudinger et al., 1995]. PICK1 interacts with the catalytic domain of PKC and becomes phosphorylated upon PKC activation. The subcellular distribution of PICK1 suggests that it docks with activated PKC-α in the perinuclear region, serving as an anchor [Staudinger et al., 1995].

In agreement with previous findings [Sanchez et al., 1998], our results strengthen the notion that ZIP/p62 is a binding/shuttling protein and not a substrate. ZIP/p62 proved to be a weak substrate of the aPKCs, as compared to other known substrates. The association between ZIP/p62 and PKC-ι is similar to that of FRS2 and the RACKs as they also serve as binding proteins but are not substrates of the aPKCs [Mochly-Rosen, 1995; Lim et al., 1999; Ron et al., 1999]. The optimal sequence required for a protein to serve as an aPKC substrate is RXGS [Nishikawa et al., 1997], as determined by peptide library screening. Collectively, hnRNPA1 [Municio et al., 1995], nucleolin [Zhou et al., 1997], and myelin basic protein

possess an RXGS/T motif, thus confirming their ability to serve as suitable substrates. This motif is not found in ZIP/p62 nor FRS2, which likely explains the inability of aPKC to phosphorylate these proteins as described in this study and others [Sanchez et al., 1998; Lim et al., 1999].

Functional regulation of aPKC is not well understood. The aPKCs bind to src [Seibenhener et al., 1999], are phosphorylated, and activated by tyrosine kinases [Gschwendt et al., 1994; Konishi et al., 1997; Wooten et al., 2001b]. Moreover, the phosphorylation of PKC has been recognized as a means whereby the subcellular localization of PKC may be regulated [Acs et al., 1997; Brodie et al., 1998; Edwards et al., 1999]. We have recently mapped the sites of tyrosine phosphorylation within aPKCs [Wooten et al., 2001b]. One site, tyrosine 116 of aPKC, lies within the domain which mediates interaction with ZIP/p62 [Puls et al., 1997]. Studies are currently underway to mutate this site and to examine the effects which tyrosine 116 has upon mediating interaction with ZIP/p62. Collectively, our findings demonstrate that a primary role for ZIP/p62 is as a shuttling protein. Our study extends the initial observations in this area [Sanchez et al., 1998] employing a different cell type, receptor and ligand and thus underscores a commonality in function for ZIP/p62 as a shuttling protein. Of significance, our findings reveal that the intracellular localization of ZIP/p62 does not depend upon PKC-ι activation, whereas the routing of aPKC is directed by ZIP/p62. This observation is similar to that reported between PKC-beta and RACK1 [Ron et al., 1999] and may thus be a common feature to PKCs and their binding proteins.

The ability of ZIP/p62 to reduce NGF-induced neurite outgrowth is in keeping with aPKC's role in mediating NGF responsiveness [Coleman and Wooten, 1994; Kuroda et al., 1999]. Recent studies have shown that internalization of TrkA to an endosomal compartment regulates NGF-induced differentiation [Zhang et al., 2000]. We find that ZIP/p62 also binds directly to the TrkA receptor in a manner that is likewise dependent upon NGF [Wooten et al., 2001a]. Collectively these findings are consistent with a model whereby p62 binding to TrkA may influence internalization as well as delivery of TrkA to the endosome. Thus, aPKC may possess a dual role in this process. Previous studies have shown that PKC can modulate both internalization and receptor routing [Formisano et al., 1998; Hipkin et al., 2000]. Internalized TrkA receptors likewise continue to signal within the endosomal compartment [Beattie et al., 1996] and are required to mediate NGF-induced differentiation [Zhang et al., 2000]. Therefore, disrupting either internalization or routing of TrkA may influence NGF's biological properties. Interestingly, trafficking of activated TrkA is decreased in neurons from individuals with Alzheimer's [Cataldo et al., 1996] and may be a contributing factor to the neuropathogenesis of the disease. ZIP/p62 has also been shown to serve as a scaffold for activation of the transcription factor NF-κB by NGF [Wooten et al., 2001a]. A challenge for future studies will be to determine the role of aPKC and ZIP/p62 in internalization as well as routing of activated TrkA receptor and receptor-associated proteins to the endosome.

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