THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 1, Issue of January 2, pp. 133–141, 2004

Printed in U.S.A.

SH2-B Is a Positive Regulator of Nerve Growth Factor-mediated Activation of the Akt/Forkhead Pathway in PC12 Cells*

Received for publication, September 10, 2003
Published, JBC Papers in Press, October 16, 2003, DOI 10.1074/jbc.M310040200

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To gain insight into the mechanism by which the adapter protein SH2-B promotes nerve growth factor (NGF)-mediated neuronal differentiation and survival, the effect of SH2-B on the serine/threonine kinase Akt/protein kinase B and downstream effector proteins was examined. PC12 cells stably overexpressing SH2-Bβ, which exhibit enhanced NGF-induced neuronal differentiation compared with control cells, showed enhanced and prolonged NGF-induced phosphorylation of Akt on Ser^{473} and Akt enzymatic activity. Surprisingly, NGF-induced phosphorylation of Akt on Ser^{473} and Akt activity were not altered in cells overexpressing SH2-Bβ(R555E) with a defective SH2 domain, despite the ability of the overexpressed SH2-Bβ(R555E) to block NGF-induced differentiation. Consistent with SH2-Bβ enhancing the activity of Akt, cells overexpressing SH2-Bβ but not SH2-Bβ(R555E) exhibited increased and/or prolonged phosphorylation of the pro-apoptotic Akt effector proteins, glycogen synthase kinase-3, and forkhead transcription factors, FKHRL1/FOXO3 and FKHR/FOXO1. Immunolocalization studies indicated that, although ectopically expressed FKHR was primarily concentrated in the cytoplasm of control cells and cells transiently overexpressing SH2-Bβ, it was concentrated in the nucleus of cells transiently overexpressing SH2-Bβ(R555E). Similarly, SH2-Bβ stimulated the accumulation of FKHR in the cytoplasm of 293T and COS-7 cells, whereas SH2-Bβ(R555E) enhanced its accumulation in the nucleus. In PC12 cells stably expressing forms of SH2-Bβ, SH2-Bβ mimicked the ability of NGF to promote redistribution of FKHR to the cytoplasm whereas SH2-Bβ(R555E) blocked this effect of NGF. Taken together, these data indicate that SH2-B is a positive regulator of NGF-mediated activation of the Akt/Forkhead pathway.

Nerve growth factor (NGF)[1] is a trophic factor essential for the development and survival of sympathetic and sensory neurons. PC12 cells, a well established cell culture model of sympathetic neurons, differentiate into a neuronal-like phenotype in the presence of NGF. In both sympathetic neurons and PC12 cells, removal of trophic support triggers retraction of neurite outgrowths and eventual apoptosis (1, 2). NGF protects PC12 cells from apoptosis induced by trophic factor withdrawal (2, 3), cytotoxic drugs (4–6), and oxidative stressors (7–9). The mechanism(s) by which NGF elicits its effects on neuronal differentiation and survival are only beginning to be understood.

A majority of the neurotrophic effects of NGF are believed to be initiated by binding of NGF to the membrane receptor tyrosine kinase TrkA. NGF binding activates TrkA, which phosphorylates itself on multiple tyrosines (10). Binding of different signaling proteins to these phosphorylated tyrosines initiates multiple signaling pathways. Several of these TrkA-binding proteins and/or their downstream effector proteins have been implicated in the regulation of neuronal differentiation and/or survival. One of the first pathways shown to be required for NGF-induced neuronal differentiation of PC12 cells is the Ras/Raf/MEK/ERK pathway. The TrkA-binding protein Shc, as well as Ras, Raf, MEK, and ERK have been implicated in neuronal differentiation of PC12 cells (11–14). Another TrkA-binding protein implicated in NGF-induced differentiation of PC12 cells is phospholipase Cγ, a protein that mediates the production of diacylglycerol and inositol trisphosphate, leading to release of intracellular Ca^{2+} stores and activation of protein kinase C (15–17).

A third pathway, involving the TrkA-binding protein phosphatidylinositol 3′-kinase and the serine/threonine kinase Akt/PKB, has been implicated in axon caliber and branching (18). However, it is primarily emerging as a transducer of survival signals. Akt activity has been linked to cell survival in several cell types (19, 20) and is both necessary and sufficient for NGF-dependent survival of sympathetic neurons (21). Inhibition of PI 3-kinase by wortmannin or LY294002 blocks the ability of NGF to prevent apoptosis in both PC12 cells and primary sympathetic neurons (21, 22). Activated forms of PI 3-kinase and AKT promote neuronal survival in the absence of external survival factors (20, 21, 23–28). Lipid products of PI 3-kinase have been shown to recruit Akt to the plasma membrane via the pleckstrin homology domain of Akt, leading to

* This work was supported in part by National Institutes of Health Grant R01-DK54222 and the Juvenile Diabetes Research Foundation Center for the Study of Complications of Diabetes. This work used the Core Facilities (Morphology and Image Analysis, and Cell and Molecular Biology Cores) of the Michigan Diabetes Research and Training Center (supported by National Institutes of Health Grant P60-DK20572). 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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‖ Supported by Predoctoral Traineeship in Cellular and Molecular Biology GM-07315 from the National Institutes of Health.

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[1] The abbreviations used are: NGF, nerve growth factor; SH2, Src homology 2; αPY, anti-phosphotyrosine antibody; FKHR, Forkhead in rhabdomyosarcoma; Foxo, Forkhead box, subgroup 0; GFP, green fluorescent protein; GSK, glycogen synthase kinase; DMEM, Dulbecco's modified Eagle's medium; PI, phosphatidylinositol; PKB, protein kinase B; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.

phosphorylation of Akt at Ser^{473} and Thr^{308} by phosphoinositide-dependent kinases (29–31). Phosphorylation of both sites is required for full activation of Akt (32). Akt can phosphorylate and modulate the activity of several proteins involved in cellular survival, including members of the FOXO family of forkhead transcription factors (33, 34), glycogen synthase kinase-3 (GSK-3) (35), the Bcl2 family members BAD and Bax (36–38), nuclear factor-κB (39, 40), and caspase 9 (41).

Phosphorylation of GSK-3 by Akt at Ser^{21} (α isoform) or Ser^{9} (β isoform) inactivates GSK-3 and is believed to be the primary mechanism responsible for growth factor inhibition of this protein kinase (42, 43). Inactivation of GSK-3 is thought to contribute to the survival effects of Akt activation and to reduce neurotrophin factor withdrawal-induced neurite retraction. Inhibition of GSK-3 by LiCl reduces NGF withdrawal-induced apoptosis in PC12 cells (44) and the degree of neurite retraction observed in wortmannin-treated SH-SY5Y neuroblastoma cells (45).

Multiple forkhead family members are targets of Akt (34, 46). Three mammalian members of this family, namely FKHR/FOXO1 (47), FKHRL1/FOXO3 (48), and AFX/FOXO4 (49), belong to the FOXO subfamily. Proteins in this subfamily have sequence similarity to the nematode homologue Daf-16, which is a downstream target of two Akt homologues in an insulin-related signaling pathway, and have been implicated in apoptosis of neuronal cells (50–52). Daf-16 as well as AFX, FKHR, and FKHRL1 contain multiple consensus Akt phosphorylation sites (47, 49), and Akt can directly phosphorylate the three mammalian forkhead proteins *in vitro* (34, 46).

Akt is thought to inhibit the activity of forkhead transcription factors primarily by regulating their subcellular localization (53, 54). In this paradigm, FKHR in the nucleus induces the expression of genes critical for cell death, such as the Fas ligand gene. When activated, Akt phosphorylates FKHR proteins in the nucleus, resulting in binding of FKHR proteins to 14-3-3 and subsequent export from the nucleus. This relocation of FKHR proteins to the cytoplasm functionally represses their transcriptional activity (53–56). Binding to 14-3-3 is also thought to help retain FKHR in the cytoplasm by masking a site necessary for nuclear reimport (56).

SH2-B was identified as a binding protein of the receptor for NGF (TrkA) (57, 58), as well as of the receptors for insulin (59), platelet-derived growth factor (60), fibroblast growth factor (61), insulin-like growth factor-1 (62), hepatocyte growth factor (61), the cytokine receptor-associated tyrosine kinase JAK2 (63), and the R1 FcεR1 receptor (64). Four SH2-B isoforms (α, β, γ, δ) have now been identified; they differ in their C termini downstream of the SH2 domain (63–66) (Fig. 1). SH2-B belongs to a family of adapter proteins that include APS and Lnk (67, 68).

We and others have shown that SH2-B (α or β isoform) is vital for NGF-induced neurite outgrowth in cultured PC12 cells and maintenance of the neuronal phenotype of primary cultured rat sympathetic neurons (57, 58). NGF stimulates the association of SH2-Bβ with TrkA via the SH2 domain of SH2-Bβ and the tyrosyl phosphorylation of SH2-Bβ. Mutating the critical Arg to Glu (R555E) within the SH2 domain of SH2-Bβ abolishes both the NGF-induced association of SH2-Bβ with TrkA and the tyrosyl phosphorylation of SH2-Bβ (57). Stable overexpression of SH2-Bβ enhances NGF-induced neuronal differentiation of PC12 cells, whereas stable expression of SH2-Bβ(R555E) blocks NGF-induced differentiation of PC12 cells (57). Interestingly, cells stably expressing SH2-Bβ(R555E) do not show impaired NGF-induced tyrosyl phosphorylation of TrkA, Shc, phospholipase C-γ, ERK1, or ERK2 (63), suggesting that impaired activation of these signaling proteins is not responsible for the dramatic overall impairment of neuronal differentiation. SH2-B has also been implicated in neuronal survival. Neuronal sympathetic neurons titrated with anti-SH2-B antibodies exhibit a reduced rate of survival when grown in NGF-containing medium. Similarly, transient expression of an N-terminally truncated form of SH2-B promotes degeneration of axons of sympathetic neurons in explants of superior cervical ganglia grown in the presence of NGF (58). In the current work, we extend these earlier studies designed to determine the role of SH2-Bβ in neuronal differentiation and survival by examining the hypothesis that SH2-Bβ positively regulates NGF-induced activation of the protein kinase Akt and its downstream targets.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Parental PC12 cells were obtained from Dr. Ben Margolis (University of Michigan, Ann Arbor, MI) or ATCC. Pools of PC12 cells stably expressing GFP, GFP-SH2-Bβ, and GFP-SH2-Bβ(R555E) were made as described previously (63) and used for Figs. 2–5. Fig. 8 used cells made in a similar fashion with the exception that all GFP-positive cells were pooled instead of only those with the top 5% expression level. The stock of 293T cells was obtained from Dr. O. A. MacDougald (University of Michigan, Ann Arbor, MI). Murine NGF was from BD Bioscience. Triton X-100, aprotinin, and leupeptin were purchased from Roche Molecular Biochemicals. The nitrocellulose membranes and enhanced chemiluminescence (ECL) detection system were from Amersham Biosciences. X-ray film came from DuPont or Eastman Kodak Co. (Fig. 8*B*), horse serum from ICN Biomedicals, fetal bovine serum from Invitrogen, and poly-L-lysine from Sigma.

Plasmids, Antibodies, and Fluorescent Probes—pcDNA-Flag-FKHR was a gift of Drs. E. Tang and K. Guan (34). cDNAs encoding GFP-tagged wild-type SH2-Bβ and SH2-Bβ(R555E) have been described previously (57). Anti-SH2-Bβ antibody (αSH2-Bβ) was prepared as described previously (63) and used at a dilution of 1:15,000 for Western blotting. Antibodies that recognize the following proteins were used for Western blotting at a dilution of 1:1000: phospho-Akt (Ser^{473}) (αpAkt-Ser^{473}) (Cell Signaling, 9276), Akt (αAkt) (Cell Signaling, 9272), FKHRL1 (αFKHRL1) (Upstate Biotechnology, 06-951), phospho-GSK-3($Ser^{21/9}$) (αpGSK-3) (Cell Signaling, 9331S), and phospho-GSK-3(Y279/Y216) (used to detect total GSK-3) (αGSK-3) (Upstate Biotechnology, 05-413). Anti-Flag M2 monoclonal antibody (αFlag) was obtained from Sigma and used at a dilution of 1:1000 for immunocytochemistry. Texas Red dye-conjugated AffiniPure goat anti-mouse IgG (H+L) was from Jackson ImmunoResearch and was used at a dilution of 1:400. Anti-rabbit IgG conjugated to horseradish peroxidase was from Santa Cruz. Antibody to FKHR (αFKHR) (Cell Signaling, 9462) recognizes only unphosphorylated FKHR.[2]

Cell Culture—PC12 cells were grown at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM L-glutamine, and 1 mM antibiotic-antimycotic (Invitrogen) (supplemented DMEM), 10% heat-inactivated horse serum, and 5% fetal bovine serum. Cells used for experiments were plated on poly-L-lysine-coated coverslips (for analysis by confocal microscopy), Lab-TekII Chamber Slide System 154941 (Nalge Nunc International Corp) (Fig. 8*A*), or on collagen-coated dishes. The latter were prepared by incubating with 0.1 mg/ml collagen (type I rat tail, Collaborative Biomedical Products, 354236) in 0.02 N acetic acid for 1 h. The confluent cells were deprived of serum overnight using supplemented DMEM containing 1% bovine serum albumin and treated as indicated with NGF. 293T and COS-7 cells were grown in supplemented DMEM and 8% calf serum.

Reverse Transcription-PCR—Total RNA was prepared from PC12 cells using TRIzol reagent as per the protocol from the manufacturer (Invitrogen). Oligonucleotide primers that recognize all four known isoforms of murine SH2-B were used to amplify 1 μg of total RNA (sense primer 5′-TCTCCCCTAGTTCTGCCTCCATTG-3′, corresponding to nucleotides 1638–1661 of murine SH2-Bα, -β, -γ, and -δ; antisense primer 5′-CGCCCCCGACGCCTCTTCT-3′, corresponding to nucleotides 2428–2410 in murine SH2-Bγ, GenBank™ accession no. AF421139 (66). Expression of SH2-Bγ was determined using the sense primer 5′-TTCAACTTCCAGGGCAAGGCTAA-3′ (corresponding to the rat equivalent of nucleotides 1991–2013 in murine SH2-Bα, -β, -γ, and -δ in which nucleotide 2011 is a T rather than an A) and an antisense primer

[2] T. Maures and C. Carter-Su, unpublished observation; Cell Signaling Technical Support, personal communication.

specific for SH2-Bγ (5′-CCGGCCTCACTTCTTGGGTGCA-3′, corresponding to nucleotides 2220–2241 in murine SH2-Bγ). Reaction products were resolved by agarose gel electrophoresis and detected by ethidium bromide staining. PCR products were extracted (Qiagen Qiaex II gel extraction kit) and sequenced to verify which isoform they represented.

Immunoblotting—Cells were solubilized in lysis buffer (50 mM Tris (pH 7.5), 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA). The lysates were centrifuged and the supernatants boiled for 5 min in a mixture (80:20) of lysis buffer plus sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Solubilized proteins were separated by SDS-PAGE. Proteins in the gel were transferred to a nitrocellulose membrane and detected by immunoblotting with desired antibodies and enhanced chemiluminescence (63). For quantification, immunoblots with signals in the linear range were scanned and the resulting images were analyzed using multi-analyst image software from Bio-Rad (Figs. 3 and 4*C*). In Fig. 5*B* quantification used the NIH Image 1.62 f software.

Immunolocalization—PC12 cells were plated onto poly-L-lysine-treated glass coverslips or Lab-TekII Chamber Slide System and transfected with various cDNA using LipofectAMINE Plus (Invitrogen). Both 293T and COS-7 cells were plated on coverslips and transfected using calcium phosphate precipitation. Twenty-four h after transfection, cells were incubated in serum-free medium overnight. As indicated, NGF was added following serum deprivation at a concentration of 100 ng/ml. Cells were then fixed in 4% formaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated with αFlag for 1 h, then for 45 min with anti-mouse IgG conjugated to Texas Red. Coverslips were mounted using mounting medium (90% glycerol and 10% phosphate-buffered saline) (Figs. 6*B* and 7 (*B* and *C*)) or Prolong Anti-fade P-7481 (Molecular Probes) (Figs. 7*A* and 8*A*) and examined using epifluorescence microscopy (Nikon Eclipse TE 200) with a 60× objective. In other experiments, coverslips under a Noran OZ laser scanning confocal microscope equipped with a 100× Nikon objective. In some experiments, fluorescently labeled antibodies and GFP-tagged proteins were imaged. Where indicated, the mean intensities of neighboring cytosolic and nuclear regions (~5 μm^2 each) were calculated using Adobe Photoshop™, corrected for background, and presented as nuclear-to-cytosol fluorescence ratios.

In Vitro Akt Kinase Assays—Immunocomplex Akt kinase assays were performed using the Akt/PKB α IP-kinase assay kit from Upstate Biotechnology. After 4–5 h of serum deprivation, NGF was added to PC12 cells stably expressing GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E) for various lengths of time. PC12 cells were placed on ice, and proteins were extracted according to the instructions from the manufacturer. Akt was immunoprecipitated from cell lysates using anti-Akt/PKB antibody. After washing, the immunoprecipitates were resuspended in 40 μl of kinase assay buffer containing 10 μCi of [γ-^{32}P]ATP and 10 μl of a specific substrate peptide (RPRAATF) and incubated at room temperature for 20 min with constant shaking. The reaction was stopped by addition of 20 μl of 40% trichloroacetic acid. The phosphorylated substrate was spotted onto P-81 phosphocellulose paper (Whatman). Residual [γ-^{32}P]ATP was removed by extensive washing. Radioactivity bound to the filter was quantified using a scintillation counter.

RESULTS

Isoforms of SH2-B Expressed in PC12 Cells—We first examined which isoforms of SH2-B are predominantly expressed in PC12 cells. RNA prepared from PC12 cells was PCR-amplified using primers that would amplify all four known isoforms (α, β, γ, and δ) (predicted products: ~637, 737, 790, and 690 bp, respectively) (Fig. 1*A*). The PCR products migrated with a size most closely resembling that predicted for the β isoform (Fig. 1*B*, *lane 1*) and co-migrated with the PCR product using SH2-Bβ cDNA as template (data not shown). The PCR products were cleaved by the restriction enzyme BsiEI, which cleaves only the 100-bp insert present in the β and γ isoforms (Fig. 1*A*). The BsiEI products (Fig. 1*B*, *lane 3*) co-migrated with the BsiEI-cleaved forms of the PCR product obtained using rat SH2-Bβ cDNA as the template (Fig. 1*B*, *lane 4*). To confirm the identity of the PCR product as SH2-Bβ, the PCR product was gel-purified and sequenced. The sequence corresponded to that predicted for the β isoform, suggesting that SH2-Bβ is the primary isoform of SH2-B expressed in PC12 cells. Because the reverse transcription-PCR products for the β and γ isoforms differ by only 53 bp in size and therefore might be difficult to separate on the gel and because the expression levels of these two isoforms could be very different, PCR primers specific for the γ isoform of SH2-B were designed to test further whether SH2-Bγ is expressed in PC12 cells. Fig. 1*B* (*lane 2*) shows that the γ isoform is also expressed in PC12 cells. Because it was possible that some δ isoform was also amplified by the primers used, even though the primer sequence was not an exact match, the PCR-amplified cDNA using γ-specific primers was gel-purified and sequenced. The sequence obtained from two different PCR products corresponded to the γ isoform. Taken together, the results of Fig. 1 are consistent with SH2-Bβ being the primary isoform of SH2-B expressed in PC12 cells. Other isoforms (*e.g.* γ isoform) appear to be expressed, but to a significantly lesser extent than the β isoform. These results are consistent with the previous finding that, after dephosphorylation by alkaline phosphatase, SH-2B in PC12 cells comigrates with the dephosphorylated SH2-Bβ expressed ectopically in COS cells (57). Based upon these findings, all subsequent experiments focused on the β isoform of SH2-B.

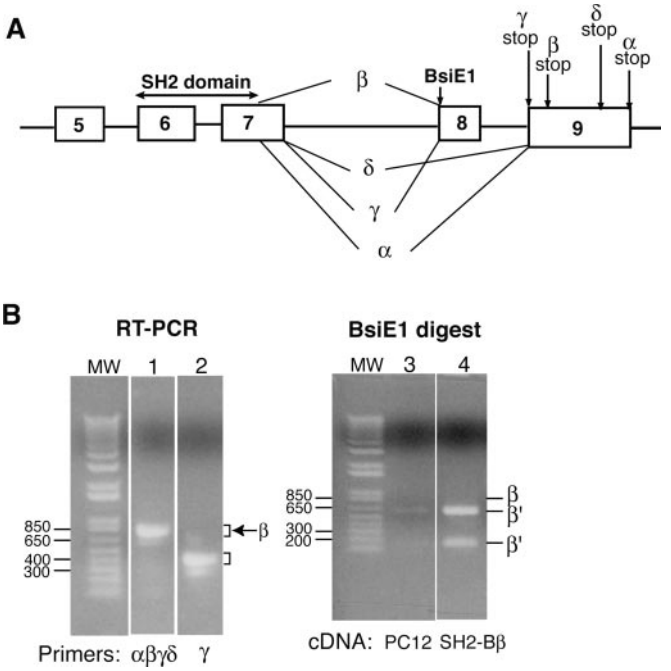

FIG. 1. **PCR reveals the expression of the β isoform of SH2-B in PC12 cells.** *A*, schematic of the four isoforms of SH2-B. Exons are denoted by *boxes* and are *numbered*. *B*, total RNA was prepared from cells. Oligonucleotide primers that recognize all four known isoforms of murine SH2-B (*lane 1*) or the γ isoform (*lane 2*) were used to amplify 1 μg of total RNA using PCR. The PCR product from a duplicate of *lane 2* (*lane 3*) or the PCR product from cDNA encoding SH2-Bβ (*lane 4*) was digested with BsiEI and digestion products separated in an agarose gel. For *lanes 3* and *4*, β denotes the predicted migration of the PCR product using the β isoform as template and β′ denotes the predicted migration of the BsiEI digestion products of the β isoform.

SH2-Bβ Enhances NGF-stimulated Activation of Akt—To determine whether SH2-Bβ is involved in the regulation of Akt by NGF, we first examined whether stable overexpression of SH2-Bβ or a mutated form of SH2-Bβ that lacks a functional SH2 domain, SH2-Bβ(R555E), affects the ability of NGF to stimulate the phosphorylation of serine 473 in Akt. Maximal activation of Akt requires phosphorylation of Ser^{473} (32). PC12 cells stably expressing GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E) were treated with 100 ng/ml NGF for various times, and cell lysates were collected. Solubilized proteins were separated by SDS-PAGE and immunoblotted with antibody that

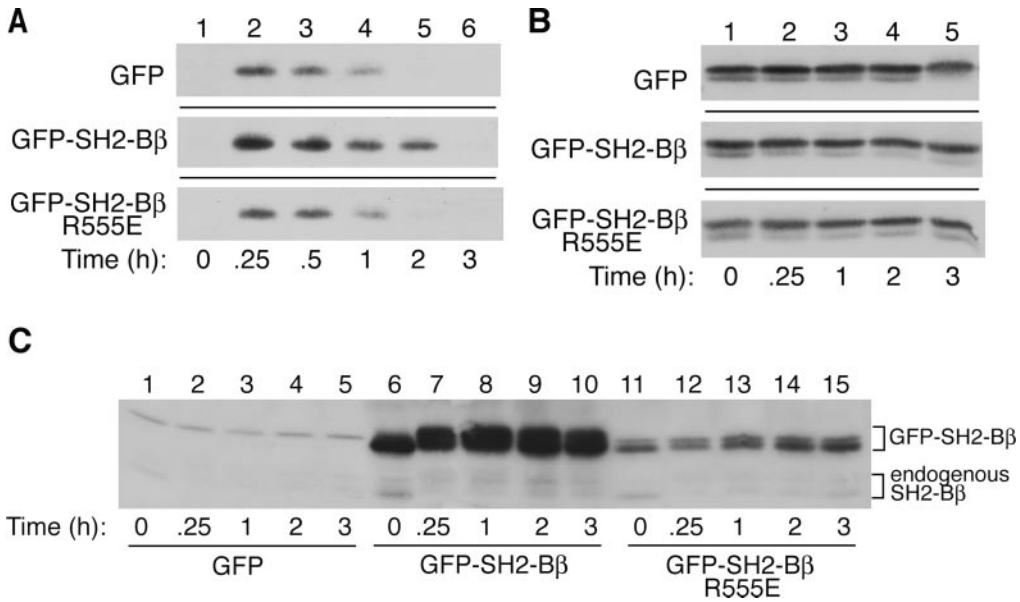

FIG. 2. **SH2-Bβ enhances and prolongs NGF-induced phosphorylation of Ser^{473} in Akt.** PC12 cells stably overexpressing GFP alone (*GFP*), GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E) as indicated were stimulated with 100 ng/ml NGF for the indicated times. In parallel experiments, an equal amount of protein in the lysates was immunoblotted with αpAkt(Ser^{473}) (*panel A*), αAkt (*panel B*), or αSH2-B (*panel C*).

specifically recognizes phosphorylated Ser^{473} (Ser(P)^{473}) within Akt. In control cells overexpressing GFP, NGF induced the appearance of Ser(P)^{473} within Akt within 15 min (Fig. 2*A*, *top panel*, *lane 2*). Phosphorylation was maximal at the 15-min time point and returned to nondetectable levels by 2 h. A similar NGF-induced increase in the amount of Ser(P)^{473}, both in terms of magnitude and time course, was observed in cells overexpressing GFP-SH2-Bβ(R555E) (Fig. 2*A*, *bottom panel*). In contrast, overexpression of GFP-SH2-Bβ both enhanced and prolonged NGF-induced phosphorylation of Ser^{473} (Fig. 2*A*, *middle panel*). The observed changes in the amount of Ser(P)^{473} were not the result of changes in the amount of Akt. No significant change in total Akt was observed after NGF treatment, and the levels of Akt in the three cell lines were indistinguishable (Fig. 2*B*). These observations demonstrate that overexpression of SH2-Bβ enhances NGF induced Akt activation, whereas overexpression of SH2-Bβ(R555E) has no effect. To verify levels of expression of GFP-SH2-Bβ in the stably expressing cell lines, cell lysates were immunoblotted with antibody to SH2-B (αSH2-B). Fig. 2*C* shows a very high level of expression (>10-fold) of both GFP-SH2-Bβ and GFP-SH2-Bβ(R555E) compared with endogenous SH2-Bβ. These levels of expression of SH2-Bβ are sufficiently high to enhance (GFP-SH2-Bβ) or block (GFP-SH2-Bβ(R555E) NGF-induced neuronal differentiation of PC12 cells (Ref. 57; data not shown).

To verify more directly the effect of SH2-Bβ on NGF-induced activation of Akt, we measured the enzymatic activity of Akt following exposure of the different cell lines to NGF. Cells were treated with NGF and solubilized. Akt was immunoprecipitated using anti-Akt/PKB antibody (αAkt) and incubated at room temperature for 20 min with [γ-^{32}P]ATP and a specific substrate peptide (RPRAATF). The reaction was stopped by addition of trichloroacetic acid, and the phosphorylated substrate was spotted onto P-81 phosphocellulose paper. Radioactivity bound to the filter was quantified by scintillation counting. The time course of Akt activation in response to 100 ng/ml NGF generally paralleled that of phosphorylation of Akt on Ser^{473}. In control PC12 cells stably expressing GFP alone, stimulation with NGF resulted in a dramatic increase in Akt activity after 3 min. Akt activity returned to near base-line levels by 2 h (Fig. 3*A*). Similar results were obtained in PC12 cells stably expressing SH2-Bβ(R555E) (data not shown). In

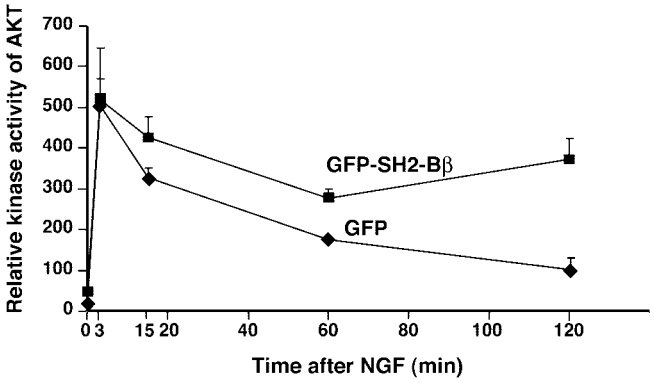

FIG. 3. **SH2-Bβ enhances and prolongs NGF-induced activity of Akt.** PC12 cells stably overexpressing GFP alone and GFP-SH2-Bβ were stimulated with 100 ng/ml NGF for the indicated times. Akt was immunoprecipitated, and kinase activity was determined. The results show the mean and range of two separate experiments.

contrast, but consistent with the Ser(P)^{473} results, Akt activity in PC12 cells stably expressing GFP-SH2-Bβ was enhanced at 15 and 60 min compared with control cells expressing GFP and remained elevated for 2 h. Overexpression of GFP-SH2-Bβ did not enhance the activity of Akt at the 3-min time point. However, overexpression of GFP-SH2-Bβ was found in preliminary studies to enhance NGF-induced Akt activity at the 3-min time point when NGF was tested at a concentration of 10 or 50 ng/ml, rather than at 100 ng/ml (data not shown), suggesting that SH2-Bβ not only prolonged NGF-activation of Akt but also shifted the peak response to an earlier time point.

SH2-Bβ Enhances Phosphorylation of GSK-3—To investigate whether SH2-Bβ affects downstream targets of Akt, we first examined whether overexpression of SH2-Bβ alters NGF-induced phosphorylation of GSK-3 on Ser-21 (α isoform) or Ser-9 (β isoform). Akt phosphorylation of the GSK-3α isoform on Ser-21 or of GSK-3β on Ser-9 results in partial inactivation of GSK-3 (43). Phosphorylation of both GSK-3α and GSK-3β in response to NGF was rapid, substantial, and transient, with maximal stimulation being observed at 15 min or 1 h (Fig. 4, *A* and *C*). As with Akt, phosphorylation of GSK-3 was prolonged in PC12 cells stably expressing SH2-Bβ compared with cells

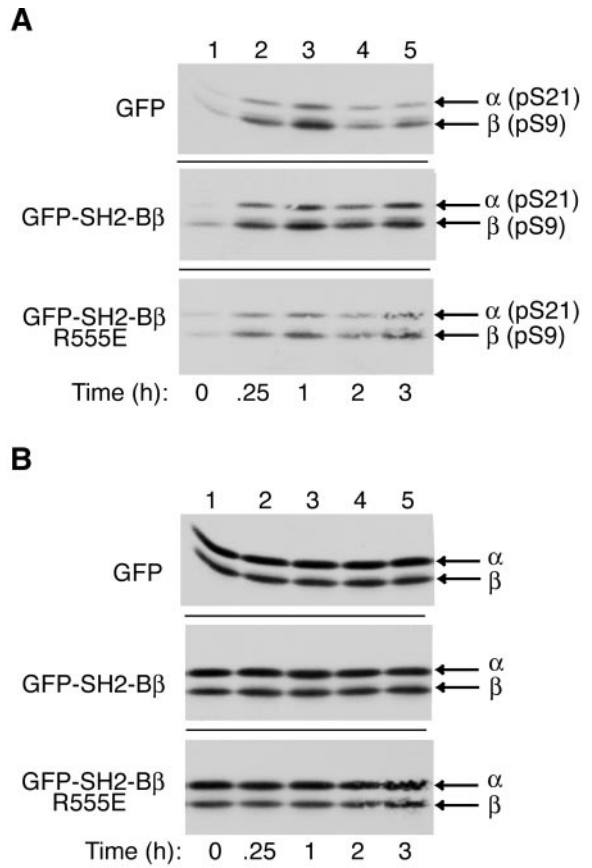

FIG. 4. **SH2-Bβ enhances and prolongs NGF-induced phosphorylation of GSK-3α/β on Ser^{21}/Ser^{9}.** PC12 cells stably overexpressing GFP alone, GFP-SH2-Bβ, and GFP-SH2-Bβ(R555E) were stimulated with 100 ng/ml NGF for the indicated times. An equal amount of protein in the lysates was immunoblotted with αpGSK-3(Ser$^{21/9}$) (*panel A*) or αGSK-3 (*panel B*). The resulting films from three separate experiments using αpGSK-3 were scanned, and the relative amounts of bound pGSK-3 were calculated (*panel C*). The results show the mean and standard error of the mean for three separate experiments, normalized to the 15-min control (*GFP*). An *asterisk* (*) denotes a p value <0.05 (two-tailed, paired Student's t test) between experimental (GFP-SH2-Bβ and GFP-SH2-Bβ(R555E)) and control (GFP) values.

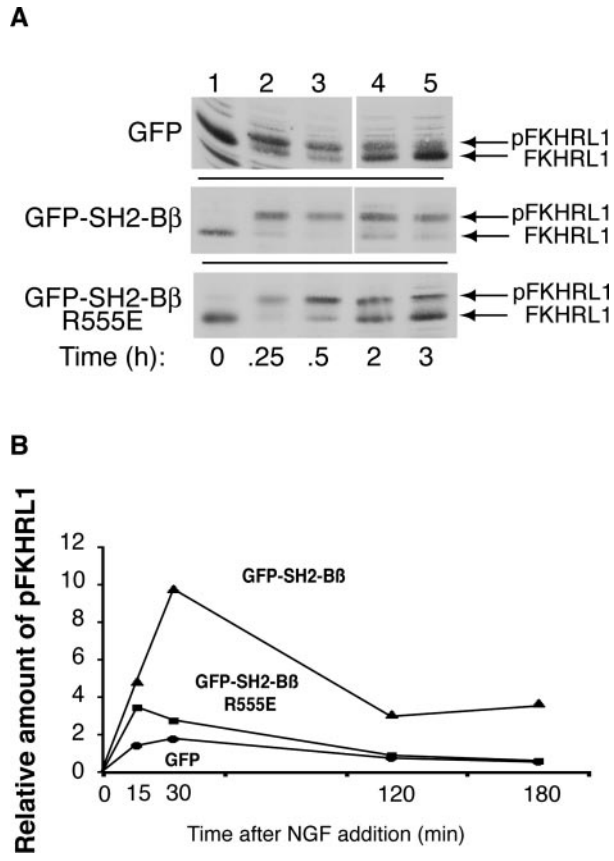

FIG. 5. **SH2-Bβ enhances and prolongs NGF-induced phosphorylation of FKHRL1.** PC12 cells stably overexpressing GFP alone, GFP-SH2-Bβ, and GFP-SH2-Bβ(R555E) were stimulated with 100 ng/ml NGF for the indicated times. An equal amount of protein in the lysates was immunoblotted with αFKHRL1 (*panel A*). The films in *panel A* were scanned, and the relative amounts of FKHRL1 were calculated (*panel B*). The results show the mean intensity of the phosphorylated FKHRL1 band (*upper band*) divided by the unphosphorylated FKHRL1 band (*lower band*).

expressing GFP alone or SH2-Bβ(R555E). The observed changes in the amount of Ser(P)$^{21/9}$ were not the result of changes in the amount of GSK-3. No significant change in total GSK-3α/β was observed after NGF treatment, and the levels of GSK-3 in the three cell lines were indistinguishable (Fig. 4*B*).

SH2-Bβ Enhances NGF-induced Phosphorylation of FKHRL1—To investigate further whether SH2-Bβ affects downstream targets of Akt, we examined whether SH2-Bβ affects NGF-induced phosphorylation of forkhead (FKHR/FOXO) family members. As shown in the anti-FKHRL1 (αFKHRL1) immunoblots in Fig. 5*A* (*upper panel*), in control cells overexpressing GFP alone, NGF induces a transient upward shift in the migration of FKHRL1, consistent with an increase in phosphorylation. The ratio of phosphorylated (upper band) to unphosphorylated (lower band) FKHRL1 was maximal at 15 or 30 min and declined thereafter (Fig. 5, *A* and *B*). Phosphorylation was significantly prolonged in PC12 cells stably expressing SH2-Bβ (Fig. 5*A*, *middle panel*) compared with control cells expressing GFP alone (Fig. 5*A*, *upper panel*) or cells expressing SH2-Bβ(R555E) (Fig. 5*A*, *lower panel*).

SH2-Bβ(R555E) Redistributes FKHR to the Nucleus—FKHR transcription factors are inactivated by Akt-mediated phosphorylation, which stimulates the redistribution of FKHR from the nucleus to the cytoplasm (53–55). Because SH2-Bβ stimulated the NGF-induced phosphorylation of FKHRL1, we examined whether overexpression of SH2-Bβ would alter the subcellular distribution of FKHR proteins. PC12 cells were transiently co-transfected with cDNAs encoding Flag-tagged FKHR and either GFP alone, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E). After 24 h, the cells were incubated in serum-free medium, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then incubated with αFlag followed by anti-mouse IgG conjugated to Texas Red. In some experiments, the location of Flag-FKHR was determined in cells that co-expressed GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E) using a Noran OZ laser-

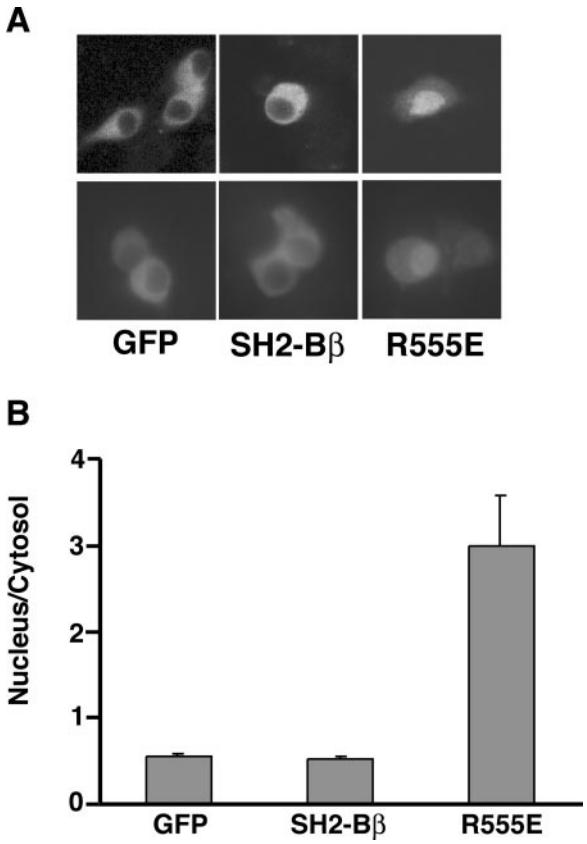

FIG. 6. **SH2-Bβ(R555E) promotes FKHR retention in the nucleus of PC12 cells.** PC12 cells (*panels A* and *B*) were transiently cotransfected with cDNA encoding Flag-FKHR and GFP alone, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E). Ectopically expressed proteins were imaged using either confocal (*panel A*, *upper*) or epifluorescence (*panel A*, *lower*) microscopy. For *panel B*, relative levels of FKHR in the cytoplasm and nucleus were determined by comparing the intensity of the signal in identically sized regions placed in the cytoplasm and nucleus of the same cell (*panel B*). The results show the mean and standard error for a total of 16–18 cells from experiments performed on 3–5 separate days.

scanning confocal microscope equipped with a 100× Nikon objective (Fig. 6*A*, *upper panel*). In other experiments, GFP- and fluorophore-conjugated antibodies were imaged by epifluorescence microscopy (Nikon Eclipse TE 200) with a 60× objective (Fig. 6*A*, *lower panel*). The mean intensities of neighboring cytosolic and nuclear regions (~5 μm^2 each) in the epifluorescent images were calculated using Adobe Photoshop™, corrected for background, and expressed as nuclear-to-cytosol fluorescence ratios (Fig. 6*B*).

Fig. 6 (*A* and *B*) shows that, in control PC12 cells co-expressing GFP and FKHR under quiescent conditions, FKHR was mainly localized in the cytoplasm and excluded from the nucleus. Co-expression of GFP-SH2-Bβ did not generally alter the subcellular localization of FKHR in these cells. In contrast, overexpression of SH2-Bβ(R555E) caused a dramatic redistribution of FKHR to the nucleus.

To determine whether this ability of SH2-Bβ(R555E) to redistribute FKHR was specific to PC12 cells, we examined the effect of expressing GFP, GFP-SH2-Bβ, and GFP-SH2-Bβ(R555E) on the subcellular distribution of Flag-FKHR in cultured COS-7 and 293T cells. In transiently transfected COS-7 cells (Fig. 7*A*), Flag-FKHR was predominantly localized in the cytoplasm and excluded from the nucleus of ~75% of COS-7 cells expressing GFP. Much like what was observed in the PC12 cells, co-expression of GFP-SH2-Bβ with Flag-FKHR did not alter the subcellular localization of FKHR. However,

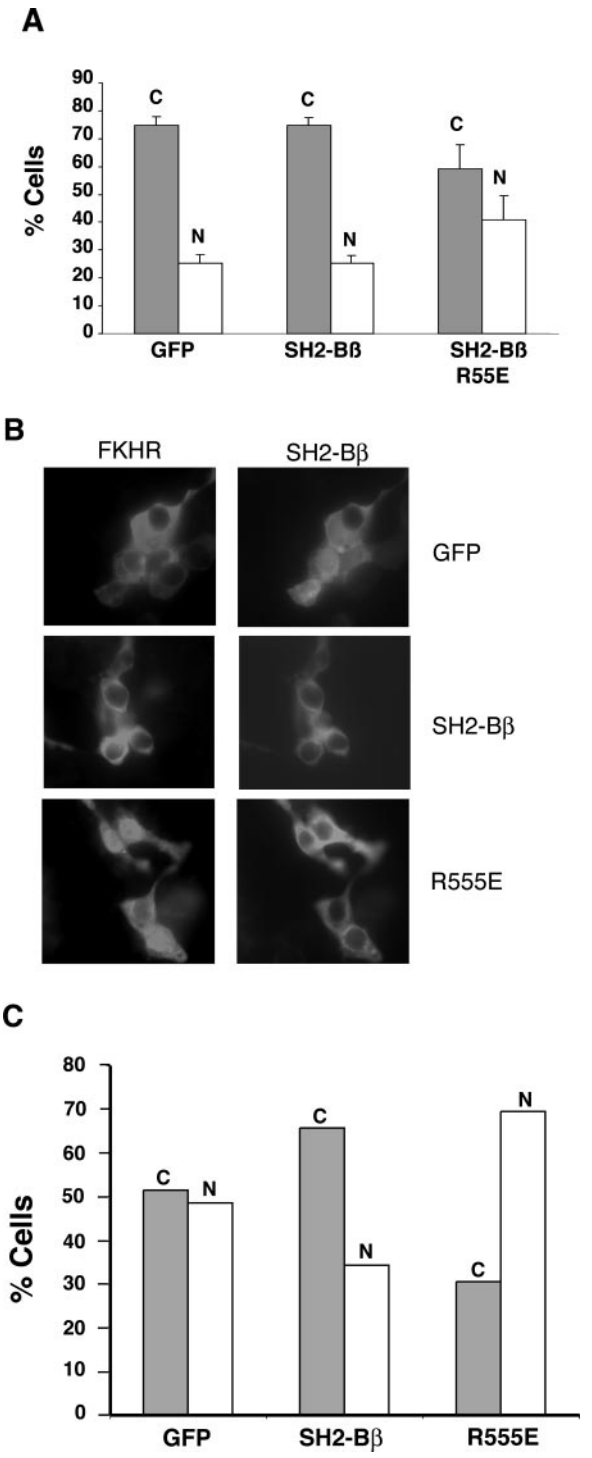

FIG. 7. **SH2-Bβ(R555E) redistributes FKHR to the nucleus in 293T and COS-7 cells.** *A*, COS-7 cells were transiently cotransfected with cDNA encoding Flag-FKHR and GFP alone, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E). The number of COS-7 cells in which FKHR was excluded from the nucleus (designated *C* for "cytoplasmic") and in which FKHR was present or concentrated in the nucleus (designated *N*) were graphed as a percentage of the total number of GFP-positive cells counted. 116, 69, and 103 cells expressing GFP alone, GFP-SH2-Bβ, and GFP-SH2-Bβ(R555E), respectively, were assessed from two trials. *B* and *C*, 293T cells were transiently cotransfected with cDNA encoding Flag-FKHR and GFP alone, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E). Ectopically expressed proteins were imaged using epifluorescence microscopy (*panel B*). The number of 293T cells in which FKHR was excluded from the nucleus (designated *C* for "cytoplasmic") and in which FKHR was present or concentrated in the nucleus (designated *N*) were graphed as a percentage of the total number of GFP-positive cells counted. 33, 35, and 49 cells expressing GFP alone, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E), respectively, were assessed.

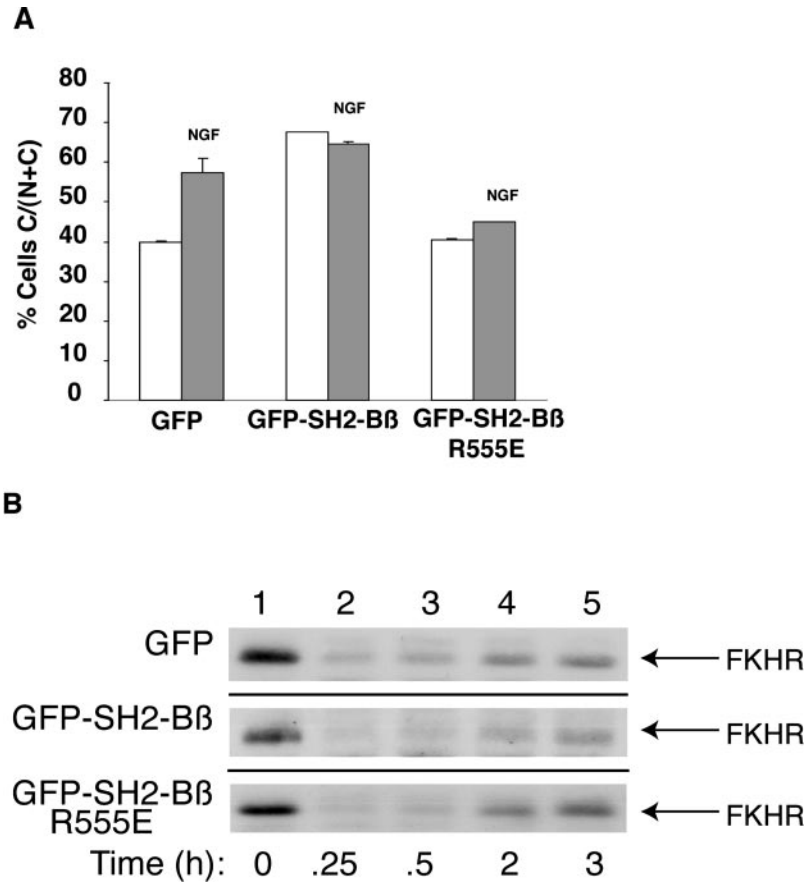

FIG. 8. **SH2-Bβ mimics and SH2-Bβ(R555E) blocks NGF-induced redistribution of FKHR from the nucleus to the cytoplasm.** PC12 cells stably expressing GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E) were transiently transfected with cDNA encoding Flag-FKHR. *A*, cells were incubated in serum free medium for 24 h and fixed (*white bars*) or treated with 100 ng/ml NGF for 1 h and fixed (*gray bars*). Units are defined as the percentage of cells with Flag-FKHR excluded from the nucleus (defined *C* for cytoplasmic) divided by the total number of cells counted of which expressed Flag-FKHR (defined *N*+*C*). 103, 100, 114, 192, 126, and 107 cells expressing GFP alone (minus and plus NGF), GFP-SH2-Bβ (minus and plus NGF), and GFP-SH2-Bβ(R555E) (minus and plus NGF), respectively, were assessed in two trials. *B*, cells were deprived for 24 h and then treated with 100 ng/ml NGF for 0, 0.25, 0.5, 2, and 3 h. An equal amount of protein in the lysates was immunoblotted with αFKHR. It is important to note that this antibody recognizes only unphosphorylated FKHR.

overexpression of SH2-Bβ(R555E) resulted in increased retention (from ~25% to ~40% of cells) of FKHR within the nucleus of COS-7 cells (40%).

293T cells ectopically expressing Flag-FKHR and GFP displayed a lower basal level of cytoplasmic FKHR (~50%) and a higher basal level of nuclear FKHR than seen in COS-7 cells (Fig. 7, *B* and *C*). In these cells, overexpression of SH2-Bβ caused an increase in the percentage of the cells (~65%) that retained FKHR in the cytoplasm and excluded it from the nucleus. As observed with PC12 and COS-7 cells, ectopic expression of SH2-Bβ(R555E) caused a substantial decrease in the percentage of 293T cells containing FKHR in the cytoplasm (from ~50% to ~30%) and corresponding increase in the percentage of cells containing FKHR in the nucleus (from ~50% to ~70%).

Thus, a substantial percentage of all three cell types exhibited a subcellular redistribution of FKHR from the cytoplasm to the nucleus when expressing SH2-Bβ(R555E). To determine whether this nuclear localization could be reversed with NGF stimulation, PC12 cells stably expressing GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E) were transiently transfected with cDNA encoding Flag-FKHR. After transfection the cells were deprived of serum overnight and treated with 100 ng/ml NGF for 1 h before fixing and staining. Fig. 8 demonstrates the characteristic subcellular shift of KKHR after NGF treatment in the GFP-expressing control cells. The addition of NGF led to an increased number of cells with Flag-FKHR excluded from the nucleus (from ~40% to ~60%). Consistent with the studies using 293T cells, which displayed a similar percentage of cells in which FKHR was excluded from the nucleus, stable expression of GFP-SH2-Bβ in PC12 cells increased the number of cells with cytoplasmic Flag-FKHR, to a level above that seen in the control cells even in the presence of NGF. Treatment with NGF did not result in a further increase in the number of cells with FKHR in the cytoplasm. In contrast, PC12 cells stably expressing GFP-SH2-Bβ(R555E) were unable to shift FKHR from the nucleus to the cytoplasm even after NGF treatment. These results suggest that overexpression of SH2-Bβ can mimic the action of NGF on the subcellular distribution of FKHR, whereas overexpression of SH2-Bβ(R555E) blocks the ability of NGF to redistribute FKHR to the cytoplasm.

Because cytoplasmic localization of FKHR is thought to be dependent on its phosphorylation, we examined whether overexpression of SH2-Bβ or SH2-Bβ(R555E) affects the serine/threonine phosphorylation of FKHR measured under the exact experimental conditions used to study the subcellular localization of FKHR in Fig. 8*A*. Cells were deprived and stimulated with NGF in the same manner as above, before collecting total cell lysates. Proteins were separated by SDS-PAGE and immunoblotted with anti-FKHR, an antibody that recognizes unphosphorylated but not phosphorylated FKHR. Thus, the degree of phosphorylation is indicated by a decrease in the signal. As shown in Fig. 8*B*, phosphorylation of ectopically expressed Flag-FKHR was identical in cells expressing GFP and SH2-Bβ(R555E). Phosphorylated Flag-FKHR peaked between 15 and 30 min in both lines and began to return to its unphosphorylated form by 2 h. Also, consistent with the FKHRL1 results (Fig. 5, *A* and *B*), cells expressing GFP-SH2-Bβ showed an increase in the duration of FKHR phosphorylation. Similar results were obtained looking at the phosphorylation of endogenous FKHR in both cells that did and cells that did not co-express Flag-FKHR (data not shown). Thus, FKHR appears to be phosphorylated normally in the GFP-SH2-Bβ(R555E) cell line in response to NGF treatment, yet still displays an inability to shift FKHR from the nucleus to the cytoplasm in response to NGF, suggesting that SH2-Bβ acts at least in part, downstream of Akt phosphorylation.

Consistent with FKHR being redistributed to the nucleus,

where it is thought to be proapoptotic by virtue of stimulating the transcription of proapoptotic proteins, co-expression of FKHR and SH2-Bβ(R555E) appeared to be deleterious to the cells. In contrast to GFP and GFP-SH2-Bβ, for which most of the cells co-expressed FKHR and either GFP or GFP-SH2-Bβ, significantly fewer cells could be found co-expressing FKHR and SH2-Bβ(R555E) in either PC12 cells or 293T cells. For example, in 293T cells deprived of serum overnight, 86% of cells expressing SH2-Bβ also expressed FKHR whereas only 16% of cells expressing SH2-Bβ(R555E) also expressed FKHR. Also consistent with nuclear FKHR being deleterious to the cell, when PC12 cells were transfected with cDNA encoding FKHR lacking its three AKT consensus phosphorylation sites, essentially no PC12 cells were found to express this FKHR mutant after 24 h (data not shown).

DISCUSSION

The results presented in this study provide strong evidence that at least one mechanism by which the putative adapter protein SH2-Bβ contributes to the ability of NGF to promote survival of sympathetic neurons is to enhance the ability of NGF to activate Akt. Using the well studied PC12 cell as a model system, we showed that NGF-induced activation of Akt is both enhanced and/or prolonged in cells overexpressing wild-type SH2-Bβ. This is most likely a consequence of the increased NGF-induced phosphorylation of Akt, because it correlates in magnitude and time course with the increased NGF-induced phosphorylation of Ser^{473}. Phosphorylation of both Ser^{473} and Thr^{308} has been shown to be obligatory for maximal activation of Akt (32). This increased activity of Akt is most likely responsible for the similarly enhanced and prolonged NGF-induced phosphorylation of the Akt effector proteins, GSK-3, FKHRL1, and FKHR. The simplest explanation for this activation of Akt is that it is a consequence of the reported ability of SH2-Bβ to enhance and prolong NGF-induced tyrosyl phosphorylation of TrkA (69). Based upon what is known about how Akt is activated by receptor tyrosine kinases (29–32, 70), we would predict that the more highly phosphorylated TrkA would recruit more PI 3-kinase, which in turn would stimulate the recruitment of more Akt to the plasma membrane, which would result in more molecules of Akt being phosphorylated on Ser^{473} and Thr^{308} by phosphoinositide-dependent kinases. The NGF-induced activation of Akt (and phosphorylation of its downstream effectors GSK-3, FKHRL1, and FKHR) reported here, like the previously reported activation of TrkA, is enhanced by overexpression of SH2-B, but is not affected by ectopic expression of a form of SH2-B expected to act as a dominant negative, such as SH2-Bβ(R555E) (57) (in the case of Akt) or an N-terminally truncated form of SH2-B (in the case of TrkA) (69). It is also possible that SH2-Bβ enhances and prolongs NGF activation of Akt by acting at a point downstream of TrkA, for instance by blocking the dephosphorylation of Akt.

Enhanced NGF-induced phosphorylation of Akt substrates presumably contributes to the ability of SH2-B to protect against axonal degeneration and/or neuron cell death. GSK-3, which exhibits increased NGF-induced phosphorylation in PC12 cells stably expressing SH2-Bβ, is inhibited upon phosphorylation by Akt (54), and inhibition of GSK-3 has been reported to reduce neuronal death caused by inhibition of Akt (71). One mechanism proposed by which inhibition of GSK-3 contributes to neuronal survival is by stabilization of β-catenin (72). Down-regulation of GSK-3 as a consequence of SH2-Bβ-enhanced phosphorylation would also be expected to prevent neurite retraction. It seems likely that SH2-Bβ enhancement of NGF-induced phosphorylation and consequent inactivation of other pro-apoptotic, Akt substrates, such as the Bcl2 family member BAD, will also be found to contribute to the survival effects of SH2-Bβ.

The lack of a negative effect of SH2-Bβ(R555E) on Akt activity was somewhat surprising to us, given the ability of this mutant to completely block NGF-induced neuronal differentiation of PC12 cells (57) and our anecdotal observation that this mutant SH2-Bβ promotes cell death. This suggested to us that the ability of SH2-Bβ to enhance and prolong NGF-induced activation of Akt may not be the only mechanism by which SH2-Bβ contributes to NGF-induced differentiation and/or survival of PC12 cells and/or sympathetic neurons. The finding that SH2-Bβ(R555E) profoundly affects the subcellular distribution of FKHR, even in the absence of NGF, provides support for the hypothesis that SH2-Bβ affects more than just Akt-mediated phosphorylation of FKHR. Based upon the current dogma (56), activated Akt moves to the nucleus where it can phosphorylate members of the FKHR family on multiple Ser/Thr. The phosphorylated FKHR binds to 14-3-3, which facilitates its export from the nucleus and may facilitate retention in the cytoplasm. Recently, the phosphorylation of two novel casein kinase 1 sites within FKHR, Ser^{322} and Ser^{325}, was shown to be required for FKHR to interact with the export machinery (73). Because FKHR members have been implicated in the regulation of a variety of apoptotic genes, including Fas ligand and cyclin-dependent kinase inhibitor $p27^{KIP1}$ (74), inhibition of FKHR activity by keeping it out of the nucleus would promote cell survival. The current results suggest that SH2-B may participate in regulating the subcellular distribution of FKHR. One scenario is that SH2-B acts as a scaffolding protein that promotes the interaction of FKHR with 14-3-3. Another possibility is that SH2-B facilitates the transport of FKHR out of the nucleus. One can envision SH2-Bβ(R555E) acting as a dominant negative by binding to some but not all of the proteins necessary for FKHR interaction with 14-3-3 or for nuclear export of FKHR. The likelihood of this possibility is suggested by our recent finding[3] that SH2-Bβ itself cycles between the cytoplasm and the nucleus. Alternatively, SH2-Bβ might take one or more of the proteins required for these events to the wrong subcellular location or affect the ability of FKHR to be phosphorylated on the casein kinase I phosphorylation sites Ser^{322} and Ser^{325}. Any of these scenarios could occur as a consequence of SH2-Bβ(R555E) not binding to TrkA (or other neurotrophic receptor tyrosine kinases) and/or not being phosphorylated on tyrosines, serines, or threonines as a consequence of not binding to activated TrkA (57, 75). However, the fact that overexpression of SH2-Bβ causes FKHR to shift to the cytoplasm in the absence of NGF suggests that modification of SH2-Bβ by TrkA is not necessary for SH2-Bβ to affect the subcellular distribution of FKHR.

In summary, using PC12 cells as a model system, we have provided substantial evidence that SH2-Bβ enhances and prolongs NGF activation of Akt and phosphorylation of its downstream effectors, including GSK-3α and -β, FKHRL1, and FKHR. This action would be expected to contribute to the observed ability of overexpressed SH2-Bβ to enhance NGF-induced neuronal differentiation of PC12 cells and survival of sympathetic neurons (57, 58). SH2-Bβ appears to have an additional effect on FKHR and potentially other members of the FKHR family, namely promoting or retaining FKHR in the cytoplasm, thereby preventing it from acting as a transcription factor. We are actively involved in determining the mechanisms by which SH2-Bβ and its mutants affect the subcellular localization of FKHR family members.

Acknowledgments—We thank B. Hawkins for assistance with the figures and the manuscript. We thank Drs. E. Tang and K. Guan for the gift of FKHR cDNA.

[3] L. Chen and C. Carter-Su, submitted for publication.

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