

Adapter Protein SH2-B β Undergoes Nucleocytoplasmic Shuttling: Implications for Nerve Growth Factor Induction of Neuronal Differentiation

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Received 22 August 2003/Returned for modification 18 September 2003/Accepted 5 February 2004

The adapter protein SH2-B has been shown to bind to activated nerve growth factor (NGF) receptor TrkA and has been implicated in NGF-induced neuronal differentiation and the survival of sympathetic neurons. However, the mechanism by which SH2-B enhances and maintains neurite outgrowth is unclear. We examined the ability of truncation mutants to regulate neuronal differentiation and observed that certain truncation mutants localized in the nucleus rather than in the cytoplasm or at the plasma membrane as reported for wild-type SH2-B β . Addition of the nuclear export inhibitor leptomycin B caused both overexpressed wild-type and endogenous SH2-B β to accumulate in the nucleus of both PC12 cells and COS-7 cells as did deletion of a putative nuclear export sequence (amino acids 224 to 233) or mutation of two critical lysines in that sequence. Deleting or mutating the nuclear export signal caused SH2-B β to lose its ability to enhance NGF-induced differentiation of PC12 cells. Neither the NGF-induced phosphorylation of ERKs 1 and 2 nor their subcellular distribution was altered in PC12 cells stably expressing the nuclear export-defective SH2-B β (L231A, L233A). These data provide strong evidence that SH2-B β shuttles constitutively between the nucleus and cytoplasm. However, SH2-B β needs continuous access to the cytoplasm and/or plasma membrane to participate in NGF-induced neurite outgrowth. These data also suggest that the stimulatory effect of SH2-B β on NGF-induced neurite outgrowth of PC12 cells is either downstream of ERKs or via some other pathway yet to be identified.

Nerve growth factor (NGF) is a trophic factor essential for the development and maintenance of sympathetic and sensory neurons. The PC12 cell line, derived from a rat pheochromocytoma arising from chromaffin cells of the adrenal medulla, is a well-established model for studying neuronal differentiation and cell signaling. Progressively, NGF-treated PC12 cells cease proliferation, exhibit somatic hypertrophy, acquire neurites, differentiate, and depend on NGF for survival (for a review, see reference 52). The signal that initiates neuronal differentiation starts with NGF binding to the receptor tyrosine kinase TrkA and activating TrkA. The activated TrkA autophosphorylates, and the phosphorylated tyrosines in the TrkA cytoplasmic domain serve as anchors for downstream signaling molecules (5, 49).

Several signaling molecules have been shown to become phosphorylated and activated in response to NGF, including the adapter protein Shc, the Src-associated neurotrophic factor-induced tyrosine-phosphorylated targets (SNTs, also called FRS2), phospholipase-C γ (PLC- γ), and phosphatidylinositol-3-kinase (PI3K) (5, 10, 16, 34, 35, 40, 60). Shc binds to phosphorylated tyrosine 490 in TrkA. Once phosphorylated by TrkA, Shc binds Grb2/SOS complexes which initiates activation of the Ras/Raf/MEK pathway that leads to activation of the mitogen-activated protein kinases (MAPKs) designated extracellular regulated kinases (ERKs) 1 and 2 (13, 14, 58, 59). SNTs are early markers for neuronal differentiation. Like Shc,

they recruit Grb2 adapter proteins in complex with SOS and Ras as well as the SH2 domain-containing protein tyrosine phosphatase SHP2. These interactions have been suggested to be important in maintaining sustained activation of ERKs 1 and 2 and thus the differentiation of PC12 cells (60). The binding of PLC- γ to TrkA regulates the production of diacylglycerol and inositol triphosphate, leading to release of intracellular Ca²⁺ stores and activation of protein kinase C (5, 8, 37, 43, 57). Mobilization of Ca²⁺ is thought to regulate growth cone function and neurite outgrowth (4, 11, 23, 64). Activation of PI3K and its downstream effector, the serine/threonine kinase AKT/PKB, has been implicated in the assembly of basic helix-loop-helix transcription factor-coactivator complexes and their ability to promote neurogenesis in P19 cells (53).

We and others identified the putative adapter/scaffold protein SH2-B as a binding partner of TrkA as well as of the receptors for insulin, insulin-like growth factor 1, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor, and the cytokine receptor-associated JAK tyrosine kinases (25, 26, 39, 41, 42, 44, 46, 54, 63). SH2-B belongs to a family of adapter proteins that include APS and Lnk (19, 21, 62, 63). The four SH2-B isoforms, α , β , γ , and δ , identified so far differ only in their C termini starting just past the SH2 domain (33, 63). SH2-B β contains three proline-rich domains, a pleckstrin homology (PH) domain and a Src homology (SH2) domain; it is the C-terminal SH2 domain that binds to the receptor tyrosine kinases (RTKs).

SH2-B α and β isoforms have been shown to be essential for NGF-induced neurite outgrowth in PC12 cells (39, 46), and SH2-B α has been implicated in the survival of rat sympathetic neurons (39). NGF enhances the association of SH2-B with

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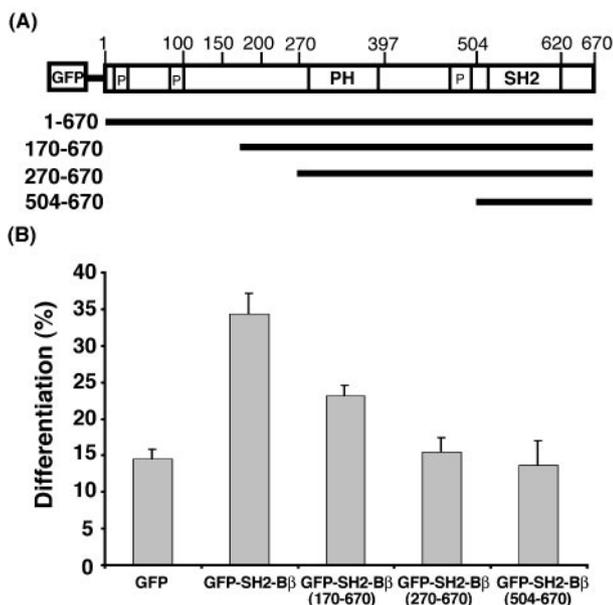


FIG. 1. Effects of N-terminal truncation mutants of SH2-B β on neuronal differentiation. (A) Schematic representations of various N-terminal truncation mutants of SH2-B β fused to the C terminus of GFP. The proline-rich domain (P), pleckstrin homology domain (PH), and Src homology domain SH2 are depicted. The numbers are the amino acid numbers in the rat sequence of SH2-B β . (B) PC12 cells were transiently transfected with cDNAs encoding either GFP alone, full-length SH2-B β , or the SH2-B β mutants depicted in panel A and then treated with 100 ng of NGF per ml for 4 days. The percentage of differentiation was determined on day 4 as the number of transfected and differentiated cells divided by the number of transfected cells. A total of 150 to 200 transfected cells were counted for each condition in each experiment. The values are averages \pm standard errors (error bars) from three different experiments.

TrkA and the tyrosine phosphorylation of SH2-B (39, 46). Deletion analysis suggests that N-terminal amino acids (100 to 235) of SH2-B α are required for forming homomultimers of SH2-B α and that multimers of SH2-B α may be required for SH2-B regulation of NGF-induced TrkA and MAPK activity (38). The ability of SH2-B β to bind TrkA via its SH2 domain appears to be critical for NGF-induced neurite outgrowth in PC12 cells. A point mutation in the FLVR motif within the SH2 domain of SH2-B β that changes Arg 555 to Glu [SH2-B β (R555E)] prevents the association of SH2-B β with TrkA as well as the tyrosine phosphorylation of SH2-B β (47). While overexpression of SH2-B β enhances NGF-induced neurite outgrowth of PC12 cells, overexpressing the SH2-B β (R555E) mutant abolishes it (46, 47). Curiously, overexpressing SH2-B β (R555E) does not inhibit the tyrosyl phosphorylation of TrkA, Shc, PLC- γ , or ERKs 1 and 2 in response to NGF stimulation (47). This latter finding raises the possibility that SH2-B β modulates neuronal differentiation via mechanisms other than through PLC- γ and ERKs 1 and 2 or downstream of those pathways.

To gain insight into the mechanism(s) by which SH2-B β enhances neurite outgrowth of PC12 cells, we set out to investigate further which domain(s) or region(s) of SH2-B β is responsible for enhancing neurite outgrowth. During this analysis, we obtained evidence suggesting that SH2-B β shuttles

between the cytoplasm and the nucleus and identified a nuclear export signal. The fact that deletion or mutation of the nuclear export signal abolished the ability of SH2-B β to enhance NGF-induced neurite outgrowth of PC12 cells suggest that cytoplasmic localization of SH2-B β is required for this stimulatory effect.

MATERIALS AND METHODS

Antibodies and reagents. Polyclonal antibody against green fluorescent protein (GFP) was from Clontech. Polyclonal antibody to rat SH2-B was raised against a glutathione *S*-transferase fusion protein containing amino acids 527 to 670 of SH2-B β as described previously (48). Polyclonal antibody against active ERKs 1 and 2 (pThr202 and pTyr204) was purchased from either Promega (for immunostaining) or from Cell Signaling (Western blot). The anti-phospho-TrkA (Y490) was from UBI, and the poly-L-lysine was from Sigma. NGF (mouse) and rat tail collagen were purchased from BD Bioscience. Lipofectamine and the Plus reagent were from Invitrogen.

Plasmids. The N-terminal truncation mutants of GFP-tagged SH2-B β (GFP-SH2-B β), GFP-SH2-B β (170-670), GFP-SH2-B β (200-670), GFP-SH2-B β (270-670), GFP-SH2-B β (397-670), and GFP-SH2-B β (504-670), were constructed by generating via PCR mutagenesis BamHI sites at amino acids 168 to 169, 267 to 268, 395 to 396, and 502 to 503, respectively, using GFP-SH2-B β as a template. These mutants were then digested with BamHI and EcoRI. The purified BamHI- and EcoRI-digested SH2-B β fragments were inserted into the BgIII- and EcoRI-digested pEGFP-C1 vector. GFP-SH2-B β (Δ 198-268) was made by generating BamHI sites at amino acids 198 and 199 and amino acids 267 to 268 using GFP-SH2-B β as a template. Fragments from NheI, BamHI, and BamHI plus EcoRI digestions of this mutant were purified. The resulting two fragments, 1.2 and 1.77 kb, were then ligated to NheI- and EcoRI-cut pEGFP-C1 vector (4 kb) to make the final GFP-SH2-B β (Δ 198-268) construct. The construction of C-terminal truncation mutants was described previously (9). Point mutant GFP-SH2-B β (L231A, L233A) was made by PCR mutagenesis. All mutant constructs were confirmed by DNA sequencing.

Cells and cell culture. The stock of PC12 cells was purchased from American Type Culture Collection. PC12 cells were plated on plates coated with 0.1 mg of rat tail collagen per ml (in 0.02 N acetic acid) and grown at 37°C in 10% CO₂ in Dulbecco modified Eagle medium (DMEM) (Invitrogen) containing heat-inactivated 10% horse serum (ICN) and 5% fetal bovine serum (Invitrogen), supplemented with 1 mM L-glutamine and 1 mM antibiotic-antimycotic (Invitrogen). PC12 cells stably overexpressing GFP, GFP-SH2-B β , GFP-SH2-B β (270-670), or GFP-SH2-B β (Δ 198-268) were made by transfecting PC12 cells with the constructs described above and then growing the cells in selection medium containing 5 mg of G418 (Invitrogen) per ml for at least 90 days. Cells resistant to G418 were subjected to fluorescence cell sorting based on GFP fluorescence. The population with the highest expression level (top 5%) was pooled to avoid clonal variation. Stable pooled cell lines were maintained in complete medium plus 2 mg of G418 per ml. COS-7 cells were cultured in DMEM containing 8% fetal bovine serum supplemented with 1 mM L-glutamine and 1 mM antibiotic-antimycotic and grown at 37°C in 5% CO₂.

Differentiation of PC12 cells. PC12 cells were plated on six-well collagen-coated plates the day before transfection. Cells were transfected using Lipofectamine and the Plus reagent with various constructs encoding SH2-B β mutants or the pEGFP vector alone. After 5 h, the medium was changed to differentiation medium (DMEM plus 2% horse serum and 1% fetal bovine serum) that contained 100 ng of NGF per ml. The NGF-containing medium was replaced every 2 days, and the percentage of differentiation was scored. Cells with neurite length at least twice the diameter of the cell body were counted as differentiated cells. The percentage of differentiated cells was determined by dividing the number of cells that were both transfected and differentiated by the total number of transfected cells.

Immunoblotting and immunoprecipitation. PC12 cells were harvested into lysis buffer (50 mM Tris [pH 7.5], 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA) and kept on ice for an additional 10 min before centrifugation at 8,250 \times g in an IEC/MicroMax centrifuge for 10 min at 4°C. The supernatant was transferred to a new tube and boiled in sample buffer. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose paper for Western blot analysis. For coimmunoprecipitation, GFP-SH2-B β was immunoprecipitated using anti-GFP antibody and then incubated with protein A agarose. The immunoprecipitated proteins were resolved by SDS-PAGE.

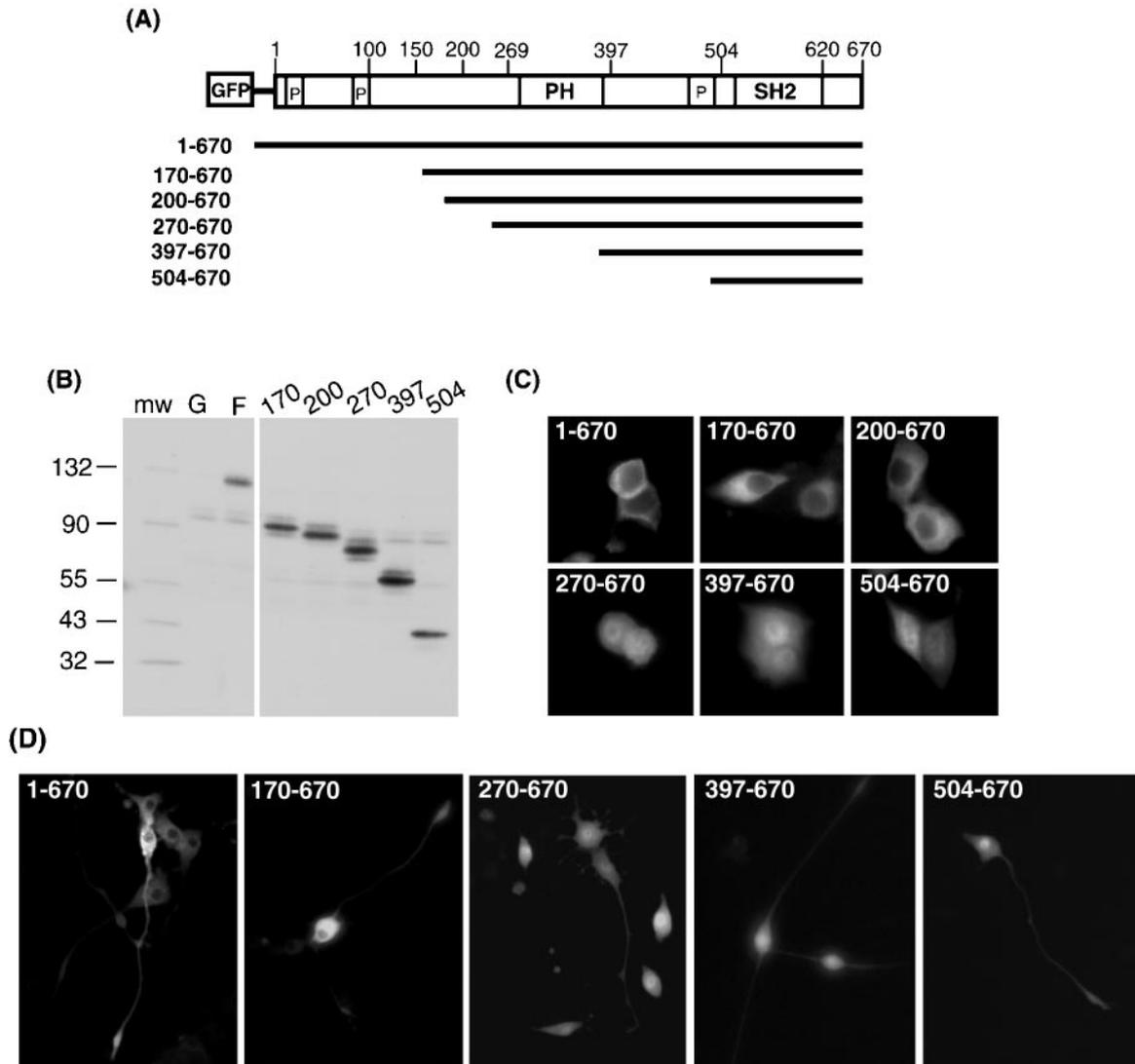


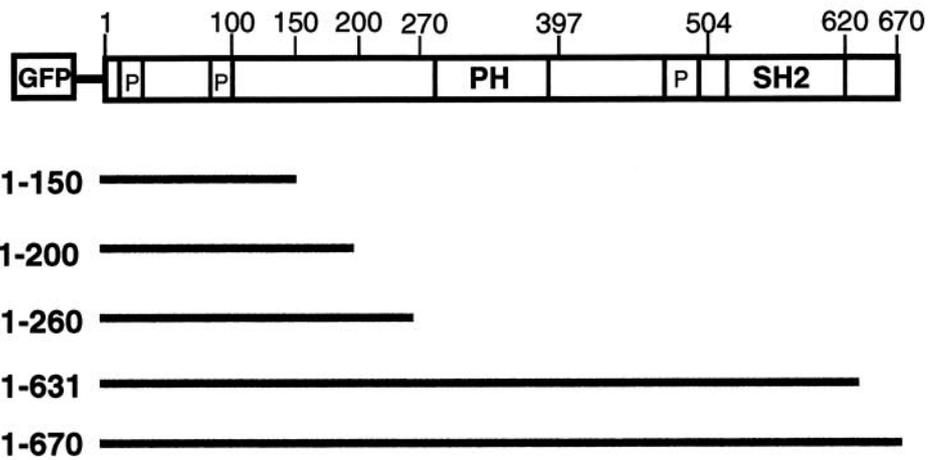
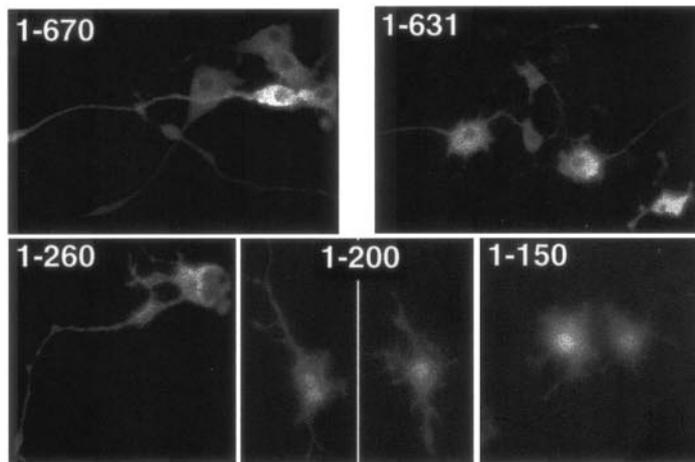
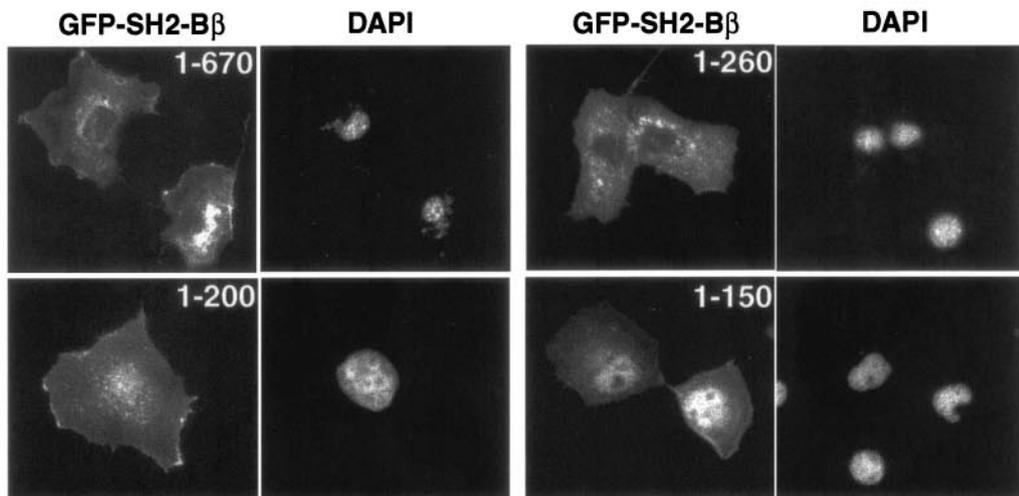
FIG. 2. Subcellular distribution of N-terminal truncation mutants of SH2-B β . (A) Schematic representations of various N-terminal truncation mutants of SH2-B β fused to the C terminus of GFP. The proline-rich domain (P), pleckstrin homology domain (PH), and Src homology domain SH2 are depicted. The numbers are the amino acid numbers in the rat sequence of SH2-B β . (B) PC12 cells were transiently transfected with cDNA encoding vector alone (G), full-length GFP-tagged SH2-B β (F), or a GFP-tagged mutant of SH2-B β shown in panel A, and designated by the number of its initial amino acid. Cells were lysed 18 h after transfection. Cell lysates were resolved by SDS-PAGE and immunoblotted with anti-SH2-B antibody. The migration positions of molecular weight (mw) standards (in thousands) are shown to the left of the gel. (C) PC12 cells were transiently transfected with cDNAs encoding GFP-tagged full-length SH2-B β [GFP-SH2-B β (1-670)] or an N-terminal truncation mutant of SH2-B β . The N-terminal truncation SH2-B β mutants GFP-SH2-B β (170-670), GFP-SH2-B β (200-670), GFP-SH2-B β (270-670), GFP-SH2-B β (397-670), and GFP-SH2-B β (504-670) were used. Cells were fixed 18 h after transfection with 4% paraformaldehyde, and the images were taken using epifluorescence microscopy. (D) PC12 cells were transiently transfected with cDNAs encoding GFP-tagged full-length SH2-B β or an N-terminal truncation mutant of SH2-B β and then treated with 100 ng of NGF per ml for 4 days. Images of live cells were taken on day 4 using epifluorescence microscopy.

Immunolocalization. PC12 cells were plated onto poly-L-lysine-coated coverslips and transfected the following day with various constructs encoding GFP-SH2-B β fusion proteins using Lipofectamine and the Plus reagents. COS-7 cells were plated on coverslips and transfected with SH2-B β -encoding constructs via calcium phosphate precipitation. After overnight expression of the transfected cDNA, cells were fixed with 4% paraformaldehyde. Coverslips were mounted onto slides with Prolong (Molecular Probe). The subcellular distribution of the various GFP-SH2-B β proteins was determined by GFP fluorescence. To detect endogenous SH2-B β , PC12 cells were plated on the coated chamber slides (Fisher) and fixed with methanol at -20°C for at least 30 min. Fixed cells were incubated with anti-SH2-B β antibody (Santa Cruz) (diluted 1:25) for 1 h, with rabbit anti-goat Alexa 488 antibody (Fab') (Molecular Probe) (diluted 1:100) for 1 h, and then with 4',6'-diamidino-2-phenylindole (DAPI) (Molecular Probe) (2

ng/ml) for 5 min. Active ERK was immunostained with active ERK antibody (diluted 1:250) (Promega) for 1 h, with goat anti-rabbit Alexa 555 antibody (Molecular Probe) (diluted 1:500) for 1 h, and with 2 ng of DAPI per ml for 5 min. The fixed and stained cells (undifferentiated PC12 cells and COS-7 cells) were visualized by fluorescence microscopy (Nikon Eclipse TE200) with either 60 \times (undifferentiated PC12 cells) or 40 \times (COS-7 cells) objectives. Live, differentiated PC12 cells were visualized using a 20 \times objective. Images were captured using a SPOT camera from Diagnostic Instruments, Inc.

RESULTS

Identification of regions of SH2-B β important for promoting NGF-induced neurite outgrowth. We and others have

(A) C-terminal truncation mutants of SH2-B β **(B) Differentiated PC12 cells****(C) COS-7 cells**

shown previously that overexpression of SH2-B (α and/or β) enhances NGF-induced neurite outgrowth of PC12 cells (38, 39, 46, 47). To determine which region of SH2-B β is required for its stimulatory effect on neuronal differentiation, we constructed a series of cDNAs encoding SH2-B β truncation mutants fused to the C terminus of GFP as depicted in Fig. 1A. PC12 cells were transiently transfected with cDNAs encoding GFP-tagged wild-type SH2-B β or various truncated forms of SH2-B β and grown for 4 days in low-serum medium containing 100 ng of NGF per ml. The differentiation percentage was determined by dividing the number of differentiated transfected cells by the total number of transfected cells on day 4. Cells were considered differentiated if they had neurites at least twice the length of their cell body. As shown in Fig. 1B, PC12 cells expressing GFP alone showed an average 14.5% of transfected cells that differentiated in response to NGF. Overexpression of GFP-SH2-B β more than doubled the number of differentiated cells (to 34.4%). Deletion of the first 169 amino acids [SH2-B β (170-670)] reduced the percentage of differentiated cells to 23.1%. Deleting the N-terminal 269 amino acids inhibited the ability of SH2-B β to enhance neuronal differentiation, with only 15.5% cells being differentiated. These results suggest that regions of SH2-B β between amino acids 1 and 270 are required for the stimulatory effect of SH2-B β on neurite outgrowth.

Subcellular distribution of SH2-B and its N-terminal truncation mutants. To our surprise, when examining the effects of the different truncated forms of SH2-B β on neurite outgrowth, a difference in the subcellular distribution of the various SH2-B β mutants was observed. Some versions of SH2-B β were found almost exclusively in the cytoplasm, while others were distributed in both the cytoplasm and nucleus. To characterize this further and to gain insight into how the subcellular distribution of SH2-B β affects its ability to enhance morphological differentiation of PC12 cells, we set out to identify the regions of SH2-B β that are responsible for its nuclear localization.

Figure 2A depicts a series of N-terminal truncation mutants of SH2-B β for which the subcellular distribution was examined. PC12 cells were transiently transfected with cDNA encoding GFP-tagged full-length SH2-B β or a GFP-tagged N-terminal truncation mutant, and the relative expression levels and sizes of the ectopically expressed SH2-B β were confirmed by Western blot analysis. As shown in Fig. 2B, the proteins expressed by the various GFP-SH2-B β fusion constructs migrated as proteins of the appropriate estimated sizes. In addition, the expression levels of these proteins were similar, suggesting that any differences in the observed subcellular distribution of these SH2-B β mutants were not a consequence of different levels of overexpression.

The subcellular distribution of N-terminal truncation mu-

tants of SH2-B β was examined 18 h after transfection via GFP green fluorescence (Fig. 2C). Full-length SH2-B β localized predominantly in the cytoplasm at steady state, as did the N-terminal truncation mutants SH2-B β (170-670) and SH2-B β (200-670). However, deletion of additional N-terminal amino acids resulted in SH2-B β (270-670), SH2-B β (397-670), and SH2-B β (504-670) in the nucleus and cytoplasm. The same pattern of subcellular distribution of the different forms of SH2-B β was also observed in the differentiated PC12 cells (Fig. 2D). In general, the nuclear-cytoplasmic transport of proteins larger than 40 kDa is thought to be via a tightly regulated and energy-dependent process (15). Thus, the nuclear distribution of these SH2-B β mutants is unlikely due to simple diffusion, because GFP-SH2-B β (270-670), GFP-SH2-B β (397-670), and GFP-SH2-B β (504-670) express as 71, 57, and 45 kDa proteins (Fig. 2B) that would not be expected to diffuse in and out of the nucleus at a significant rate. Thus, deleting the N-terminal 269 amino acids shifted the location of SH2-B β from primarily cytoplasm to both the cytoplasm and nucleus. We also examined the subcellular distribution of myc-tagged SH2-B β constructs and observed the same localization pattern (data not shown), suggesting that the subcellular distribution pattern of the various forms of SH2-B β is not an artifact of using the GFP tag. These localization results obtained using the SH2-B β N-terminal truncation mutants suggest that SH2-B β can enter the nucleus and that amino acids 200 to 270 are responsible for maintaining SH2-B β in the cytoplasm.

Subcellular distribution of C-terminal truncation mutants of SH2-B β . To confirm that sequence between amino acids 200 and 270 constitute the region that causes a shift in the steady-state subcellular distribution of SH2-B β , a series of constructs encoding C-terminal truncation mutants of SH2-B β fused to the C terminus of GFP were used as depicted in Fig. 3A. When cells transiently expressing these proteins were treated with 100 ng of NGF per ml for 4 days, full-length SH2-B β (1-670) and SH2-B β (1-631) expressed in differentiated PC12 cells localized primarily in the cytoplasm (Fig. 3B). While SH2-B β (1-260) was also seen in the cytoplasm of differentiated PC12 cells, in contrast, SH2-B β (1-200) concentrated in the nucleus. When only the N-terminal 150 amino acids of SH2-B β were expressed, SH2-B β (1-150) also localized to both the cytoplasm and nucleus.

To visualize the subcellular distribution of SH2-B β better, we took advantage of the larger and flatter morphology of COS-7 cells. Both full-length SH2-B β (1-670) and SH2-B β (1-631) showed a primarily cytoplasmic distribution in COS-7 cells (Fig. 3C). The shift in the location of SH2-B β from primarily in the cytoplasm to the cytoplasm and nucleus occurred when amino acids 200 to 260 were deleted, as with the PC12 cells. Both SH2-B β (1-200) and SH2-B β (1-150) were found in the nucleus and cytoplasm (Fig. 3C). Together, these results

FIG. 3. Subcellular localization of the C-terminal truncation mutants of SH2-B β . (A) Schematic representations of various C-terminal truncation mutants of SH2-B β fused to the C terminus of GFP. (B) PC12 cells were transiently transfected with cDNAs encoding GFP-tagged full-length SH2-B β [GFP-SH2-B β (1-670)] or a C-terminal truncation mutant of SH2-B β shown in panel A. The C-terminal truncation SH2-B β mutants GFP-SH2-B β (1-631), GFP-SH2-B β (1-260), GFP-SH2-B β (1-200), and GFP-SH2-B β (1-150) were used. The cells were then treated with 100 ng of NGF per ml for 4 days. Images of live cells were taken on day 4. (C) COS-7 cells were transiently transfected with cDNAs encoding GFP-tagged full-length SH2-B β or a C-terminal truncation mutant of SH2-B β as indicated. Cells were fixed 18 h after transfection with 4% paraformaldehyde, and the images were taken using epifluorescence microscopy. DAPI images show nuclei.

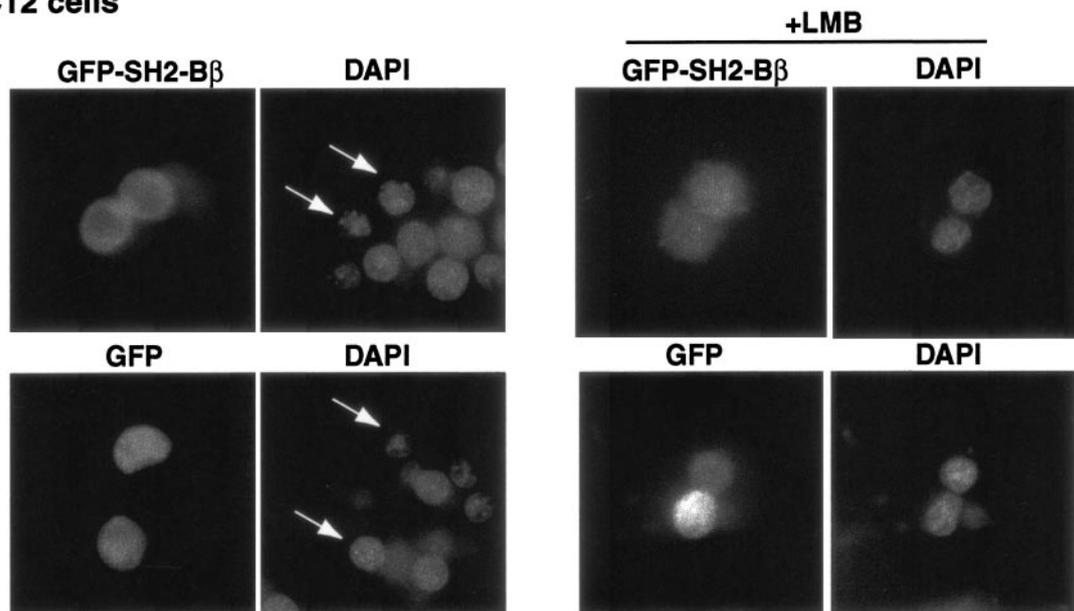
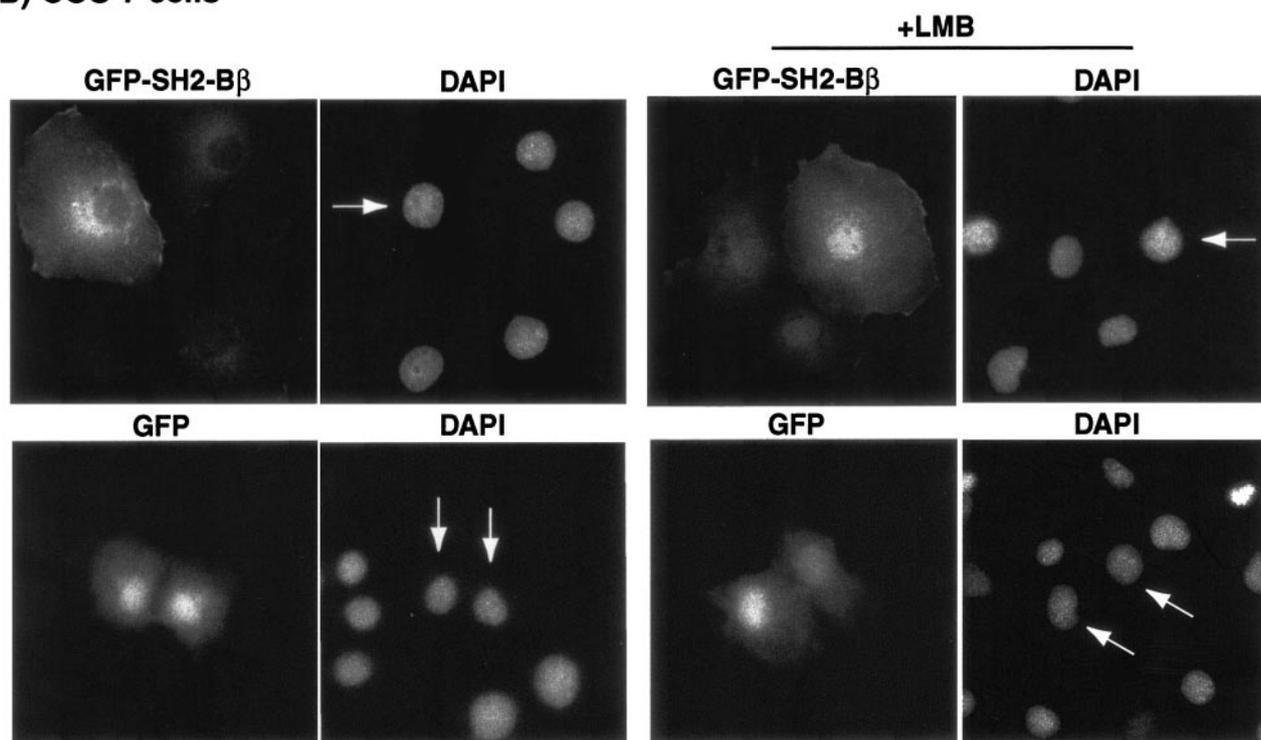
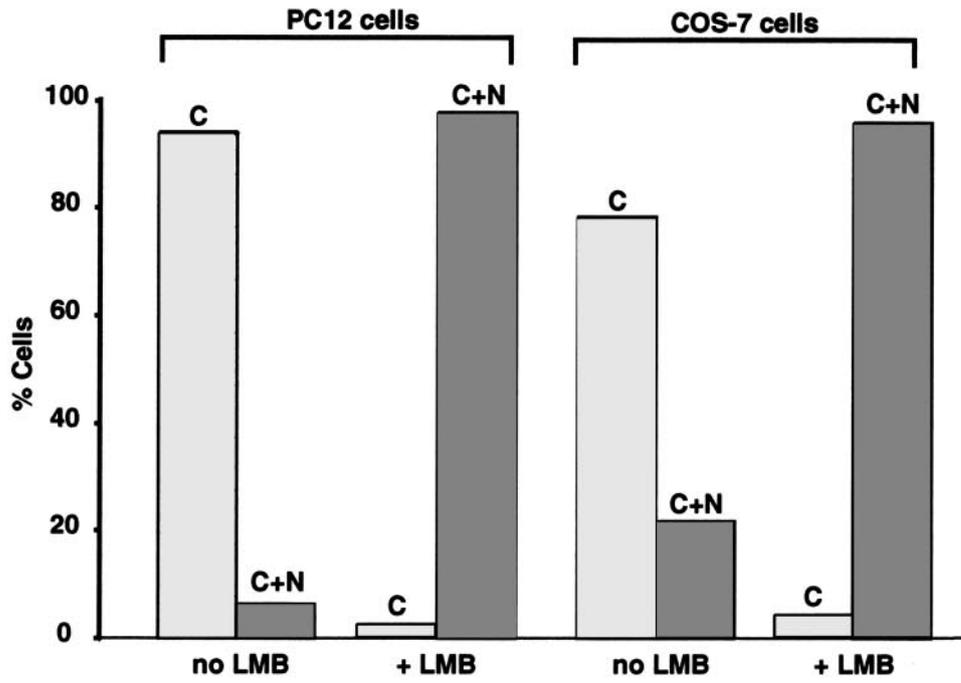
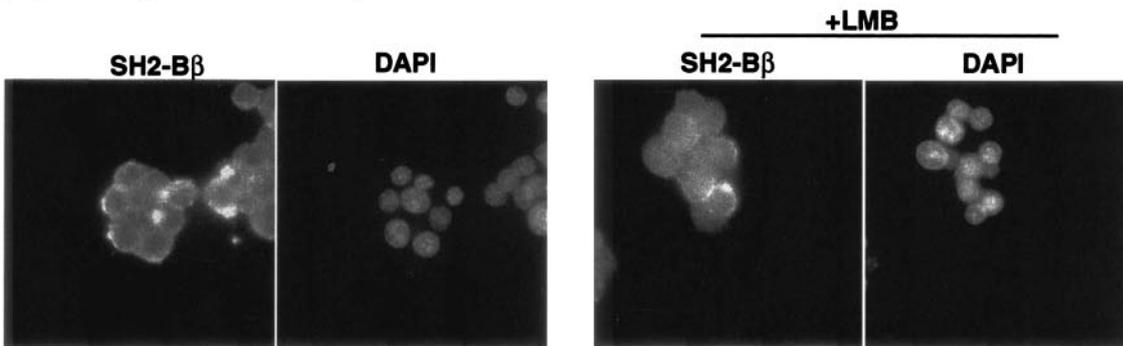
(A) PC12 cells**(B) COS-7 cells**

FIG. 4. SH2-B β shuttles between the cytoplasm and nucleus. (A) PC12 cells were transiently transfected with cDNA encoding GFP-SH2-B β or GFP. Eighteen hours after transfection, cells were either mock treated (left panels) or treated with 20 nM leptomycin B (+LMB) for 3 h (right panels) before fixation. Images were then taken to determine the subcellular distribution of GFP-SH2-B β . DAPI images next to the fluorescence images showed the localization of the nucleus. (B) COS-7 cells were transiently transfected with GFP-SH2-B β or GFP. Eighteen hours after transfection, cells were either mock treated (left panels) or treated with 20 nM LMB for 5 h (right panels) before fixation. Images were then taken using epifluorescence microscopy. DAPI images showed the nucleus localization. (C) Quantification of GFP-SH2-B β distribution in the cytoplasm (C) or in the cytoplasm and nucleus (C+N) in either PC12 cells or COS-7 cells without or with LMB (+ LMB) treatment. A total of 17 to 43 cells were counted from two experiments per condition. (D) PC12 cells were incubated without or with LMB for 3h before fixation. Endogenous SH2-B β was detected by incubating cells with goat anti-SH2-B β antibody and then with rabbit anti-goat Alexa 488. The images were visualized using epifluorescence microscopy. The corresponding DAPI images show the nuclei. (E) COS-7 cells were incubated without or with LMB for 28 h before fixation. Endogenous SH2-B β was detected and visualized as described for panel D.

(C)



(D) Endogenous SH2-B β in PC12 cells



(E) Endogenous SH2-B β in COS-7 cells

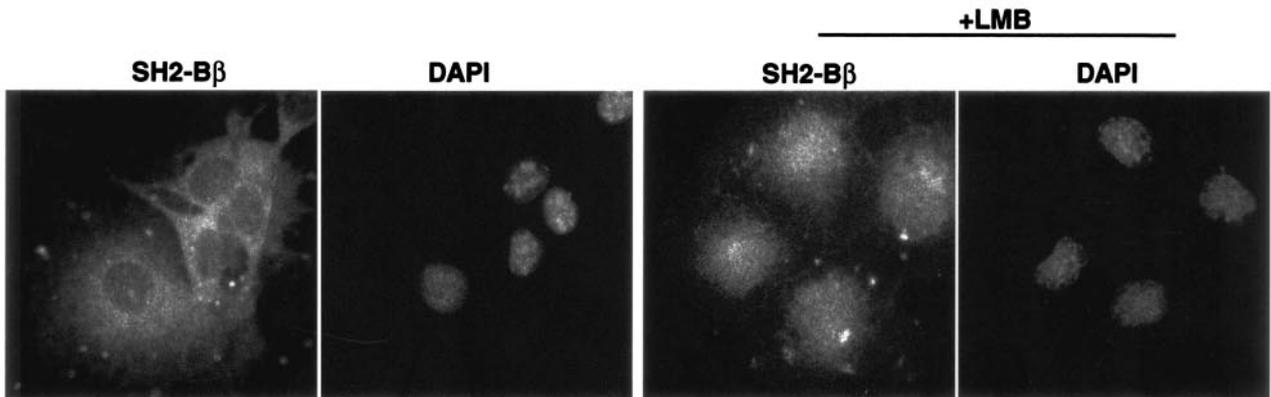


FIG. 4—Continued.

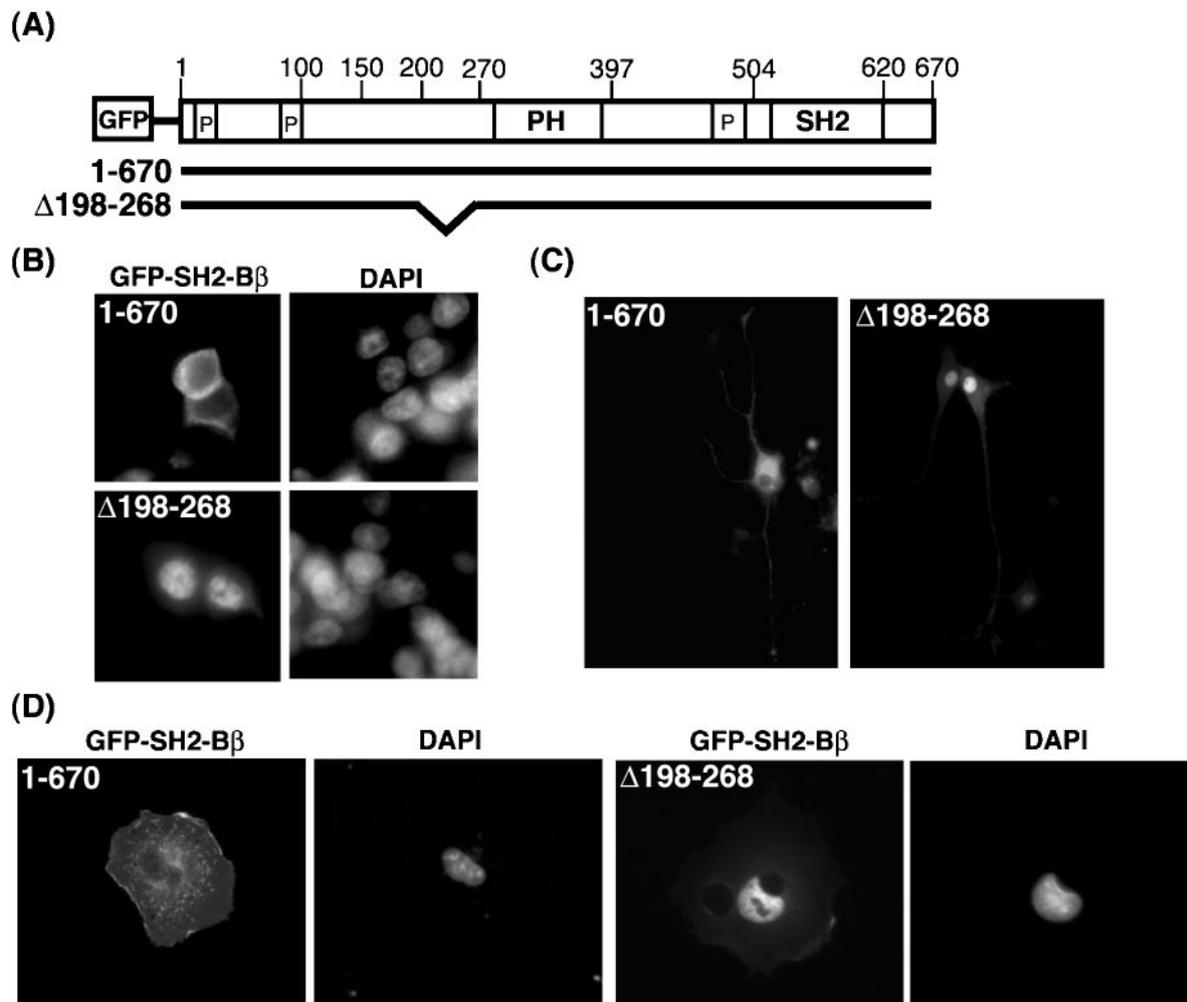


FIG. 5. Subcellular distribution of GFP-SH2-B β (Δ 198-268). (A) Schematic representation of the internal deletion mutant of SH2-B β fused to the C terminus of GFP, GFP-SH2-B β (Δ 198-268). (B) PC12 cells were transiently transfected with cDNAs encoding either GFP-SH2-B β or GFP-SH2-B β (Δ 198-268). Cells were fixed, and images were taken using epifluorescence microscopy. DAPI images next to the fluorescence images showed the nucleus localization. (C) PC12 cells were transiently transfected with cDNA encoding either GFP-SH2-B β or GFP-SH2-B β (Δ 198-268) and then treated with 100 ng of NGF per ml for 4 days. Live images of differentiated PC12 cells were taken on day 4 to show the localization of GFP-SH2-B β or GFP-SH2-B β (Δ 198-268). (D) COS-7 cells were transiently transfected with cDNA encoding either GFP-SH2-B β or GFP-SH2-B β (Δ 198-268). Cells were fixed 18 h after transfection, and images were taken using epifluorescence microscopy. DAPI images next to the fluorescence images were used to show the nucleus localization.

suggest that amino acids 200 to 260 are essential for SH2-B β to concentrate in the cytoplasm, consistent with the conclusion drawn from analyzing the N-terminal truncation mutants of SH2-B β (Fig. 2).

Nucleocytoplasmic shuttling of SH2-B β . The subcellular distribution studies of the N- and C-terminal truncation mutants reveal an intriguing possibility: SH2-B β shuttles between the cytoplasm and nucleus. Transport of proteins in and out of the nucleus is generally thought to rely on their recognition by soluble shuttling receptors, importins and exportins. The majority of nuclear export depends on the nuclear export receptor, Crm1 (also called exportin 1). Leptomycin B (LMB) is a specific inhibitor of Crm1 and is commonly used to prevent Crm1-dependent nuclear export (15).

To determine whether wild-type SH2-B β shuttles between the cytoplasm and nucleus, the distribution of transiently expressed full-length GFP-tagged SH2-B β was examined in

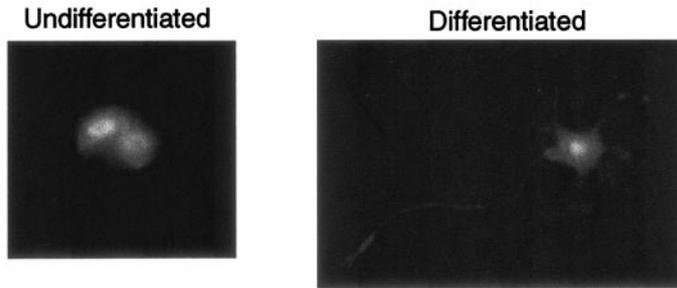
PC12 cells with or without LMB treatment. Despite its predominantly cytoplasmic localization, if SH2-B β is continuously shuttling between the cytoplasm and nucleus and is exported from the nucleus in a Crm1-dependent manner, it would be expected to accumulate in the nucleus when cells are treated with LMB. In Fig. 4A, undifferentiated PC12 cells transiently expressing GFP-SH2-B β were treated with 20 nM LMB for 3 h or not treated with LMB, and the cells were fixed for imaging. In 94% of the PC12 cells, SH2-B β distributed predominantly in the cytoplasm in the absence of LMB (Fig. 4A and C). In contrast, in the presence of LMB, SH2-B β appeared in both the cytoplasm and the nucleus in 98% of the cells (Fig. 4A and C).

To visualize the nuclear localization of SH2-B β better, possible cytoplasmic-nuclear shuttling of SH2-B β was also examined in COS-7 cells. Similar to what was found in PC12 cells, GFP-SH2-B β was found mainly in the cytoplasm at steady

(A) NES motif

	↓ ↓
SH2-B	GERWTHR F ERLRLSR
Mk2	KEEMTSALATMRVDY
PKI	SNELALKLAGLDINK
P53	FEMFRELNEALELKD
HIV/REV	VPLQLPPLERLTLDC
RCK2/CLK	DEQLEQNMFQLTLDT
Cyclin B	DLCQAFSDVLI A
14-3-3	TLIMQLLRDNLTLWT
TFIIIA	ETNGSLVLDKLT I Q
IKBα	STRIQQQLGQLTLEN
STAT1	SLAARFRHLQ L K
STAT2	GLIWDFGYLTLV
STAT3	SLSAEFKHLTLR
STAT4	SLSVEFRHLQ P K
STAT5a	TL S AHFRNMSL K
STAT5b	CCSALFKNLL L K

(B) GFP-SH2-B β (L231A, L233A) in PC12 cells



(C)

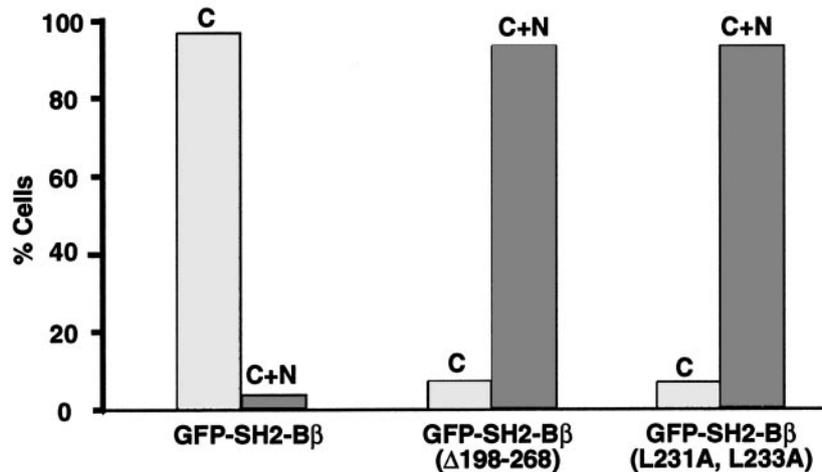


FIG. 6. Point mutations in the putative NES motif in SH2-B β inhibit the nuclear export of SH2-B β . (A) Known NESs in various classes of proteins and the putative NES in SH2-B. The arrows point to the two leucines in SH2-B β being mutated. (B) PC12 cells were transiently transfected with the cDNA encoding the GFP-fused SH2-B β point mutant, SH2-B β (L231A, L233A). Cells were fixed 18 h after transfection (undifferentiated). Another set of cells was treated with 100 ng of NGF per ml for 4 days, and live images of the differentiated PC12 cells were taken. (C) Quantification of the localization of GFP-SH2-B β , GFP-SH2-B β (Δ 198-268), or SH2-B β (L231A, L233A) in either the cytoplasm (C) or in both the cytoplasm and nucleus (C+N). A total of 61 to 173 differentiated PC12 cells from three experiments were counted for each construct.

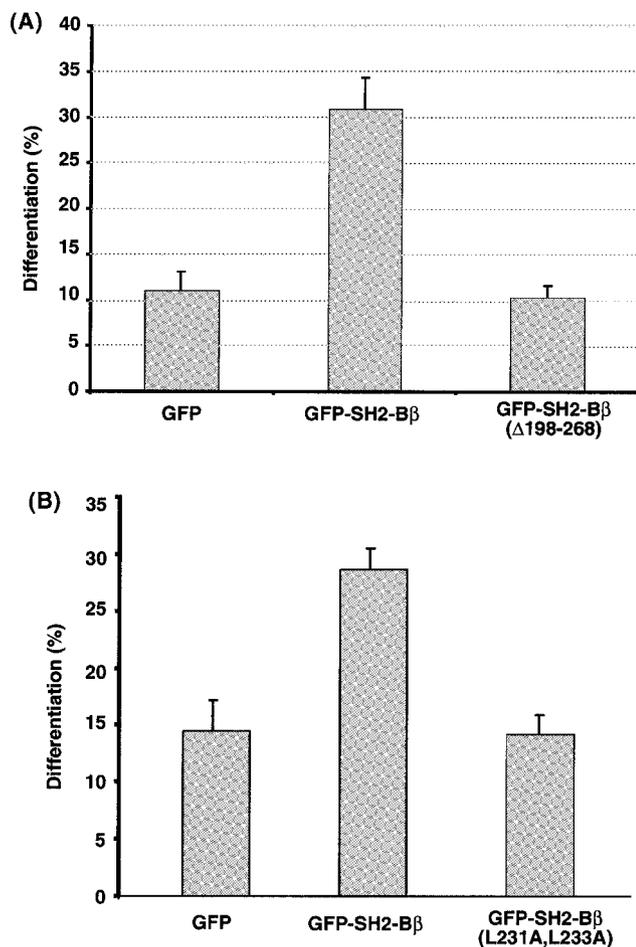


FIG. 7. Effect of SH2-B β (Δ 198-268) and SH2-B β (L231A, L233A) on neuronal differentiation. (A) PC12 cells were transiently transfected with the cDNA encoding the vector alone (GFP), GFP-SH2-B β , or GFP-SH2-B β (Δ 198-268) as indicated. Cells were then treated with 100 ng of NGF per ml for 4 days, and the percentage of differentiation was determined as described in the legend to Fig. 1. A total of 300 to 450 transfected cells were counted for each condition in each experiment, and the values are averages \pm standard errors (error bars) from three different experiments. (B) PC12 cells were transiently transfected with the cDNA encoding the vector alone, GFP-SH2-B β , or GFP-SH2-B β (L231A, L233A) as indicated. Cells were then treated with 100 ng of NGF per ml for 4 days, and the percentage of differentiation was determined as described in the legend to Fig. 1. A total of 333 to 870 transfected cells were counted for each condition in each experiment, and the values are averages \pm standard deviations (error bars) from two different experiments.

state in 78% of the cells but accumulated in the nucleus after 5 h of LMB treatment in 96% of cells (Fig. 4B and C). These results strongly suggest that SH2-B β constitutively shuttles between the cytoplasm and nucleus. The constitutive cytoplasmic-nuclear shuttling of SH2-B β was also observed using myc-tagged SH2-B β (data not shown).

The experiments presented thus far used overexpressed wild-type or mutant forms of SH2-B β . We asked whether endogenous SH2-B β also undergoes nucleocytoplasmic shuttling. Again, we treated both PC12 and COS-7 cells with LMB for various times and determined the subcellular distribution of endogenous SH2-B β by performing indirect immunofluores-

cence microscopy. As predicted from the overexpression studies, we observed SH2-B β being excluded from the nucleus of PC12 cells at steady state and distributed in both the cytoplasm and nucleus after LMB treatment (Fig. 4D). In COS-7 cells, SH2-B β was also shown to be excluded from the nucleus in the absence of LMB and to appear in the nucleus after 8 h or longer of LMB treatment (Fig. 4E).

Nuclear localization of SH2-B β lacking the region required for nuclear export. Both N-terminal and C-terminal truncation analysis of SH2-B β point to a region between amino acids 200 and 260 being required for the nuclear export of SH2-B β . To determine whether this region is necessary for SH2-B β to exit the nucleus, we next examined the subcellular distribution of an internal deletion mutant, SH2-B β (Δ 198-268), lacking this region (Fig. 5A). As shown in Fig. 5B to D, SH2-B β (Δ 198-268) localized almost exclusively in the nucleus. This nuclear distribution was pronounced in undifferentiated PC12 cells (Fig. 5B) as well as in differentiated PC12 cells (Fig. 5C) and in COS-7 cells (Fig. 5D). Thus, it is very likely that amino acids 198 to 268 contain a signal for the nuclear export of SH2-B β .

SH2-B contains a conserved NES. Closer examination of the sequence between amino acids 200 and 260 in SH2-B β revealed a conserved nuclear export sequence (NES). The NES identified thus far has a relatively loosely defined motif, LX₂₋₃LX₂₋₄LXL (2, 3, 32). As shown in Fig. 6A, a putative NES motif of SH2-B is present between amino acids 224 and 233. As previously shown for 14-3-3, a scaffolding protein that shuttles between the cytoplasm and nucleus and participates in multiple signaling pathways, the C-terminal amino acids LXL are essential for nuclear export activity (3). Therefore, point mutations were introduced to change amino acids L231 and L233 of SH2-B β to alanines, and the subcellular distribution of GFP-SH2-B β (L231A, L233A) was examined. When GFP-SH2-B β (L231A, L233A) was transiently expressed in PC12 cells, it accumulated in the nucleus, consistent with it being defective in nuclear export. As expected, this nuclear localization was observed in both undifferentiated and differentiated PC12 cells (Fig. 6B). Comparing the distribution in differentiated PC12 cells of wild-type SH2-B β and the nuclear export-deficient mutants, wild-type SH2-B β was found to reside predominantly in the cytoplasm in 97% of the cells at steady state, whereas both SH2-B β (Δ 198-268) and SH2-B β (L231A, L233A) were concentrated in the nucleus in 93% of the cells (Fig. 6C).

Effects of export-impaired SH2-B β mutants on neuronal differentiation. As shown in Fig. 1, PC12 cells overexpressing SH2-B β and SH2-B β (170-670) showed an increased percentage of differentiated cells compared to control cells (transfected with vector only) when the cells were treated with NGF. In contrast, cells overexpressing the N-terminal truncation mutants SH2-B β (270-670) and SH2-B β (504-670) did not show increased neurite outgrowth. Full-length SH2-B β (1-670) and SH2-B β (170-670) are found predominantly in the cytoplasm, whereas SH2-B β (270-670) and SH2-B β (504-670) are found in both the cytoplasm and nucleus (Fig. 2). These findings led us to speculate that the "proper" locale of SH2-B β is vital for the ability of SH2-B β to enhance NGF-induced neurite outgrowth.

To test this hypothesis, we investigated the effects of the nuclear export-impaired SH2-B β (Δ 198-268) and SH2-B β (L231A,

L233A) mutants on NGF-induced neurite outgrowth. Similar to cells expressing the N-terminal deletion mutants SH2-B β (270-670) and SH2-B β (504-670), PC12 cells transiently expressing the internal deletion mutant GFP-SH2-B β (Δ 198-268) or the point mutant GFP-SH2-B β (L231A, L233A) showed no enhancement in NGF-induced neurite outgrowth compared to cells expressing GFP alone (Fig. 7B). These results suggest that SH2-B β needs to reside predominantly in the cytoplasm to enhance NGF-induced neurite outgrowth in PC12 cells.

Effects of SH2-B β mutations on NGF-induced activation of TrkA and ERKs 1 and 2. One mechanism by which the NES of SH2-B β may contribute to NGF-induced morphological differentiation is by enhancing the ability of SH2-B β to activate one or more NGF signaling pathways. When NGF binds to its receptor TrkA, TrkA rapidly becomes activated and phosphorylated on tyrosines with tyrosine 490 being a primary phosphorylation site (34, 35). We and others have shown that NGF stimulation enhances the interaction between SH2-B β and TrkA (39, 46). SH2-B has also been reported to enhance and prolong NGF stimulation of TrkA phosphorylation on Tyr 490 and activation of Akt and ERKs 1 and 2 (38, 55).

To determine whether the nuclear export-impaired SH2-B β mutants lose or retain the ability to regulate NGF signaling via TrkA, we made PC12 cell lines stably overexpressing GFP, GFP-SH2-B β , GFP-SH2-B β (Δ 198-268), or GFP-SH2-B β (L231A, L233A). To avoid clonal variation, we pooled the clones with the highest (5%) level of expression. The subcellular distribution of SH2-B β and its mutants in these stable cell lines was similar to that seen in the transiently transfected cells (data not shown). The relative abilities of these stably transfected cell lines to differentiate in response to NGF mirrored the relative abilities of the transiently transfected cells to differentiate, with SH2-B β enhancing NGF-induced neurite outgrowth and the nuclear export-deficient forms of SH2-B β having no effect (data not shown).

First, we examined whether nuclear export SH2-B β mutants, such as SH2-B β , interacted with activated TrkA. PC12 cells stably expressing GFP, GFP-SH2-B β , GFP-SH2-B β (Δ 198-268), or GFP-SH2-B β (L231A, L233A) were incubated in serum-free medium overnight. Cells were then treated with sodium vanadate (a phosphatase inhibitor) for 1 h prior to NGF stimulation (100 ng/ml, 10 min) and GFP-SH2-B β was immunoprecipitated using anti-GFP antibody. The immunoprecipitated proteins were resolved by SDS-PAGE and blotted first with antiphosphotyrosine antibody and then reprobed with anti-SH2-B β antibody. As shown in Fig. 8A, GFP-SH2-B β (Δ 198-268) and GFP-SH2-B β (L231A, L233A), like wild-type GFP-SH2-B β , were capable of binding to phosphorylated TrkA, though to a lesser extent. This result suggests that these two nuclear export mutants are functional proteins. It also suggests that failure to interact with TrkA is not the reason why the nuclear export-defective mutants are loss-of-function mutants.

Next we investigated the effect of mutating the NES of SH2-B β on its ability to enhance NGF activation of ERKs 1 and 2. Phosphorylation of ERKs 1 and 2 in cells expressing GFP alone was highest 10 min after NGF stimulation and then declined rapidly. Overexpression of SH2-B β showed substantially enhanced phosphorylation of ERKs 1 and 2 at both 90

and 240 min after NGF challenge. Like cells expressing GFP-SH2-B β , cells expressing the GFP-SH2-B β (L231A, L233A) mutant showed elevated stimulation 240 min after the addition of NGF (Fig. 8B), but not after 90 min. NGF promotes the nuclear localization of ERKs 1 and 2 (18, 52).

To determine whether SH2-B β (L231A, L233A) might alter the subcellular distribution on ERKs 1 and 2, we examined the localization of the active forms of ERKs 1 and 2 in stable cell lines. PC12 cells stably expressing GFP, GFP-SH2-B β , or GFP-SH2-B β (L231A, L233A) were incubated in serum-free medium overnight. After 1 h of treatment with 100 ng of NGF per ml, cells were fixed, and the distribution of endogenous active ERKs 1 and 2 was visualized using indirect immunofluorescence microscopy (Fig. 8C). After 1 h of NGF treatment, active ERKs 1 and 2 were seen in the nucleus in cells expressing GFP, GFP-SH2-B β , or GFP-SH2-B β (L231A, L233A). There was no obvious localization defect of active ERKs 1 and 2 in cells expressing GFP-SH2-B β (L231A, L233A). Therefore, we conclude that the nuclear export mutant GFP-SH2-B β (L231A, L233A) does not appreciably alter the subcellular distribution of ERKs 1 and 2. Therefore, the loss-of-function phenotype of SH2-B β (L231A, L233A) on NGF-induced neurite outgrowth is unlikely to be attributable to decreased NGF activation of ERK proteins or to impaired transport of ERK proteins into or out of the nucleus.

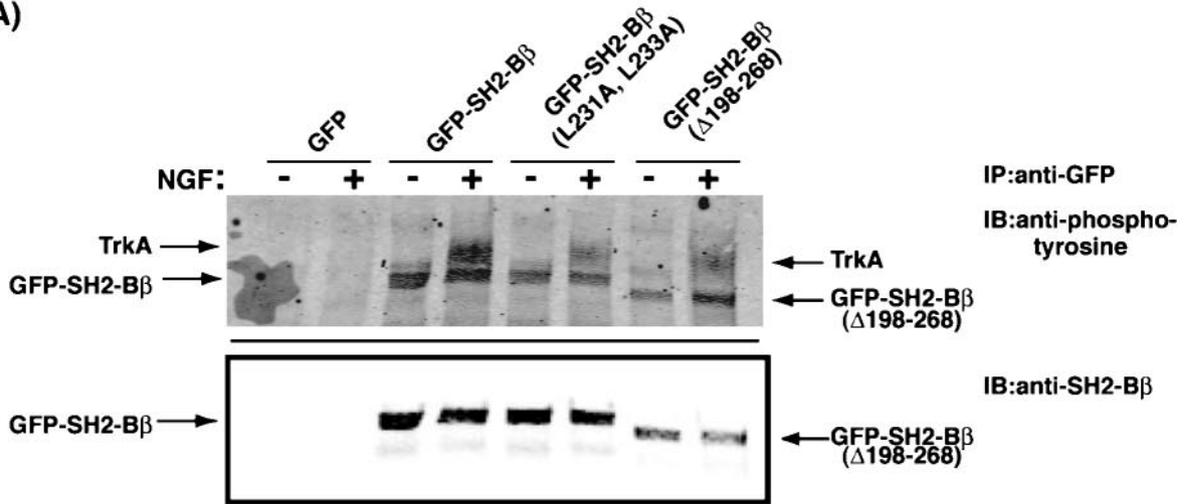
DISCUSSION

Members of the SH2-B family of proteins were originally identified as binding proteins of receptor tyrosine kinases, the cytokine receptor-associated JAK2, or phosphorylated tyrosines within receptors of the immune system (39, 45, 48). The facts that this binding was ligand dependent and that SH2-B family members lack a transmembrane domain sequence but contain an SH2 domain led to the hypothesis that SH2-B family members were most likely cytoplasmic and recruited to phosphorylated tyrosines within membrane receptor/tyrosine kinase complexes. The results of immunolocalization studies supported the hypothesis that SH2-B localized to the plasma membrane before and after ligand stimulation, to membrane ruffles after ligand stimulation, and to the cytoplasm (17, 47).

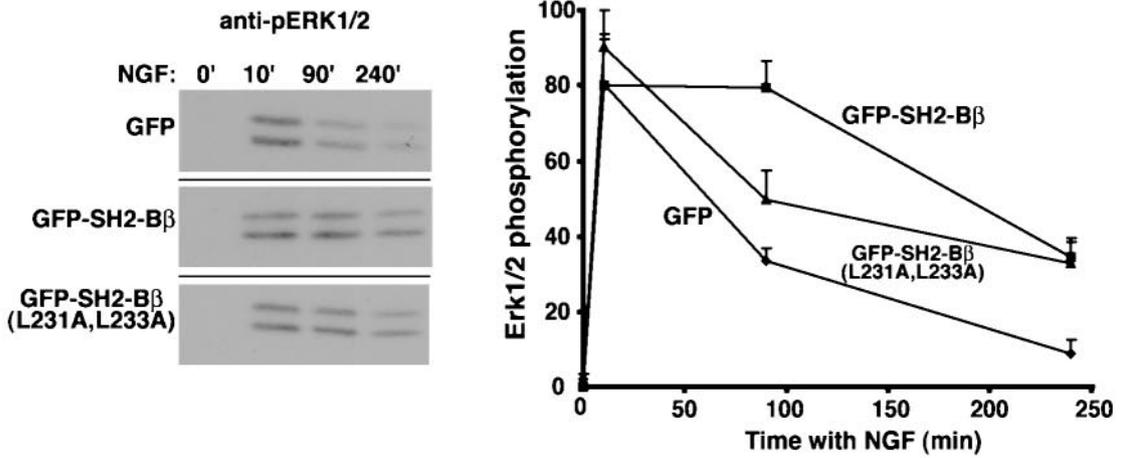
In the present study in which SH2-B β was overexpressed in both undifferentiated and differentiated PC12 cells and in COS-7 cells, some SH2-B β could be visualized at the plasma membrane, though most of the SH2-B β was found in the cytoplasm. However, while wild-type SH2-B β as well as SH2-B β lacking its first 170 or 200 amino acids localized predominantly in the cytoplasm, surprisingly, truncation mutants SH2-B β (270-670), SH2-B β (397-670), and SH2-B β (504-670) were found in both the cytoplasm and nucleus (Fig. 2). These results suggest that while equilibrium favors SH2-B β being primarily in the cytoplasm, SH2-B β shuttles continuously between the cytoplasm and nucleus.

These results further suggest that the nuclear export signal responsible for the export of SH2-B β out of the nucleus is located between amino acids 200 and 270 of SH2-B β . In the absence of these amino acids, SH2-B β can enter the nucleus but is not capable of exiting. In support of amino acids 200 to 270 containing the nuclear export signal, an internal deletion

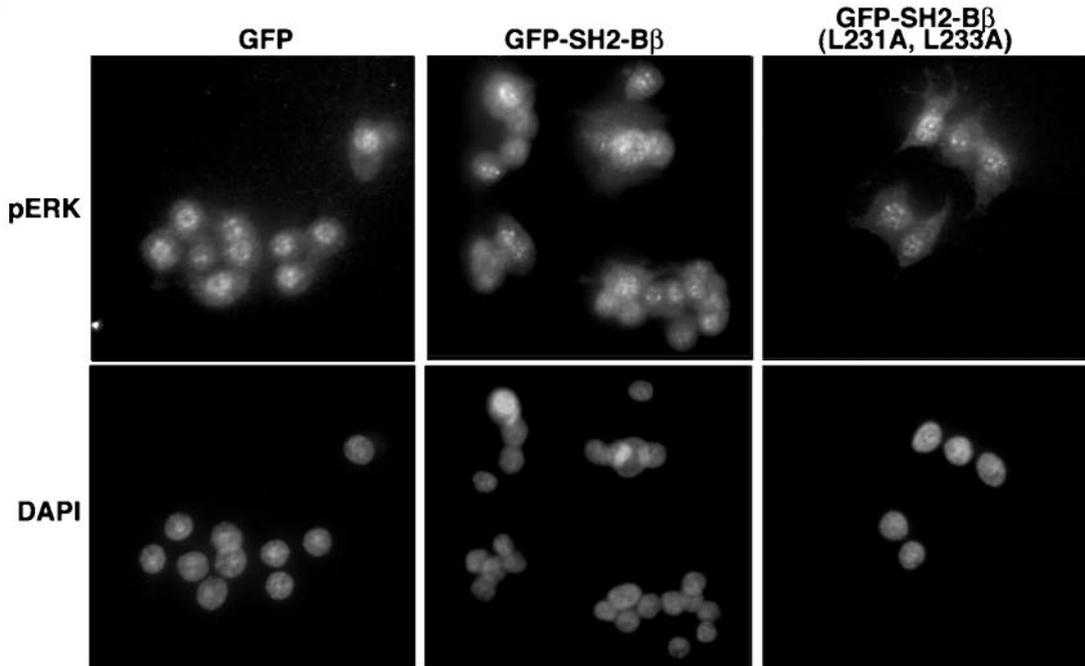
(A)



(B)



(C)



mutant of SH2-B β lacking these amino acids was located in the nucleus. Mutating two leucines in a putative nuclear export signal within this region in the context of full-length SH2-B β also caused SH2-B β to localize to the nucleus. These results further support the hypothesis that SH2-B β shuttles between the cytoplasm and nucleus. The sequences containing the nuclear export motif are identical among the four known isoforms of SH2-B, so it is likely that nucleocytoplasmic shuttling is common for all four isoforms. The fact that both overexpressed wild-type SH2-B β and endogenous SH2-B β are found predominantly in the cytoplasm suggests that SH2-B β is continuously cycling between the nucleus and cytoplasm, with the ratio of nuclear export to import favoring most of the SH2-B β residing in the cytoplasm at any given time. The nuclear export inhibitor LMB causes both overexpressed wild-type SH2-B β and endogenous SH2-B β to accumulate in the nucleus, supporting the notion that endogenous SH2-B β shuttles constitutively between the cytoplasm and nucleus. NGF did not noticeably affect the steady-state distribution of SH2-B β (data not shown); however, it is possible that NGF affects the kinetics of nucleocytoplasmic shuttling of SH2-B β .

The finding of SH2-B β shuttling between the cytoplasm and nucleus raises the question of whether this nucleocytoplasmic shuttling is required for SH2-B β to enhance NGF-induced neurite outgrowth. Mutating the NESs, either by truncation [SH2-B β (270-670)], internal deletion [SH2-B β (Δ 198-268)], or point mutations [SH2-B β (L231A, L233A)], resulted in a loss of the ability of SH2-B β to enhance NGF-induced neurite outgrowth (Fig. 1 and 7). One possible explanation for the loss of this ability is that enhancement of neurite outgrowth requires SH2-B β to reside some of the time in the cytoplasm and/or plasma membrane and some of the time in the nucleus. One can envision cytoplasmic SH2-B β providing a reservoir of SH2-B β to bind to TrkA in response to NGF, which upon the appropriate posttranslational modification, subsequently goes to the nucleus either as a nuclear import shuttle for other signaling proteins or as a scaffolding protein for transcription factor complexes. Alternatively, one can envision SH2-B β being required to facilitate the export of proteins that mediate or regulate transcription. ERKs 1 and 2 were possible candidates for such a shuttling event, because of the key role they are known to play in NGF-induced neurite outgrowth of PC12 cells and their nuclear import and export mechanism is still controversial (7, 12, 24, 31, 61). However, our results indicate that cells expressing the nuclear export-defective forms of SH2-B β do not show substantially reduced NGF-induced ac-

tivation and nuclear localization of ERKs 1 and 2 compared to cells expressing wild-type SH2-B β . We also think it unlikely that the export-deficient mutants sequester vital NGF signaling proteins in the nucleus, because unlike the SH2 domain-defective mutant SH2-B β (R555E), they do not act as dominant negatives and block NGF-induced neurite outgrowth. One possible explanation is that after SH2-B binds to TrkA and initiates one or more signaling pathways important for neurite outgrowth, it moves to the nucleus. Eventually, however, it recycles back to the cytoplasm where it can repeat the cycle. In contrast, while the nuclear export-deficient mutants would be able to initiate one round of signaling, they would have difficulty recycling back to the cytoplasm from the nucleus. This latter hypothesis is consistent with our recent results indicating that nuclear import SH2-B β mutants enhance the morphological differentiation of PC12 cells more than wild-type SH2-B β does (T. J. Maures and C. Carter-Su, unpublished data).

Although the exact mechanism by which SH2-B β enhances neurite outgrowth, as well as the role of nuclear SH2-B β , remains unclear, our results strongly suggest a mechanism that involves SH2-B β interacting with extranuclear components participating in the process of neurite outgrowth. Several cytoplasmic proteins have been shown to participate in neuronal differentiation, including the actin regulatory proteins, LIM kinases, and their downstream effectors, such as the Rho family of GTPases, Cdc42, Rac, and Rho (27, 28). It is possible that SH2-B β affects the function of some of these proteins by affecting their subcellular localization or by serving as a scaffolding protein that brings them into close proximity to appropriate regulatory or effector proteins, similar to how the *Saccharomyces cerevisiae* scaffolding protein Ste5 organizes and localizes the MAPK cascade of the mating pathway to respond appropriately to a pheromone stimulus (1, 6, 20, 29, 50, 56). Rac was a logical candidate for several reasons. It has been shown to regulate neuronal differentiation via modulating growth cone collapse during neurite outgrowth (27, 30, 51) and to bind constitutively, either directly or indirectly, to the N-terminal second proline-rich region of SH2-B β (amino acids 85 to 106) (9). However, while the SH2-B β truncation mutant SH2-B β (170-670) lacking this proline-rich region showed a reduced ability to promote NGF-induced neurite outgrowth compared to wild-type SH2-B β (Fig. 1B), neurite outgrowth was not as reduced as in the cells overexpressing the mutants lacking NES (Fig. 7). This finding implies that Rac is not the only player in SH2-B-mediated neuronal differentiation, if it is one. Other potential players include microtubule-associated proteins

FIG. 8. Effect of a nuclear export-defective mutant of SH2-B β on NGF signaling. (A) PC12 cells stably expressing either GFP, GFP-SH2-B β , GFP-SH2-B β (L231A, L233A), or GFP-SH2-B β (Δ 198-268) as indicated were incubated in serum-free medium overnight and then pretreated with sodium vanadate for 1 h before stimulation without (–) with (+) 100 ng of NGF per ml for 10 min. Cell lysates were incubated with anti-GFP antibody and then with protein A agarose. The immunoprecipitated complex was resolved by SDS-PAGE and immunoblotted with antiphosphotyrosine antibody (top gel) and then reprobed with anti-SH2-B antibody (bottom gel). IP, immunoprecipitation; IB, immunoblotting. (B) PC12 cells stably expressing either GFP, GFP-SH2-B β , or GFP-SH2-B β (L231A, L233A) were incubated in serum-free medium overnight and stimulated with 100 ng of NGF per ml for 0, 10, 90, and 240 min (240'). Lysates from these cell lines expressing GFP, GFP-SH2-B β , or GFP-SH2-B β (L231A, L233A) were resolved by SDS-PAGE, and the blot was incubated with anti-phospho-ERKs 1 and 2 (anti-pERK1/2). The highest level of phosphorylated ERKs 1 and 2 in each experiment was set at 100. The values are the averages \pm standard errors (error bars) from three different experiments. (C) PC12 stable cell lines were treated with 100 ng of NGF per ml for 1 h as described for panel B and then fixed and incubated with a rabbit antibody against active phosphorylated ERK (pERK) and then with goat anti-rabbit Alexa 555 antibody. DAPI images reveal the nuclei. The images were taken using epifluorescence microscopy.

and focal adhesion molecules. Paxillin and its binding partner Pyk2 (a focal adhesion kinase) have been shown to participate in NGF-induced Ca^{2+} mobilization important for cytoskeletal reorganization and neurite and growth cone functions (22, 36). Whether SH2-B β affects the activity or subcellular distribution of any of these proteins remains to be determined.

The fact that the nuclear import-deficient forms of SH2-B β promote neuronal differentiation of PC12 cells while nuclear export-deficient forms of SH2-B β do not rule out the possibility that nuclear SH2-B β functions in some other cellular functions or even helps to fine tune the differentiation program. For example, nuclear SH2-B β may contribute to the antiapoptotic effects of NGF. We have recently shown that SH2-B β can affect the distribution of FKHR transcription factors, FKHR (FOXO 1) and FKHL1 (FOXO 3) (55). By enhancing the retention of FKHR in the cytoplasm or promoting its nuclear export, SH2-B β may contribute to cell survival.

In summary, the present study provides the first evidence that the ubiquitously expressed adapter protein SH2-B β shuttles between the cytoplasm and nucleus, making SH2-B β one of a relatively few examples of a membrane receptor binding partner that undergoes nucleocytoplasmic shuttling. In addition, we identify a nuclear export motif, GERWTHRFRERLRLSR, required for the nuclear export of SH2-B β . This sequence is conserved in the four known isoforms of SH2-B, thus implying that all isoforms of SH2-B undergo nucleocytoplasmic shuttling. The cytoplasmic localization of SH2-B β appears to be important for the ability of SH2-B β to enhance morphological differentiation of PC12 cells. The finding of nucleocytoplasmic shuttling of SH2-B β raises the interesting possibility that SH2-B β might facilitate shuttling of other proteins into and/or out of the nucleus or help assemble specific transcriptional complexes involved in the regulation of NGF-induced neuronal differentiation and/or neuronal survival. SH2-B β was previously thought to be restricted to the cytoplasm and plasma membrane. Therefore, the results of this study also emphasize that caution must be used when searching for a function for a protein on the basis of its steady-state subcellular localization.

ACKNOWLEDGMENTS

We thank Barbara Hawkins for assistance with this manuscript. We thank all members of Carter-Su laboratory for advice. We thank Liangyou Rui for helpful comments on this manuscript.

This work was supported in part by NIH grant (RO1-DK54222) and the Juvenile Diabetes Research Foundation Center for the Study of Complications of Diabetes. DNA sequencing was performed with financial support from the Cellular and Molecular Biology Core of the Michigan Diabetes Research and Training Center (P60-DK20572), and fluorescence cell sorting was performed with financial support from the University of Michigan Comprehensive Cancer Center (P30-CA46592).

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