SH2-B, a Membrane-associated Adapter, Is Phosphorylated on Multiple Serines/Threonines in Response to Nerve Growth Factor by Kinases within the MEK/ERK Cascade*

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SH2-B has been shown to be required for nerve growth factor (NGF)-mediated neuronal differentiation and survival, associate with NGF receptor TrkA, and be tyrosyl-phosphorylated in response to NGF. In this work, we examined whether NGF stimulates phosphorylation of SH2-B on serines/threonines. NGF promotes a dramatic upward shift in mobility of SH2-B, resulting in multiple forms that cannot be attributed to tyrosyl phosphorylation. Treatment of SH2-B with protein phosphatase 2A, a serine/threonine phosphatase, reduces the many forms to two. PD98059, a MEK inhibitor, dramatically inhibits NGF-promoted phosphorylation of SH2-B on serines/threonines, whereas depletion of 4β-phorbol 12-myristate 13-acetate-sensitive protein kinase Cs does not. ERKs 1 and 2 phosphorylate SH2-Bβ primarily on Ser-96 in vitro. However, NGF still stimulates serine/threonine phosphorylation of SH2-Bβ(S96A). SH2-Bβ(S96A), like wild-type SH2-Bβ, enhances NGF-induced neurite outgrowth. In contrast, SH2-Bβ(R555E) containing a defective SH2 domain blocks NGF-induced neurite outgrowth and displays greatly reduced phosphorylation on serines/threonines in response to NGF. SH2-Bβ(R555E), like wild-type SH2-Bβ, associates with the plasma membrane, suggesting that the dominant negative effect of SH2-Bβ(R555E) cannot be explained by an abnormal subcellular distribution. In summary, NGF stimulates phosphorylation of SH2-B on serines/threonines by kinases downstream of MEK, which may be important for NGF-mediated neuronal differentiation and survival.

Neurotrophins, including NGF, brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4/5, and neurotrophin-6, play a crucial role in differentiation, survival, and plasticity of developing neurons. NGF is essential for development and survival of sympathetic neurons and a subpopulation of sensory neurons (1–4). NGF binds with high affinity to TrkA, a member of the Trk family of receptor tyrosine kinases, and with low affinity to p75NTR, a member of the tumor necrosis factor receptor family (5). TrkA appears to be the major mediator of NGF signaling in developing and adult neurons (6). In rat pheochromocytoma (PC12) cells, NGF promotes neuronal differentiation (e.g., extension of neurite outgrowth and expression of neuronal specific genes) through activation of TrkA (7–10).

NGF stimulates the dimerization of TrkA (11), resulting in the activation of the intrinsic tyrosine kinase of TrkA and autophosphorylation of multiple tyrosines within the cytoplasmic domain of TrkA (12). The phosphorylated tyrosines recruit to the TrkA complex signaling molecules containing Src homology 2 (SH2) or phosphotyrosine-interacting domains, including Shc (12–14), phospholipase Cγ (12, 13), and SH2-B (15, 16). These signaling molecules then initiate the activation of multiple signaling pathways that mediate the biological responses to NGF.

One such pathway required for NGF-induced neuronal differentiation is the Ras/Raf/MEK/ERK pathway. NGF stimulates Shc binding to phosphorylated Tyr-490 in TrkA and the tyrosyl phosphorylation of Shc by TrkA (12, 14, 17, 18), which then enables Shc to recruit Grb2-SOS complexes to the plasma membrane. This results in the activation of the Ras/Raf/MEK/ERK pathway (19). Microinjection of antibody against Ras (20) or overexpression of dominant negative mutant Ras (21) or dominant negative mutant MEK (22) abrogates NGF-promoted neuronal differentiation of PC12 cells. Furthermore, overexpression of oncogenic Shc (23), oncogenic Ras (24, 25), oncogenic Raf (26), or constitutively active MEK (22) is sufficient to promote neuronal differentiation of PC12 cells similar to that induced by NGF. These observations suggest that serine/threonine phosphorylation of proteins by the Ras/Raf/MEK/ERK pathway plays an essential role in NGF signaling.

SH2-B, a recently described adapter protein containing SH2 and pleckstrin homology (PH) domains (27, 28), has been shown to be required for NGF-induced neuronal differentiation of PC12 cells (16) and implicated in NGF-mediated axonal growth and survival of primary sympathetic neurons (15). NGF stimulates association of SH2-B with TrkA in PC12 cells (16) as well as in primary sympathetic neurons (15). NGF promotes tyrosyl phosphorylation of SH2-B in primary sympathetic neurons (15) and PC12 cells overexpressing TrkA or SH2-B (16). NGF-induced tyrosyl phosphorylation of SH2-B was also observed in untransfected PC12 cells but only in the presence of a tyrosine phosphatase inhibitor (16), suggesting that any phosphorylated tyrosines in SH2-B are rapidly dephosphorylated.

In addition to its 9 tyrosines, SH2-Bβ has a large number of serines (82 serines) and threonines (29 threonines) including many that lie within consensus sequences for phosphorylation sites for protein kinase C, ERKs 1 and 2, cAMP- and cGMP-dependent protein kinases, and casein kinase II. We previously
NGF-induced Phosphorylation of SH2-B on Serines/Threonines

NGF, GFP-SH2-B

immunoprecipitation and immunoblotting.

min and were treated for various times with NGF at 37 °C at the serum. The confluent cells were deprived of serum overnight using photericin, 10% heat-inactivated horse serum, and 5% fetal bovine serum. The mutation was verified by DNA sequencing (Sequenase 2.0; US. Biochemical Corp.). Wild-type and SH2-B were subcloned into BsnHI/EcoRI sites into pEX-ERG to produce GST fusion proteins. The construction of other plasmids was described previously (16, 27) and used at a dilution of 1:100 for immunoprecipitation and 1:15,000 for immunoblotting. Monoclonal anti-phosphotyrosine 4G10 (aPY) was purchased from Upstate Biotechnology Inc. and was used at a dilution of 1:7,500 for immunoblotting. Anti-ERK2 was from Santa Cruz Biotechnology and used at a dilution of 1:100 for immunoprecipitation.

Plasmid Construction—Ser-96 in vitro, but SH2-B(S96A) is still phosphorylated on multiple serines/threonines in cells in response to NGF and enhances NGF-induced neurite outgrowth. These findings indicate that kinase(s) downstream of MEK, other than ERKs 1 and 2, phosphorylate SH2-B and that phosphorylation of Ser-96 by ERKs 1 and 2 is not required for its action in NGF-mediated neurite outgrowth. A dominant negative SH2-B(βR555E), which is unable to bind to TrkA, exhibits a profound defect in its serine/threonine phosphorylation, suggesting that association with TrkA and/or subsequent tyrosyl phosphorylation by TrkA may be a prerequisite for serine/threonine phosphorylation of SH2-B in response to NGF.

EXPERIMENTAL PROCEDURES

Cells and Reagents—PC12 cells were provided courtesy of Drs. D. Meyer and B. Margolis (University of Michigan, Ann Arbor, MI). Murine NGF and EGF were from Collaborative Biomedical Products. Recombinant glutathione-agarose beads and polylysine were from Sigma. Protein A-agarose was from Repligen. Alkaline phosphatase, aprotinin, leupeptin, and Triton X-100 were purchased from Roche Molecular Biochemicals. Santa Cruz Biotechnology and used at a dilution of 1:20,000 for immunoblotting. Anti-ERK2 was from a gift of Dr. A. R. Saltiel (Parke-Davis). 4-

Phosphorylation of SH2-B contributes to the NGF-induced shift in p60. The presented images are representative of at least three separate experiments. In Vitro Kinase Assay—PC12 cells were untreated or treated with 100 ng/ml NGF or EGF for 10 min and lysed as described above. ERKs 1 and 2 were immunoprecipitated with aERK2 (recognizing both ERKs 1 and 2) from the cell lysates. After extensive washing with lysis buffer, aERK2 immunoprecipitates were incubated at 30 °C for 30 min with 10 µCi of [γ-32P]ATP and 10 µg of GST fusion protein containing SH2-B(S96A) or SH2-B(J96A) in kinase reaction buffer (50 mM HEPES, pH 7.4, 10 mM MgCl2, 0.5 mM dithiothreitol, 50 µM ATP, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 5 mM Na3VO4). GST fusion proteins containing SH2-B-J96A or SH2-B(S96A) were prepared as described previously (27). Following the in vitro kinase assay, the GST fusion proteins were precipitated with glutathione agarose beads. SH2-B-J96A or SH2-B(S96A) was released from the beads by incubation at 30 °C for 40 min with 10 units of thrombin in digestion buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2, 0.1% β-mercaptoethanol). Isolated wild-type or mutant SH2-B-J96A or SH2-B(J96A) was resolved on SDS-PAGE and transferred onto nitrocellulose. The membrane was subjected to autoradiography and then immunoblotted with aSH2-B.

Confocal Fluorescence Microscopy—Confocal imaging was performed with a Nikon OZ laser scanning confocal microscope equipped with a × 60 Nikon objective. GFP was excited at 488 nm by a krypton-argon laser, and fluorescence above 500 nm was captured. Cells were grown on collagen-coated glass coverslips attached to the bottom of a 60-mm culture dish and imaged at room temperature in Krebs-Ringer phosphate buffer (125 mM NaCl, 7 mM KCl, 1 mM CaCl2, 1.2 mM MgSO4, 1 mM NaHPO4, 10 mM glucose, pH 7.4) containing 0.1% bovine serum albumin. The contribution of cellular autofluorescence was judged to be less than 1%. The presented images are representative of at least three separate experiments.

RESULTS

NGF Stimulates Phosphorylation of SH2-B on Multiple Serines/Threonines—We have shown previously that NGF stimulates a large shift in mobility of SH2-B as well as tyrosyl phosphorylation of SH2-B in PC12 cells overexpressing TrkA. SH2-B(J96A) or SH2-B(J96A) were pretreated with Na3VO4, a threonine phosphatase inhibitor (16). To test whether serine/threonine phosphorylation of SH2-B contributes to the NGF-induced shift in mobility of SH2-B, PC12 cells were treated with NGF in the absence of Na3VO4. SH2-B was immunoprecipitated with aSH2-B and immunoblotted with aPY. No detectable tyrosyl phosphorylation of SH2-B was observed using this experiment.
NGF-induced Phosphorylation of SH2-B on Serines/Threonines

**NGF stimulates phosphorylation of SH2-B on multiple serines and threonines.** A, PC12 cells were stimulated with 100 ng/ml NGF for 10 min. SH2-B was immunoprecipitated (IP) with αSH2-B and immunoblotted (IB) with αPY (lanes 1 and 2). The same blot was reprobed with αSH2-B (lanes 3 and 4). B, PC12 cells were stimulated with 100 ng/ml NGF for 10 min, and SH2-B was immunoprecipitated with αSH2-B. The precipitated SH2-B was incubated with dephosphorylation buffer in the absence (lanes 1 and 2) or presence of alkaline phosphatase (AP, lanes 3 and 4) or PP2A (lanes 5 and 6) and immunoblotted with αSH2-B. C, PC12 cells were incubated with 100 ng/ml NGF for indicated times (upper panel) or for 10 min with the indicated concentration of NGF (lower panel). SH2-B was immunoprecipitated with αSH2-B and immunoblotted with αSH2-B.

**MEK Is Critical for NGF-induced Phosphorylation of SH2-B on Serines/Threonines—NGF activates MEK, a kinase that plays an essential role in NGF-induced neuronal differentiation of PC12 cells (22).** PD98059, a MEK inhibitor (32), inhibited NGF-induced neurite outgrowth (Fig. 2b) consistent with previous work (33). Interestingly, overexpression of SH2-Bβ, which has been shown to enhance NGF-induced neurite outgrowth (16), was unable to overcome the inhibition of NGF-induced neuronal differentiation by the MEK inhibitor (Fig. 2d), raising the possibility that phosphorylation of SH2-B by kinases within the MEK/ERK cascade may be involved in regulation of neurite outgrowth in response to NGF.

**To determine whether MEK plays a role in NGF-induced serine/threonine phosphorylation of SH2-B, PC12 cells were**

![Fig. 1. NGF stimulates phosphorylation of SH2-B on multiple serines and threonines.](image1)

![Fig. 2. PD98059 inhibits NGF-induced neurite outgrowth of PC12 cells.](image2)
pretreated for 30 min with 100 μM PD98059 prior to stimulation with 50 ng/ml NGF for 10 min. SH2-B was immunoprecipitated (IP) with αSH2-B and immuno-blotted (IB) with αSH2-B (top panel). Proteins (50 μg) in cell lysates were immunoblotted with αSH2-B (middle panel) or α-active mitogen-activated protein kinase (MAPK) (bottom panel). B, PC12 cells were pretreated without (lanes 1–3) or with 1 μM PMA (lanes 4–6) for 2 days prior to stimulation for 10 min with 1 μM PMA or 100 ng/ml NGF. Proteins in cell lysates were immunoblotted with αSH2-B (upper panel) or α-active mitogen-activated protein kinase (lower panel).

Because multiple isoforms of PKC are proposed to play a role in NGF signaling (34–37), we also examined whether PMA-sensitive PKCs are involved in NGF-induced phosphorylation of SH2-B. PMA, a robust activator of multiple isoforms of conventional PKCs (38), stimulated a mobility shift of SH2-B in PC12 cells (Fig. 3B, upper panel, lane 1 versus 2). PMA also stimulated activation of ERKs 1 and 2 (Fig. 3B, lower panel, lanes 1 and 2), consistent with the previous observations that PKC activates the Raf/MEK/ERK pathway (39–41). Therefore, it is unclear whether PKC activated by PMA phosphorylates SH2-B directly or indirectly via MEK or kinases downstream of MEK. Chronic PMA pretreatment abolished the ability of PMA to activate ERKs 1 and 2 (Fig. 3B, lower panel, lanes 4 and 5) and to stimulate a mobility shift of SH2-B (Fig. 3B, upper panel, lanes 4 and 5), consistent with chronic treatment of cells with PMA depleting PMA-sensitive PKCs (36). In contrast, chronic treatment of PC12 cells with PMA did not alter the NGF-induced mobility shift of SH2-B (Fig. 3B, upper panel, lane 3 versus 6) or activation of ERKs 1 and 2 (Fig. 3B, lower panel, lane 3 versus 6), suggesting that PMA-sensitive PKCs are not responsible for the NGF-stimulated serine/threonine phosphorylation of SH2-B that is responsible for its shift in mobility.

SH2-Bβ Is Phosphorylated at Ser-96 in Vitro by Activated ERKs 1 and 2—Because ERKs 1 and 2 lie downstream of MEK and SH2-B has a consensus phosphorylation site (Pro-Leu-Ser96-Pro) for mitogen-activated protein kinase, we tested whether Ser-96 is a target of activated ERKs 1 and 2. ERKs 1 and 2 from either control or NGF-stimulated cells were immunoprecipitated with αERK2 (αERK2 recognizes both ERKs 1 and 2) and used for an immunocomplex kinase assay with GST-SH2-Bβ fusion protein as an exogenous substrate. ERKs 1 and 2 precipitated from NGF-stimulated but not control cells phosphorylated SH2-Bβ in this in vitro kinase assay (Fig. 4A, upper panel, lanes 3 and 4). When Ser-96 was mutated to Ala (S96A), phosphorylation of SH2-Bβ (S96A) by activated ERKs 1 and 2 in vitro was reduced dramatically (Fig. 4A, upper panel, lane 4 versus 5), suggesting that Ser-96 is the primary phosphorylation site within SH2-Bβ for ERKs 1 and 2. Similarly, ERKs 1 and 2 from EGF-treated cells phosphorylated SH2-Bβ in vitro (Fig. 4B). Surprisingly, the mobility of SH2-Bβ did not detectably change after its phosphorylation by ERKs 1 and 2 (Fig. 4A, lower panel, lanes 3 and 4), suggesting that phosphorylation of Ser-96 in SH2-Bβ by ERKs 1 and 2 does not account for the NGF-induced mobility shift of SH2-B observed in PC12 cells. Combined with data from the MEK inhibitor experiments, these results suggest that in addition to ERKs 1 and 2, MEK or kinases downstream of MEK phosphorylate SH2-B on serines/threonines in response to NGF.

Phosphorylation of Ser-96 in SH2-B by ERKs 1 and 2 Is Not

**Fig. 3.** NGF stimulates phosphorylation of SH2-B by MEK or kinases downstream of MEK. A, PC12 cells were pretreated without (lanes 1 and 2) or with a MEK inhibitor PD98059 (PD, lane 3) prior to stimulation with 50 ng/ml NGF for 10 min. SH2-B was immunoprecipitated (IP) with αSH2-B and immunoblotted (IB) with αSH2-B (top panel). Proteins (50 μg) in cell lysates were immunoblotted with αSH2-B (middle panel) or α-active mitogen-activated protein kinase (MAPK) (bottom panel). B, PC12 cells were pretreated without (lanes 1–3) or with 1 μM PMA (lanes 4–6) for 2 days prior to stimulation for 10 min with 1 μM PMA or 100 ng/ml NGF. Proteins in cell lysates were immunoblotted with αSH2-B (upper panel) or α-active mitogen-activated protein kinase (lower panel).
Required for NGF-induced Neurite Outgrowth—To investigate whether phosphorylation of SH2-B on Ser-96 by ERKs 1 and 2 plays a role in NGF-induced neuronal differentiation, GFP-tagged SH2-B(S96A) was stably overexpressed in PC12 cells as described previously (16). In agreement with our previous observation, overexpression of GFP-SH2-B significantly enhanced NGF-induced neurite outgrowth, whereas overexpression of GFP-SH2-B(S96A) blocked neurite outgrowth induced by NGF (Fig. 5), indicating that SH2-B is an essential signaling molecule for NGF-induced neurite outgrowth. SH2-B(R555E) is a dominant negative form of SH2-B that inhibits the function of endogenous SH2-B in neuronal differentiation induced by NGF. Interestingly, overexpression of GFP-SH2-B(R555E) enhanced NGF-induced neurite outgrowth of PC12 cells to a similar extent as wild-type GFP-SH2-B (Fig. 5), indicating that phosphorylation of SH2-B on Ser-96 by ERKs 1 and 2 is not required for NGF-induced neurite outgrowth.

The SH2 Domain Is Required for Full Phosphorylation of SH2-B in Response to NGF.—To examine whether the dominant negative SH2-B(R555E) inhibits activation of kinases that phosphorylate SH2-B on serines/threonines, cells overexpressing either GFP-SH2-B or GFP-SH2-B(R555E) were stimulated with NGF, and endogenous SH2-B was immunoprecipitated with αSH2-B and immunoblotted with αSH2-B. Expression of neither GFP-SH2-B nor GFP-SH2-B(R555E) altered the ability of NGF to induce serine/threonine phosphorylation of endogenous SH2-B (Fig. 6A). Thus, overexpression of GFP-SH2-B(R555E) seems not to interfere with the activation of TrkA and kinases that phosphorylate SH2-B on serines/threonines, consistent with our previous observation that GFP-SH2-B(R555E) does not alter NGF-induced tyrosyl phosphorylation of TrkA, Shc, and phospholipase Cγ and activation of ERKs 1 and 2 (16).

Because the SH2 domain of SH2-B is required for its association with TrkA and subsequent tyrosyl phosphorylation by TrkA (16), we examined whether it also plays a role in serine/threonine phosphorylation of SH2-B. PC12 cells overexpressing GFP, GFP-SH2-B, GFP-SH2-B(R555E), or GFP-SH2-B(S96A) were stimulated with NGF, and proteins in cell lysates were immunoprecipitated with αSH2-B. NGF stimulated a substantial shift in mobility of both GFP-SH2-B (Fig. 6B, lanes 4 and 8) and GFP-SH2-B(S96A) (Fig. 6B, lane 10), resembling the NGF-induced mobility shift of endogenous SH2-B (Fig. 1A, lane 4; Fig. 1C and Fig. 3A, lane 2, upper two panels). This supports our previously proposed hypothesis that kinase(s) other than ERKs phosphorylate SH2-B on multiple serines/threonines, resulting in multiple forms with different migrations. In contrast, NGF stimulated only a marginal shift in mobility of GFP-SH2-B(R555E) (Fig. 6B, lane 6), indicating that a functional SH2 domain is crucial for NGF-induced serine/threonine phosphorylation of SH2-B. Because the kinase(s) that phosphorylate endogenous SH2-B are activated normally (Fig. 6A), these results suggest that association with TrkA or/and tyrosyl phosphorylation of SH2-B is required for NGF-dependent phosphorylation of SH2-B on serines/threonines.

Mutation of Neither Ser-96 to Ala Nor Arg-555 to Glu Changes the Association of SH2-B with the Plasma Membrane—To gain more insight into the action of SH2-B, we examined the subcellular distribution of SH2-B. PC12 cells were lysed in a hypotonic buffer without detergent and frac-
DISCUSSION

SH2-B has been shown to be tyrosyl-phosphorylated by TrkA and required for NGF-mediated neuronal differentiation and survival (15, 16). In this work, we provide strong evidence that SH2-B is phosphorylated on multiple serines/threonines in response to NGF. In support of NGF promoting phosphorylation of SH2-B on multiple sites, NGF stimulates a dramatic shift in mobility of SH2-B, resulting in multiple forms with different migrations. When treated with alkaline phosphatase that dephosphorylates phosphotyrosines, phosphoserines, and phosphothreonines, these multiple forms revert to a single form of SH2-B migrating similarly to SH2-B from control cells. In support of SH2-B being phosphorylated on multiple serines/threonines, the multiple forms of SH2-B induced by NGF in the absence of a tyrosine phosphatase inhibitor are not recognized by anti-phosphotyrosine. Furthermore, treatment of SH2-B from NGF-stimulated cells with PP2A, which specifically dephosphorylates phosphoserines and phosphothreonines, dramatically reduces the migration of SH2-B.

Our results indicate that kinase(s) within the MEK/ERK cascade play a critical role in this NGF-induced phosphorylation of SH2-B on serines/threonines. PD98059 inhibits the NGF-induced shift in mobility of SH2-B. NGF promotes prolonged phosphorylation of SH2-B, whereas EGF stimulates transient phosphorylation (data not shown), consistent with sustained activation of the MEK/ERK cascade by NGF and transient activation of this pathway by EGF. It is unlikely that MEK phosphorylates SH2-B, because there is no consensus sequence in SH2-B for phosphorylation by MEK. Obvious candidates downstream of MEK are ERKs 1 and 2, since ERKs 1 and 2 are known to be required for NGF-induced neuronal differentiation of PC12 cells (22), and there is a consensus sequence for ERKs 1 and 2 within SH2-B. In vitro studies identified Ser-96 of SH2-B as a phosphorylation site for ERKs 1 and 2, although whether ERKs phosphorylate Ser-96 in intact cells remains to be determined. However, phosphorylation of SH2-B on Ser-96 by ERKs 1 and 2 in vitro did not detectably change its migration in SDS-PAGE gels, and SH2-B (S96A) ectopically expressed in PC12 cells exhibited a large NGF-induced shift in its mobility indistinguishable from that of wild-type SH2-B. This suggests that besides ERKs 1 and 2 another kinase downstream of MEK or an as yet unidentified PD98059-sensitive kinases phosphorylate SH2-B in serines/threonines in response to NGF. A PD98059-sensitive kinase(s) other than ERKs 1 and 2 has been reported to phosphorylate SOS in insulin signaling (43). One candidate kinase is p90rsk, which lies downstream of MEK and phosphorylates MAPK-response element binding protein and Fos in response to NGF in PC12 cells (44–48).

In contrast, deletion of PMA-sensitive isoforms of PKCs does not affect NGF-promoted phosphorylation of SH2-B on serines/threonines, although SH2-B has multiple potential phosphorylation sites for PKC. These results indicate that PMA-sensitive isoforms of PKC may not play a significant role in the NGF-induced phosphorylation of SH2-B.

Interestingly, NGF-induced serine/threonine phosphorylation of the dominant negative SH2-B (R555E) was dramatically reduced, although the activity of kinases that phosphorylate endogenous SH2-B in PC12 cells overexpressing SH2-B (R555E) appeared to be normal. These findings indicate that the SH2 domain of SH2-B is necessary for SH2-B to be phosphorylated on serines/threonines. We observed previously that SH2-B (R555E) is unable to bind to TrkA and be tyrosyl-phosphorylated by TrkA (16). We speculate that association with TrkA or/and tyrosyl phosphorylation of SH2-B is required for NGF-induced phosphorylation of SH2-B on serines/threonines.

SH2-B has been proposed as an adapter for a variety of hormones, cytokines, and growth factors (15, 16, 27, 28, 49). In addition to its SH2 domain, it has multiple proline-rich motifs, a PH domain, and multiple potential serine, threonine, and tyrosine phosphorylation sites. PH domains and proline-rich motifs are present in many signaling molecules and are thought to target these proteins to the plasma membrane by binding to phospholipids (50–52) and constitutively associate with SH3 or WW domain-containing proteins, respectively (53–59). Therefore, we think it likely that its PH domain targets SH2-B to the plasma membrane, and its proline-rich motifs interact constitutively with signaling molecules containing...
SH2 domains. NGF-induced phosphorylation of SH2-B on tyrosines may recruit downstream effectors containing SH2 domain. Thus, one could envision that SH2-B acts as an adapter or a scaffold protein that assembles a large protein complex (signalingosome) of multiple signaling proteins. The interaction of the SH2 domain of SH2-B with phosphorylated tyrosine(s) in the cytoplasmic domain of TrkA recruits this signalingosome to TrkA in response to NGF. Phosphoserines and phosphothreonines have also been shown to form binding sites for various signaling molecules (60–65). One domain that has been shown to bind specifically to phosphoserines and phosphothreonines is the WW domain that is present in many signaling molecules (66). Thus, phosphoserines and phosphothreonines in SH2-B may recruit to this signalingosome downstream effectors in response to NGF. Alternatively, NGF-induced serine/threonine phosphorylation of SH2-B may change the conformation of SH2-B, thereby regulating the composition of this signalingsome and/or activity of some components of this signalingsome. Consistent with these ideas, SH2-Bβ(R555E), which is defective in its association with TrkA and its phosphorylation on tyrosines and serines/threonines, blocks NGF-induced neurite outgrowth, presumably by constitutively binding to and sequestering critical downstream effectors away from endogenous SH2-B. The C-terminal part of SH2-B also acts as a dominant negative mutant in NGF signaling (15). This mutant contains the entire SH2 domain but lacks most proline-rich motifs, tyrosines, and serines/threonines. It would be expected to compete with endogenous SH2-B for TrkA but not to bind all of the signaling molecules needed for the actions of NGF.

SH2-B has been reported to associate constitutively with Grb2 and to mediate NGF-stimulated activation of ERKs 1 and 2 via a mutant TrkA lacking its Shc-binding site (15). However, our previous data indicate that overexpression of neither wild-type SH2-B nor a dominant negative SH2-Bβ(R555E) alters NGF-induced activation of ERKs 1 and 2 in PC12 cells (16), suggesting that SH2-B does not play a significant role in NGF-induced activation of ERKs via endogenous TrkA, at least in PC12 cells. When the primary phosphorylation site Ser-96 for ERKs was mutated, SH2-B(S96A) was still able to enhance NGF-induced neurite outgrowth, suggesting that phosphorylation of SH2-B by ERKs is also not required for its action in promoting NGF-induced neurite outgrowth.

In summary, we show that SH2-B resides at the plasma membrane, which presumably positions it to bind rapidly to TrkA in response to NGF. We also show that upon NGF stimulation, SH2-B is phosphorylated on multiple serines/threonines by kinase(s) downstream of MEK. ERKs are known to be preferentially activated downstream of MEK, which presumably positions it to bind rapidly to downstream effectors away from endogenous signaling molecules needed for the actions of NGF.
NGF-induced Phosphorylation of SH2-B on Serines/Threonines

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