SH2-B Is Required for Nerve Growth Factor-induced Neuronal Differentiation*

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Nerve growth factor (NGF) is essential for the development and survival of sympathetic and sensory neurons. NGF binds to TrkA, activates the intrinsic kinase activity of TrkA, and promotes the differentiation of pheochromocytoma (PC12) cells into sympathetic-like neurons. Several signaling molecules and pathways are known to be activated by NGF, including phospholipase Cγ, phosphatidylinositol-3-kinase, and the mitogen-activated protein kinase cascade. However, the mechanism of NGF-induced neuronal differentiation remains unclear. In this study, we examined whether SH2-Bβ, a recently identified pleckstrin homology and SH2 domain-containing signaling protein, is a critical signaling protein for NGF. TrkA bound to glutathione S-transferase fusion proteins containing SH2-Bβ, and NGF stimulation dramatically increased that binding. In contrast, NGF was unable to stimulate the association of TrkA with a glutathione S-transferase fusion protein containing a mutant SH2-Bβ(R555E) with a defective SH2 domain. When overexpressed in PC12 cells, SH2-Bβ co-immunoprecipitated with TrkA in response to NGF. NGF stimulated tyrosyl phosphorylation of endogenous SH2-Bβ as well as exogenously expressed GFP-SH2-Bβ but not GFP-SH2-Bβ(R555E). Overexpression of SH2-Bβ(R555E) blocked NGF-induced neurite outgrowth of PC12 cells, whereas overexpression of wild type SH2-Bβ enhanced NGF-induced neurite outgrowth. Overexpression of either wild type or mutant SH2-Bβ(R555E) did not alter tyrosyl phosphorylation of TrkA, Shc, or phospholipase Cγ in response to NGF or NGF-induced activation of ERK1/2, suggesting that SH2-Bβ may initiate a previously unknown pathway(s) that is essential for NGF-induced neurite outgrowth. Taken together, these data indicate that SH2-Bβ is a novel signaling molecule required for NGF-induced neuronal differentiation.

PC12 cells, a rat adrenal pheochromocytoma cell line, are a widely used paradigm for studying NGF-induced neuronal differentiation. NGF stimulates PC12 cells to differentiate into sympathetic-like neurons by activating the NGF receptor TrkA, a member of the Trk family of receptor tyrosine kinases (1–3). Upon NGF binding, TrkA dimerizes (4) and autophosphorylates multiple tyrosines within its cytoplasmic domain (5). Signaling molecules containing Src homology 2 (SH2) or phosphotyrosine-binding domains, such as phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase, and Shc proteins, interact with tyrosyl phosphorylated TrkA and transmit NGF signals (5, 6). Phosphatidylinositol 3-kinase is essential for NGF protection of PC12 cells from apoptosis (7) but is not required for NGF-induced neuronal differentiation (8). In contrast, the Sh/Ras/MEK/ERK pathway appears to be essential for NGF-induced neuronal differentiation of PC12 cells (5, 8–10). ERK1/2 can be activated by both NGF and epidermal growth factor (EGF), but the biological responses to NGF and EGF are opposite: NGF promotes differentiation, whereas EGF stimulates proliferation of PC12 cells (11). It was thought that sustained activation of ERK1/2 by NGF compared with transient activation by EGF contributes to the specificity of the NGF differentiation signal (11). Prolonged activation of ERK1/2 by NGF has been shown to be mediated by Rap1 and required for the expression of neuronal specific genes (12). Surprisingly, sustained activation of ERK1/2 is not required for NGF-induced neurite outgrowth (12), demonstrating that morphological differentiation of PC12 cells is mediated by other as yet unidentified signaling proteins/pathway(s).

SH2-Bβ, a predicted adapter protein with multiple potential protein-protein interaction domains/motifs (e.g. pleckstrin homology, SH2, and proline-rich), has recently been shown to be regulated by a variety of ligands that activate receptor tyrosine kinases or receptor-associated tyrosine kinases (13–15). Three alternatively spliced isoforms of SH2-B (α, β, and γ) have been described (13, 16, 17) that differ in their C termini down-stream of the SH2 domain. No cellular function had been ascribed to any of these isoforms. In this study, we show that NGF stimulates association of SH2-Bβ with TrkA via the SH2 domain of SH2-Bβ and tyrosyl phosphorylation of SH2-Bβ. The mutation of the conserved Arg to Glu within the FLVR motif within the SH2 domain of SH2-Bβ abolishes the association of the mutant SH2-Bβ with TrkA and tyrosyl phosphorylation of the mutant SH2-Bβ in response to NGF. Furthermore, this mutant SH2-Bβ acts as a dominant negative SH2-Bβ to block NGF-induced neurite outgrowth when overexpressed in PC12 cells. These results suggest that SH2-Bβ is an essential component of a signaling pathway(s) that is vital for NGF-induced neurite outgrowth.

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Requirement of SH2-Bβ for NGF-induced Neuronal Differentiation

EXPERIMENTAL PROCEDURES

Materials—Murine NGF and EGF were from Collaborative Biomedical Products. Sodium orthovanadate was from Sigma. Recombinant protein A-agarose was from Repligen. Antibody to rat SH2-Bβ (αSH2-B) was raised against a GST fusion protein containing the C-terminal portion of SH2-Bβ (13). Monoclonal anti-phosphotyrosine antibody 4G10 (αpY) and monoclonal antibody to PLCγ (αPLCγ) were from Upstate Biotechnology Inc. Polyclonal antibody to Shc (αShc) was from Transduction Labs. Polyclonal antibody against the extracellular domain of TrkA (αTrkA) (18) was kindly provided by Dr. Louis F. Reichardt (University of California, San Francisco). Polyclonal antibody against the cytoplasmic domain of TRKα was purchased from Santa Cruz Biotechnology, Inc. (C-14). Monoclonal antibody to GFP (αGFP) was from CLONTECH. Anti-active MAPK (active MAPK) was from Promega.

Plasmids and Transfection—cDNA encoding rat SH2-Bβ was subcloned in-frame at BglII/EcoRI sites into pEGFP-C1 (CLONTECH), which encodes a red-shifted variant of GFP. Arg-555 in SH2-Bβ was mutated to Glu, using QuickChange™ site-directed mutagenesis kit (Stratagene) with the primer 5′-GTCTCTCTTGATACAGAGTCAGAGGTCAAGA-3′. PC12 cells were transfected with pEGFP-C1 encoding GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ (R555E), using LipofectAMINE Plus (Life Technologies, Inc.). After 72 hr at 37 °C in 5% CO2 in standard medium (Dulbecco’s modified Eagle’s medium supplemented with 1 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, 10% heat-inactivated horse serum, and 5% fetal bovine serum), the transfectants were cultured for 40 additional days in medium supplemented with 1 mg/ml G418. The G418-resistant transfected cells were pooled, and the top 2% of cells in terms of expression of GFP fusion proteins were selected by flow cytometry (Elite, ESP). Neurite outgrowths were monitored and photographed every 12 h using phase contrast microscopy. NGF was added every 2 days without changing the culture medium.

RESULTS

NGF Stimulates the Association of SH2-Bβ with TrkA via the SH2 Domain of SH2-Bβ—SH2-B in PC12 cells is thought to be the β isomorph because after dephosphorylation by alkaline phosphatase, it comigrates with dephosphorylated SH2-Bβ expressed ectopically in COS cells (data not shown). To determine whether SH2-Bβ plays a role in NGF signaling, we first examined whether SH2-Bβ interacts with TrkA. Wild type or mutant SH2-Bβ was fused to GST. PC12 cells overexpressing TrkA (19) were treated with NGF, and the cell extracts were incubated with the indicated GST fusion proteins immobilized on agarose beads. GST fusion proteins containing either wild type (GST-WT) or the C-terminal 20% of SH2-Bβ with the entire SH2 domain (GST-SH2) bound weakly to TrkA from control cells (Fig. 1a). NGF treatment dramatically increased the binding of TrkA to both GST-SH2-Bβ and GST-SH2 (Fig. 1a). Densitometric analysis revealed that NGF stimulated the association of TrkA with GST-SH2-Bβ by more than 5-fold (n = 3) and with GST-SH2 by more than 4.5-fold (n = 3). In contrast, only a residual amount of TrkA bound to GST fusion proteins containing mutant SH2-Bβ (R555E) in which Glu replaced the critical Arg within the FLVR motif of the SH2 domain of SH2-Bβ (GST-RE) (Fig. 1a). NGF treatment did not increase the binding of TrkA to GST-RE (Fig. 1a). GST alone did not bind to TrkA, even when cell extracts had been treated with NGF (Fig. 1a). These results suggest that the SH2 domain of SH2-Bβ is sufficient and necessary for NGF-promoted association of TrkA with SH2-Bβ. In addition, the weak binding of GST-WT or GST-SH2 to TrkA from control cells suggests that the SH2 domain of SH2-Bβ may have a low affinity for nontyrosyl phosphorylated, inactive TrkA. Alternatively, there may be other binding site(s) in the C-terminal portion of SH2-Bβ that bind with low affinity to nontyrosyl phosphorylated TrkA.

To determine whether SH2-Bβ associates with TrkA in cells in response to NGF, PC12 cells overexpressing GFP-tagged SH2-Bβ were treated with NGF, and proteins in the cell lysates were immunoprecipitated with αTrkA (from Dr. Louis F. Reichardt, 1:500 dilution). b, PC12 cells stably overexpressing GFP-SH2-Bβ were stimulated with 100 ng/ml NGF for 6 min. Proteins in the cell lysates were immunoprecipitated (IP) with αTrkA (from Dr. Louis F. Reichardt, 1:100 dilution) and immunoblotted sequentially with αSH2-B and αGFP as indicated. The same blot was stripped and reprobed with αTrkA.

Fig. 1. NGF promotes the interaction of SH2-Bβ with TrkA. a, PC12 cells overexpressing TrkA were stimulated with 100 ng/ml NGF for 10 min. Cell lysates were incubated with GST fusion proteins containing wild type (GST-WT), the SH2 domain of (GST-SH2), or R555E mutant (GST-RE) SH2-Bβ. Proteins bound to GST fusion proteins were immunoblotted (IB) with αTrkA from PC12 (from Dr. Louis F. Reichardt, 1:500 dilution). b, PC12 cells stably overexpressing GFP-SH2-Bβ were stimulated with 100 ng/ml NGF for 6 min. Proteins in the cell lysates were immunoprecipitated (IP) with αTrkA (from Dr. Louis F. Reichardt, 1:100 dilution) and immunoblotted sequentially with αSH2-B and αGFP as indicated. The same blot was stripped and reprobed with αTrkA.
cells overexpressing TrkA were treated with 100 ng/ml NGF for 1 min, a
with type (m) were immunoprecipitated with aPH (upper panel, 1:7500 dilution). The same blot was reprobed with aSH2-B (lower panel, b), PC12 cells overexpressing TrkA were treated with 100 ng/ml NGF for 1 min, and SH2-Bβ was immunoprecipitated with aSH2-B and immunoblotted with aPY (upper panel). The same blot was reprobed with aSH2-B (lower panel, c), PC12 cells were pretreated with 100 μM NaN3VO4 for 60 min prior to 100 ng/ml NGF or 125 ng/ml EGF for 10 min. SH2-Bβ was immunoprecipitated with aSH2-B and immunoblotted with aPY.

Fig. 2. NGF stimulates tyrosyl phosphorylation of SH2-Bβ. a, PC12 cells were pretreated with 100 μM NaN3VO4 for 60 min prior to 100 ng/ml NGF for 10 min. SH2-Bβ was immunoprecipitated (IP) with aSH2-B and immunoblotted (IB) with aPY (upper panel, 1:7500 dilution). The same blot was reprobed with aSH2-B (lower panel, b), PC12 cells overexpressing TrkA were treated with 100 ng/ml NGF for 10 min. Proteins in lysates were immunoprecipitated with aSH2-B and immunoblotted with aPY (upper panel). The same blot was reprobed with aSH2-B (lower panel, c). PC12 cells were pretreated with 100 μM NaN3VO4 for 60 min prior to 100 ng/ml NGF or 125 ng/ml EGF for 10 min. SH2-Bβ was immunoprecipitated with aSH2-B and immunoblotted with aPY.

SH2-Bβ without a dramatic change in the mobility of SH2-Bβ (Fig. 2b, lower panel). This is in contrast to the large mobility change observed in endogenous SH2-Bβ in PC12 cells treated with NGF for 10 min (Fig. 2a, lower panel). This difference in mobility shift in the two figures is likely due to the different times of incubation with NGF, because a large mobility change of SH2-Bβ was observed when PC12 cells overexpressing TrkA were treated with NGF for 10 min (data not shown). These data support the hypothesis that the shift in the mobility of SH2-Bβ is caused by phosphorylation of SH2-Bβ on serines/threonines rather than on tyrosines. The results also suggest that NGF-stimulated tyrosyl phosphorylation of SH2-Bβ may precede serine/threonine phosphorylation of SH2-Bβ.

In contrast to NGF, EGF did not stimulate tyrosyl phosphorylation of SH2-Bβ in PC12 cells even in the presence of phosphatase inhibitor (Fig. 2c). Because EGF stimulates proliferation while NGF promotes neuronal differentiation of PC12 cells, this differential response of SH2-Bβ to these two growth factors might contribute to the specificity of biological response following the activation of receptors for these two growth factors. Taken together, the results raise the possibility that NGF-stimulated association of SH2-Bβ with TrkA and/or the subsequent tyrosyl phosphorylation of SH2-Bβ initiates one or more novel signaling pathways that may be specific for NGF and required for NGF-induced neuronal differentiation.

SH2-Bβ Is Required for NGF-induced Neurite Outgrowth of PC12 Cells—To examine the function of SH2-Bβ in cellular responses to NGF, we generated PC12 cells that stably overexpress GFP, GFP-tagged SH2-Bβ, or GFP-SH2-Bβ(R555E) that lacks a functional SH2 domain. To eliminate differences due to clonal variation of PC12 cells, we pooled all G418-resistant clones. The GFP tag enabled us to use flow cytometry to isolate cells expressing high levels of SH2-Bβ. GFP-SH2-Bβ and GFP-SH2-Bβ(R555E) are present at ~60 and 20 times, respectively, the level of endogenous SH2-Bβ (Fig. 3a).

We first examined whether GFP-SH2-Bβ, like endogenous SH2-Bβ, is tyrosyl phosphorylated in response to NGF. PC12 cells overexpressing GFP-SH2-Bβ (designated as GFP-WT) were stimulated with NGF. Proteins in the cell lysates were immunoprecipitated with aSH2-B and then immunoblotted with aPY. Significant tyrosyl phosphorylation of GFP-SH2-Bβ was detected (Fig. 3b, upper panel). Reprobing the same blot with aSH2-B showed that NGF also stimulated a shift in the mobility of GFP-SH2-Bβ (Fig. 3b, lower panel). In contrast, NGF did not stimulate tyrosyl phosphorylation of GFP-SH2-Bβ(R555E) (Fig. 3b, upper panel) as anticipated. These results suggest that like endogenous SH2-Bβ, GFP-tagged SH2-Bβ binds to activated and tyrosyl phosphorylated TrkA and is tyrosyl phosphorylated by TrkA in response to NGF.

To study the role of SH2-Bβ in NGF-induced neuronal differentiation of PC12 cells, we examined whether SH2-Bβ is required for NGF-induced neurite outgrowth, a hallmark of neuronal differentiation. In the presence of NGF, cells overexpressing GFP alone developed neurite outgrowths in a manner similar to parental PC12 cells (Fig. 4, a and b, and data not shown). Strikingly, overexpression of GFP-SH2-Bβ(R555E) blocked neurite outgrowth induced by NGF (Fig. 4, c and d). Even treatment of cells with a supramaximal concentration of NGF (100 ng/ml) for 8 days or longer did not induce neurite outgrowth (data not shown). This observation suggests that SH2-Bβ is required for NGF-induced morphological differentiation of PC12 cells.

Consistent with SH2-Bβ playing an essential role in NGF-induced differentiation of PC12 cells, overexpression of GFP-SH2-Bβ enhanced NGF-induced neurite outgrowth when NGF was tested at a submaximal concentration. For example, 25 ng/ml NGF for 4 days promoted differentiation of the majority of cells expressing GFP-SH2-Bβ, whereas only a very few of the cells expressing GFP alone differentiated in response to NGF (Fig. 4, e–h). Neurite outgrowth (twice cell diameter) was observed in about 60% of cells expressing GFP-SH2-Bβ after 2 days of incubation with 15 ng/ml NGF, whereas fewer than 1% of the cells expressing GFP had neurites. By day 5, approximately 95% of the cells expressing GFP-SH2-Bβ had neurite outgrowth, whereas only about 40% of cells expressing GFP had neurite outgrowth. In the absence of NGF, overexpression of GFP-SH2-Bβ did not induce neuronal differentiation (Fig. 4g), suggesting that SH2-Bβ must be activated in some way by

4 The apparent difference in the amount of GFP-SH2-Bβ in control and NGF-treated cells in Fig. 3b (lower panel) is an artifact of this mobility shift and the large amount of GFP-SH2-Bβ. Experiments in which a smaller amount of GFP-SH2-Bβ was electrophoresed revealed similar amounts of GFP-SH2-Bβ from control and NGF-stimulated cells.
NGF, presumably by being phosphorylated, to mediate neurite outgrowth.

NGF-induced Tyrosyl Phosphorylation of TrkA, Shc, and PLCγ and Activation of ERK1/2 Are Not Affected by Overexpression of SH2-Bβ(R555E) — To explore the mechanism of action of SH2-Bβ, we asked whether overexpression of SH2-Bβ or SH2-Bβ(R555E) affects tyrosyl phosphorylation of TrkA, Shc, or PLCγ, all of which are known to play important roles in NGF signaling (20). Cells stably overexpressing GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E) were treated with NGF, and proteins in the cell lysates were immunoprecipitated with the antibodies against TrkA, Shc, or PLCγ and immunoblotted with anti-phosphotyrosine antibody. NGF induced tyrosyl phosphorylation of TrkA (Fig. 5a), PLCγ (Fig. 5c), and all three isoforms of Shc (Fig. 5b). Levels of induction were unchanged in cells overexpressing SH2-Bβ or SH2-Bβ(R555E) (Fig. 5), suggesting that TrkA activation and signaling events immediately downstream of TrkA are not compromised by overexpression of SH2-Bβ(R555E).

Because the kinetics of the activation of ERK1/2 have been hypothesized to play an important role in NGF-induced neuronal differentiation of PC12 cells, we examined the time course of activation of ERK1/2 by NGF by immunoblotting cell lysates with antibody recognizing only activated ERK1/2 that is phosphorylated on both tyrosine and threonine. Neither the extent nor the duration of activation of ERK1/2 by NGF was affected by overexpression of SH2-Bβ or SH2-Bβ(R555E) (Fig. 6), consistent with the previous observation that sustained activation of ERK1/2 induced by NGF is not sufficient for neuronal differentiation of PC12 cells (21, 22).

**DISCUSSION**

In this study, we show that NGF promotes the association of SH2-Bβ with activated TrkA and stimulates tyrosyl phosphorylation of SH2-Bβ. Mutating the SH2 domain of SH2-Bβ abolishes its ability to bind activated TrkA and to be phosphorylated in response to NGF, suggesting that SH2-Bβ binds via its SH2 domain to tyrosyl phosphorylated TrkA and that this interaction is required for subsequent phosphorylation of SH2-Bβ. Overexpression of a GFP-tagged SH2-Bβ(R555E) lacking a functional SH2 domain blocks NGF-induced neurite outgrowth of PC12 cells, whereas overexpression of GFP-tagged wild type SH2-Bβ enhances NGF-induced neurite outgrowth. SH2-Bβ represents only the second signaling molecule, the first being Shc, known to bind to activated TrkA, to be phosphorylated, and to be required for NGF-induced neuronal differentiation.

SH2-Bβ has been shown to associate with receptors for insulin, insulin-like growth factor-1 (14), platelet-derived growth factor (15), and EGF (data not shown), in addition to TrkA. Of the hormones and growth factors that bind to these receptors, only NGF and platelet-derived growth factor have been shown
to stimulate tyrosyl phosphorylation of SH2-Bβ (14, 15). In PC12 cells, both NGF and platelet-derived growth factor (23) promote neurite outgrowth and neuronal differentiation. In contrast, EGF, insulin, and insulin-like growth factor-1 stimulate cell proliferation (11, 24). This correlation raises the possibility that ligand-dependent tyrosyl phosphorylation of SH2-Bβ plays a critical role in promoting neurite outgrowth of PC12 cells. Consistent with this idea, NGF stimulates robust tyrosyl phosphorylation of GFP-SH2-Bβ (Fig. 3b), whose expression enhances NGF-induced neurite outgrowth. However, NGF is unable to stimulate the tyrosyl phosphorylation of GFP-SH2-Bβ(R555E) (Fig. 3b), which acts as a dominant negative SH2-Bβ to block NGF-induced neurite outgrowth. Because SH2-Bβ but not SH2-Bβ(R555E) binds to TrkA (Fig. 1a), it is likely that binding of SH2-Bβ to TrkA is required for its phosphorylation and that SH2-Bβ is tyrosyl phosphorylated directly by activated TrkA.

SH2-Bβ(R555E) neither binds to TrkA nor interferes with phosphorylation of endogenous SH2-Bβ (data not shown) in response to NGF. Therefore, it seems likely that SH2-Bβ(R555E) functions as a dominant negative mutant to interfere with the action of endogenous SH2-Bβ by competing with endogenous SH2-Bβ for downstream effector(s) and sequestering these putative effector(s) in an inactive state. Phosphorylation of SH2-Bβ does not appear to be required for sequestration of effectors, because GFP-SH2-Bβ(R555E) is not significantly phosphorylated in response to NGF (Fig. 2b). However, phosphorylation of SH2-Bβ may be important for activation of downstream effector(s). Alternatively, the downstream effectors of SH2-Bβ may be activated when recruited to TrkA-containing complexes by the interaction of SH2-Bβ with TrkA.

Interestingly, overexpression of either GFP-SH2-Bβ(R555E) nor GFP-SH2-Bβ affect NGF-induced tyrosyl phosphorylation of TrkA, PLCγ, three isoforms of Shc and activation of ERK1/2, indicating that the signaling events immediately downstream of TrkA and the Shc/Ras/MEK/ERKs cascade are not compromised by overexpression of wild type or dominant negative mutant SH2-Bβ. This observation demonstrates that the dominant negative effect of SH2-Bβ(R555E) on NGF-induced neurite outgrowth is not secondary to a defect in the activation of TrkA, the ability of TrkA to tyrosyl phosphorylate its substrates, or the activation of the Shc/Ras/MEK/ERK cascade by NGF. Thus, SH2-Bβ is likely to initiate a novel pathway required for NGF-induced neurite outgrowth.

In conclusion, we show that the putative adapter protein SH2-Bβ binds via its SH2 domain to activated TrkA and is tyrosyl phosphorylated in response to NGF. We also demonstrate that overexpression of SH2-Bβ enhances NGF-induced neuronal differentiation, whereas overexpression of a dominant negative SH2-Bβ blocks that differentiation. These findings provide the first insight into the cellular function of SH2-B. They also suggest that SH2-Bβ may be one of the hypothesized “missing links” parallel to or downstream of ERKs that are thought to be required for NGF-induced differentiation.

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