Growth Factors Differentially Stimulate the Phosphorylation of Shc Proteins and Their Association with Grb2 in PC-12 Pheochromocytoma Cells*

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Growth factor receptor tyrosine kinases can form stable associations with intracellular proteins that contain src homology (SH) 2 domains, including two proteins, Shc and Grb2, that are thought to lie upstream from the ras protooncogene in a signaling cascade. The phosphorylation and molecular associations of these proteins were evaluated in PC-12 pheochromocytoma cells treated with nerve growth factor (NGF), epidermal growth factor (EGF), and insulin. Both NGF and EGF stimulated the tyrosine phosphorylation of Shc proteins and their subsequent association with the receptors. In contrast, insulin had no effect on Shc phosphorylation, despite the expression of functional insulin receptors in these cells at levels comparable to those observed for NGF and EGF. NGF and EGF also induced the association of Shc proteins with a Grb2 fusion protein or endogenous Grb2, whereas insulin had no effect. All of the tyrosine-phosphorylated Shc proteins associated with the Grb2 fusion protein, although only about half of the endogenous Shc was phosphorylated in response to NGF or EGF. However, all three hormones induced the association of several additional tyrosine phosphorylated proteins with Grb2, some of which also coprecipitated with antiserum against the 85-kDa subunit of phosphatidylinositol-3 kinase. Moreover, these growth factors stimulated the association of phosphatidylinositol-3 kinase activity with the Grb2 fusion protein, although this activity was not detected in anti-Shc immunoprecipitates. These results provide further evidence for the divergence of signaling pathways in insulin action, and suggest that Grb2 forms separate complexes with tyrosine-phosphorylated proteins in PC-12 cells.

Neurotrophins and other growth factors promote the survival, growth, and development of a number of sympathetic and sensory neurons. Although the precise biochemical mechanisms involved remain elusive, it is recognized that early changes in protein phosphorylation play an important role. Indeed, a number of these growth factors, such as nerve growth factor (NGF),1 epidermal growth factor (EGF), and insulin, bind to receptors that contain tyrosine kinase activity (1-6). The ligand-dependent activation of these receptor tyrosine kinases is subsequently translated into stimulation of a series of serine/threonine kinases thought to be activated by a linear cascade (7-9). In this pathway, activated tyrosine kinases can stimulate the p21ras protooncogene, followed by the downstream stimulation of rasf-1 kinase (10-13), mitogen-activated protein kinase kinase (15, 16), mitogen activated protein (MAP) kinase (14), and ribosomal S6 kinase II (17). Some evidence suggests that these serine/threonine phosphorylations are stimulated sequentially following growth factor-stimulated GTP binding to p21ras, although the possibility remains that multiple divergent and convergent pathways are involved (16).

The precise receptor tyrosine kinase substrates that initiate the growth and differentiative responses of neurons have yet to be identified. However, the many similarities in early phosphorylation events produced by differentiative agents like NGF and mitogenic agents like EGF and insulin suggest that subtle differences in signal initiation are required to distinguish different cellular fates. One mechanism for early signal divergence may involve differential interactions of receptors with effector proteins containing src homology 2 (SH2) domains. Many of these proteins can form stable associations with receptors in a growth factor-dependent manner via an interaction with the SH2 domain (3, 18-20), although these interactions can be differentiated on the basis of affinity (21, 22). Recently two new genes that encode proteins which have SH2 domains were identified that are thought to reside upstream of p21ras activation by tyrosine kinases (23). One gene, src, encodes two overlapping proteins of molecular masses of 46 and 52 kDa (24). Another Shc protein of molecular mass 66 kDa is thought to be encoded by a related gene (24). Overexpression of these Shc proteins induces malignant transformation in 3T3 cells (24). Moreover, Shc proteins can associate with tyrosine-phosphorylated receptors and are themselves phosphorylated on tyrosine in response to growth factors (24). The second gene encodes an abundant 23-kDa polypeptide known as growth factor receptor-bound protein 2 (Grb2) or ASH (25-27). This protein contains SH2 and SH3 domains, although its state of phosphorylation does not appear to be increased by growth factors (25, 28). The association of Shc with Grb2/ASH has been implicated in activation of the ras pathway by tyrosine kinases (23). Additionally, the Drasophila homolog of Grb2, drk, may activate the guanyl nucleotide exchange factor of ras via its SH3 domains (28, 29).

We have recently identified a PC-12 pheochromocytoma cell line that differentially responds to NGF, EGF, and insulin, gel electrophoresis; GST, glutathione S-transferase; MAP, mitogen-activated protein; IRS1, insulin receptor substrate-1.
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despite the presence of equal numbers of receptors for these three hormones (16). In these cells, both NGF and EGF produce the substantial stimulation of p21^{\text{W40}}, raf, MAP kinase kinase, and MAP kinase, whereas insulin is totally without effect. However, insulin does bind to and activate its own receptor kinase and stimulates the tyrosine phosphorylation of its major 185-kDa substrate and subsequent activation of phosphatidylinositol (PI)-3 kinase. In this report we describe the differential phosphorylation of Shc proteins by NGF, EGF, and insulin, as well as their association with the Grb2 protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—125I-Labeled protein A (30 mCi/mg) and 125I-labeled antiamouse Ig (3000 Ci/mmol) were from Amersham Corp. \(\gamma\)-32P]ATP (3000 Ci/mmol) was from DuPont NEN. NGF 2.5 S was from Bioproducts for Science (Indianapolis, IN). Receptor grade EGF was from Collaborative Research (Lexington, MA). Insulin was from Lilly. Glutathione-agarose CL-4B was from Fluka Chemical. Silica gel thin-layer chromatography plates were from EM Separations (Gibbstown, NJ). Anti-phosphotyrosine, anti-Shc, anti-Grb2, and anti-p85 subunit of PI-3 kinase antisera were from Upstate Biotechnology (Lake Placid, NY). All other reagents were purchased from Sigma and were the highest quality available. The pGEX plasmid expressing Grb2 sequence in this lacks the NH2-terminal 14 amino acids and was expressed in a glutathione S-transferase (GST) fusion protein (27).

**Immunoblots**—PC-12 cells were grown in 150-mm dishes. Before hormonal treatment, the medium was replaced with serum-free medium and incubated for 1 h. 10 nm NGF, 10 nm EGF, or 100 nm insulin was directly added to the medium and incubated for 1 min at 37 °C. After hormonal treatment, the medium was removed, and cells were washed twice with 12 ml of ice-cold phosphate-buffered saline before the addition of 1 ml of HNTG buffer (50 mM HEPEs, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 100 mM NaF, 10 

**Preparation of Protein Extracts**—This was performed as described elsewhere (3, 30). In some experiments, binding to the bacterially expressed GST-Grb2 fusion protein was assayed. Supernatants were incubated for 90 min with glutathione agarose, to which the GST-Grb2 fusion protein had been bound. The beads were washed three times with 1 ml of HNTG buffer, and samples were resolved on 8% SDS-gels. Transfer to nitrocellulose paper, immunoblotting with anti-Shc antiserum, as described under "Experimental Procedures.

**Phosphotyrosine Assay**—To determine the extent of tyrosine phosphorylation of Shc proteins, PC-12 cells (18) were treated with NGF, EGF, or insulin for 1 min, and lysates were precipitated with anti-Shc antiserum. The resulting immunoprecipitates (Fig. 1A) were electrophoresed on 8% SDS-polyacrylamide gels, and tyrosine-phosphorylated proteins were detected by immunoblotting with anti-phosphotyrosine antisera. Both NGF and EGF stimulated the tyrosine phosphorylation of Shc proteins migrating at 46, 52, and 66 kDa (Fig. 1A, lanes 2 and 3). In addition, tyrosine phosphorylation of the 140-kDa NGF and 170-kDa EGF receptors was also detected in these immunoprecipitates, confirmed by Western blot analysis with antireceptor antisera (data not shown). In contrast, insulin had no detectable effect on the phosphorylation of the three Shc proteins in these cells. Variation of the time of incubation or concentration of insulin failed to produce any detectable tyrosine phosphorylation of Shc proteins. Moreover, immunoblotting of anti-Shc immunoprecipitates from NGF-, EGF- and insulin-treated cells revealed no difference in the amount of immunoprecipitated Shc proteins recovered from cells exposed to the different growth factors (data not shown).

**Western Blot Analysis**—Immunoblotting of anti-Shc immunoprecipitates from NGF-, EGF- and insulin-treated cells revealed no difference in the amount of immunoprecipitated Shc proteins recovered from cells exposed to the different growth factors (data not shown).

**RESULTS**

**NGF and EGF, but Not Insulin, Stimulate the Tyrosine Phosphorylation of Shc Proteins in PC-12 Cells**—We have previously reported that the receptors for NGF, EGF, and insulin can
differentially associate with a variety of SH2-containing proteins in PC-12 cells (18). In some cases, these interactions occur via a direct association between receptor and the SH2 domain, as in the case of phospholipase C-yl and the NGF receptor (3, 18). In other cases, these growth factor-dependent interactions occur via an indirect mechanism, typified by the association of the 85-kDa regulatory subunit of PI-3 kinase with the insulin receptor substrate-1 (IRS1) or the NGF receptor substrate pp100 (32). To explore these interactions in more detail, we have examined the growth factor-dependent phosphorylation of Shc proteins in these cells. PC-12 cells were treated with NGF, EGF, or insulin for 1 min, and lysates were precipitated with anti-Shc antisera. The resulting immunoprecipitates (Fig. 1A) or whole cell lysates (Fig. 1B) were electrophoresed on polyacrylamide gels, and tyrosine-phosphorylated proteins were detected by immunoblotting with anti-phosphotyrosine antisera. Both NGF and EGF stimulated the tyrosine phosphorylation of Shc proteins migrating at 46, 52, and 66 kDa (Fig. 1A, lanes 2 and 3). In addition, tyrosine phosphorylation of the 140-kDa NGF and 170-kDa EGF receptors was also detected in these immunoprecipitates, confirmed by Western blot analysis with antireceptor antisera (data not shown). In contrast, insulin had no detectable effect on the phosphorylation of the three Shc proteins in these cells. Variation of the time of incubation or concentration of insulin failed to produce any detectable tyrosine phosphorylation of Shc proteins. Moreover, immunoblotting of anti-Shc immunoprecipitates from NGF-, EGF- and insulin-treated cells revealed no difference in the amount of immunoprecipitated Shc proteins recovered from cells exposed to the different growth factors (data not shown).

**Some tyrosine phosphorylation of the 95-kDa \(\beta\) subunit of the insulin receptor was detected in anti-Shc immunoprecipitates (Fig. 1A, lane 4), however, the amount of phosphorylated insu-
lin receptor observed in anti-Shc immunoprecipitates was much less than even that detected in whole cell lysates from insulin-treated cells (Fig. 1B, lane 4), indicating only a minor interaction between these proteins.

Although the tyrosine phosphorylated NGF and EGF receptors could be precipitated from growth factor-treated cells with anti-Shc antiserum, we were unable to detect Shc proteins by western blotting of anti-receptor immunoprecipitates from NGF- or EGF-treated cells. This discrepancy may have resulted from the relatively low number of NGF and EGF receptors in these PC-12 cells. However, it is also possible that the binding of the Shc protein to these receptors is of relatively low affinity. Indeed, this latter possibility is supported by direct analysis of the Shc-Grb2 complex in PC-12 cells. These studies revealed only low affinity interactions, whereas this SH2 domain bound to the PDGF receptor with higher affinity.  

NGF and EGF, but Not Insulin, Induce the Formation of an Shc-Grb2 Complex in PC-12 Cells—It has been reported that Grb2/ASH associates with the tyrosine-phosphorylated EGF and PDGF receptors via its SH2 domain (26). To evaluate the growth factor-dependent interactions of this protein, PC-12 cells were treated with NGF, EGF, or insulin for 1 min, and lysates were incubated with an immobilized GST fusion protein containing both the SH3 and SH2 domains of Grb2 prior to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-phosphotyrosine antiserum (Fig. 2A). Treatment of cells with EGF induced the association of its tyrosine phosphorylated receptor with the Grb2 fusion protein (Fig. 2A, lane 3). NGF- or insulin-induced association of their receptors with GST-Grb2 was barely detectable (lanes 2 and 4), although these differences might reflect differences in phosphotyrosine content or ability to detect these receptors with anti-phosphotyrosine antiserum. However, both NGF and EGF induced the association of several tyrosine phosphorylated proteins with the Grb2 fusion protein, including major species with molecular masses of 46, 52, 66, 100, and 110 kDa. Treatment of cells with insulin (Fig. 2A, lane 4) resulted in the association of the Grb2 fusion protein with a single tyrosine phosphorylated protein migrating at 185 kDa, subsequently identified by sequential immunoprecipitation with anti-Shc and anti-IRS1 antiserum as IRS1 (data not shown).

Because three of the phosphoproteins detected in the Grb2 precipitates from cells treated with NGF or EGF migrated with an apparent mobility identical to the Shc proteins, we directly tested for the formation of an Shc-Grb2 complex by immunoblotting with anti-Shc antiserum. Following treatment with NGF, EGF, or insulin for 1 min, lysates were incubated with the Grb2 fusion protein prior to SDS-PAGE and immunoblotting with anti-Shc antiserum (Fig. 2B). All three Shc proteins were detected in Grb2 fusion protein precipitates from cells treated with NGF or EGF. In contrast, treatment of cells with insulin had no effect.

To evaluate whether Shc proteins associated with endogenous Grb2 in response to the growth factors, we attempted to coimmunoprecipitate the complex with anti-Grb2 antiserum. Following treatment with NGF, EGF, or insulin for 1 min, lysates were immunoprecipitated with anti-Grb2 antiserum, followed by immunoblotting with anti-phosphotyrosine antiserum (Fig. 2C). Tyrosine phosphorylated Shc proteins migrating at 52 and 46 kDa were detected in the anti-Grb2 precipitate from cells treated with NGF or EGF, although insulin had no effect. In addition, the EGF receptor was also detected in these immunoprecipitates, although neither NGF or insulin receptors were found. Interestingly, despite the detection of IRS-1 using Grb2 fusion protein, little IRS1 was found in the anti-Grb2 immunoprecipitates.

The fraction of tyrosine phosphorylated Shc proteins that associate with Grb2 in NGF-treated cells was evaluated by comparison of the tyrosine phosphorylated proteins in sequential precipitations with the Grb2 fusion protein (Fig. 3). A majority of Shc proteins that were tyrosine phosphorylated in response to NGF were found in the first Grb2 fusion protein precipitate as detected by anti-phosphotyrosine immunoblotting (Fig. 3A). Subsequent incubation precipitated only a trace of these proteins, and the resulting post-precipitate supernatant showed no detectable signal (data not shown). The fusion protein precipitations were also probed with anti-Shc antiserum (Fig. 3B). Interestingly, approximately equal signals were detected in Grb2 precipitates and the post-precipitate supernatants, indicating that while virtually all of the tyrosine phosphorylated Shc proteins can associate with Grb2, only about half of the total cellular Shc proteins are phosphorylated in

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*G. Zhu and A. R. Saltiel, unpublished observations.*
response to NGF.

**Grb2 Forms Separate Complexes with Tyrosine Phosphorylated Proteins**—We have previously reported that both NGF and EGF stimulate the tyrosine phosphorylation of two proteins, with molecular masses of 100 and 110 kDa, that specifically bind to the 85-kDa subunit of PI-3 kinase via its SH2 domain (32). Because we could detect no association of the NGF or EGF receptors with p85 in these cells, we speculated that these two p85 binding proteins might serve to activate PI-3 kinase as surrogate phosphorylated docking proteins, as has been proposed for the IRS1 (33). Thus, we compared the pattern of phosphoprotein binding of p85 with Grb2 in cells treated with EGF and NGF (Fig. 4). Lysates from control, NGF, or EGF-treated cells were precipitated with anti-p85 antisem (lanes 1–3) or with the immobilized Grb2 fusion protein (lanes 4–6). Alternatively, lysates were cleared with the Grb2 fusion protein, and subsequently immunoprecipitated with anti-p85 antisem (lanes 7–9). As previously described (32), both NGF and EGF stimulated the coprecipitation of pp100 and 110 with anti-p85 immunoprecipitates. Clearance of lysates with the Grb2 fusion protein depleted greater than 90% of the 110-kDa phosphoprotein, suggesting that both NGF and EGF separately induce the formation of a Grb2-pp100/110 complex.

The detection of these two phosphoproteins (pp100/110) is known to be associated with the regulatory subunit of PI-3 kinase suggested that Grb2 might itself form a complex with the enzyme in response to NGF and EGF. Moreover, the association of tyrosine phosphorylated IRS1 with this fusion protein (Fig. 2) suggested that PI-3 kinase might also complex with Grb2 in response to insulin. To explore this possibility, we examined the effect of the three growth factors on PI-3 kinase activity assayed in Grb2 fusion protein precipitates. PC-12 cells were treated with NGF, EGF, or insulin for the indicated times, and lysates were incubated with the immobilized fusion protein, followed by the assay of PI-3 kinase (Fig. 5). As was seen previously in antiphosphotyrosine immunoprecipitates (32), NGF, EGF and insulin rapidly stimulated the association of PI-3 kinase activity with the Grb2 fusion protein. In contrast, none of these growth factors stimulated the association of PI-3 kinase activity with the Shc proteins, as measured in anti-Shc immunoprecipitates (data not shown). Although it remains possible that this antisem allosterically prevents the activation of PI-3 kinase, these results indicate that Shc does not associate with PI-3 kinase, further suggesting that separate complexes are formed with Grb2.

![Image of Grb2 fusion protein precipitates](image-url)
DISCUSSION

Although the tyrosine phosphorylation of growth factor receptors clearly plays an important part in triggering the growth and differentiation of neurons, the downstream regulation of many key enzymes or transcription factors is often controlled by changes in serine or threonine phosphorylation. Moreover, differentiative factors such as NGF and growth factors such as EGF and insulin frequently induce identical or similar phosphorylation events in model systems like the PC-12 cell (16). Thus, the search for early biochemical events that allow cells to differentiate in response to NGF and EGF in these cells, there was no observed response to insulin, despite the insulin-dependent tyrosine phosphorylation of its receptor and IRS1, increased glycosgen, lipid and protein synthesis, and the stimulation of PI-3 kinase activity detected in anti-phosphotyrosine (16, 32), anti-IRS1 (16), and GST-Grb2 fusion precipitates. Although the inability of insulin to stimulate Shc phosphorylation or its association with Grb2/ASH in this clonal PC-12 cell line provides further evidence for receptor diversity in signal transduction, the mechanistic basis for the uncoupling of the insulin receptor to this pathway is unclear. Insulin-stimulated phosphorylation of Shc proteins has been observed in fibroblasts (35). However, these cells were transfected with over 10^6 receptors/cell, a concentration at which the specificity of the receptor may be decreased (35). Although it is possible that the PC-12 cells described here express mutant receptors that are differentially coupled to signaling pathways, it is more likely that these results reflect the inherent inefficiency of the insulin receptor in the phosphorylation of Shc, and stimulation of the subsequent ras-mediated phosphorylation pathways. One possible clue to the relative ineffectiveness of insulin may lie in recent observations that the carboxyl-terminal tail of its receptor may exert a negative influence on mitogenic signaling (36). Indeed, mutation of the two carboxyl-terminal tyrosines resulted in the enhanced sensitivity of mitogenesis to insulin (37). Additionally, the lack of sequence homology between the insulin and IGF-1 receptors in the carboxyl-terminal domain may perhaps account for the relative difference in growth-promoting activities of these two hormones (38). Finally, the pp140^-cr NGF receptor does not contain a carboxyl-terminal domain corresponding to that of the insulin receptor, despite extensive sequence homology between these receptors in the remainder of their intracellular domains (39).

Although these observations provide further evidence that the Shc/Grb2-ras pathway is not universally required for insulin action, they do support a correlation between the tyrosine phosphorylation of Shc, its association with Grb2 and activation of p21ras (23). Interestingly, both NGF and EGF induced the association of several different tyrosine phosphorylated proteins with Grb2, although these complexes appeared to form separately. In addition to the Shc-Grb2 complex, PI-3 kinase activity was detected with the Grb2 protein in growth factor-treated cells, but was not found in anti-Shc immunoprecipitates from NGF-treated cells, further indicating the separate roles of these different SH2 proteins in signal transduction.

Although the phosphorylation of Shc proteins was easily detected in response to NGF and EGF in these cells, there was no observed response to insulin, despite the insulin-dependent tyrosine phosphorylation of its receptor and IRS1, increased glycosgen, lipid and protein synthesis, and the stimulation of PI-3 kinase activity detected in anti-phosphotyrosine (16, 32), anti-IRS1 (16), and GST-Grb2 fusion precipitates. Although the inability of insulin to stimulate Shc phosphorylation or its association with Grb2/ASH in this clonal PC-12 cell line provides further evidence for receptor diversity in signal transduction, the mechanistic basis for the uncoupling of the insulin receptor to this pathway is unclear. Insulin-stimulated phosphorylation of Shc proteins has been observed in fibroblasts (35). However, these cells were transfected with over 10^6 receptors/cell, a concentration at which the specificity of the receptor may be decreased (35). Although it is possible that the PC-12 cells described here express mutant receptors that are differentially coupled to signaling pathways, it is more likely that these results reflect the inherent inefficiency of the insulin receptor in the phosphorylation of Shc, and stimulation of the subsequent ras-mediated phosphorylation pathways. One possible clue to the relative ineffectiveness of insulin may lie in recent observations that the carboxyl-terminal tail of its receptor may exert a negative influence on mitogenic signaling (36). Indeed, mutation of the two carboxyl-terminal tyrosines resulted in the enhanced sensitivity of mitogenesis to insulin (37). Additionally, the lack of sequence homology between the insulin and IGF-1 receptors in the carboxyl-terminal domain may perhaps account for the relative difference in growth-promoting activities of these two hormones (38). Finally, the pp140^-cr NGF receptor does not contain a carboxyl-terminal domain corresponding to that of the insulin receptor, despite extensive sequence homology between these receptors in the remainder of their intracellular domains (39).

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