Insulin Receptor Substrate 2 And Shc Play Different Roles In Insulin-like Growth Factor I Signaling*

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The major substrates for the type I insulin-like growth factor (IGF-I) receptor are Shc and insulin receptor substrate (IRS) proteins. In the current study, we report that IGF-I induces a sustained tyrosine phosphorylation of Shc and its association with Grb2 in SH-SY5Y human neuroblastoma cells. The time course of Shc tyrosine phosphorylation parallels the time course of IGF-I-stimulated activation of extracellular signal-regulated kinase (ERK). Transfection of SH-SY5Y cells with a p52 Shc mutant decreases Shc tyrosine phosphorylation and Shc-Grb2 association. This results in the inhibition of IGF-I-mediated ERK tyrosine phosphorylation and neurite outgrowth. In contrast, IGF-I induces a transient tyrosine phosphorylation of IRS-2 and an association of IRS-2 with Grb2. The time course of IRS-2 tyrosine phosphorylation and IRS-2-Grb2 and IRS-2-p85 association closely resembles the time course of IGF-I-mediated membrane ruffling. Treating cells with the phosphatidylinositol 3'-kinase inhibitor PD98059, as well as transfection with the p52 Shc mutant, has no effect on IGF-I-mediated membrane ruffling. Immunolocalization studies show IRS-2 and Grb2, but not Shc, concentrated at the tip of the extending growth cone where membrane ruffling is most active. Collectively, these results suggest that the association of Shc with Grb2 is essential for IGF-I-mediated neurite outgrowth, whereas the IRS-2-Grb2-phosphatidylinositol 3'-kinase complex may regulate growth cone extension and membrane ruffling.

Binding of insulin-like growth factor I (IGF-I) to the extracellular α-subunits of the type I insulin-like growth factor receptor (IGF-IR) results in autophosphorylation of the cytoplasmic β-subunits (1, 2). This autophosphorylation of the IGF-IR initiates a cascade of cellular signal transduction pathways. One key event is the binding of insulin receptor substrates (IRS) 1 and 2 to phosphoryrosine residues on the receptor β-subunits (3). After binding by activated receptors, IRS-1 and -2 are tyrosine phosphorylated and act as docking proteins for downstream signaling molecules containing Src homology 2 domains, such as the 85-kDa regulatory (p85) subunit of phosphatidylinositol 3'-kinase (PI3K) and the adapter protein Grb2 (reviewed in Refs. 3 and 4). Recent reports suggest IRS proteins play important and distinctive roles in IGF and insulin signaling. For example, IRS-1 is essential for IGF-I-stimulated mitogenesis (5). Mice that are made IRS-1 deficient have retarded growth and reduced glucose metabolism when stimulated by insulin or IGF-I (6, 7). In contrast, IRS-2 is more tightly linked to glucose regulation. Mice that are made IRS-2 deficient have the typical phenotype of type 2 diabetes (8).

Another substrate for activated IGF-IR is Shc, which exists in three isoforms: p66, p52, and p46 (9). Shc is composed of a C terminus Src homology 2 domain, an N terminus phosphotyrosine binding domain, and a central collagen homologous domain (10). Like IRS-1 and -2, Shc proteins are tyrosine phosphorylated upon binding to IGF-IR (11, 12) and can associate with Grb2 (13, 14). Shc has been implicated in the mitogenic signaling of IGF-I (15), the maintenance of cell-cell interactions, and transformation in breast cancer cells (16).

The binding of IRS or Shc proteins to Grb2 induces the Grb2-associated son of sevenless (SOS) protein to activate p21ras by stimulating GDP/GTP exchange (14, 17, 18). Stimulation of p21ras leads to activation of the mitogen-activated protein (MAP) kinase pathway, which plays an important role in cellular differentiation and growth (19, 20). Activation of one of the MAP kinases, the extracellular signal-regulated kinase (ERK), is required for IGF-I-mediated neurite outgrowth in SH-SY5Y human neuroblastoma cells (21).

There is continued controversy surrounding the relative importance of Shc compared with the IRS proteins in activation of the MAP kinase pathway. Sasaoka et al. (22) reported that the majority of p21ras guanine nucleotide exchange activity was present in Shc immunoprecipitates, whereas only negligible amounts were found in IRS-1 immunoprecipitates. Studies with mutant insulin receptors show that insulin can mediate the tyrosine phosphorylation of Shc, the association of Shc and Grb2, and p21ras-GTP formation, all without detectable tyrosine phosphorylation of IRS-1 or enhancement of the interaction between IRS-1 and Grb2 (23). Taken together, these studies suggest that the Shc-Grb2 complex is the key intermediate in downstream signaling of the MAP kinase pathway. In contrast, several reports implicate a central role for IRS proteins in MAP kinase signaling. SOS can be found in IRS-1 immunoprecipitates (17), and the expression of IRS-1 induces cellular transformation with the activation of MAP kinases (24). Furthermore, mutated insulin receptors that tyrosine phosphorylate IRS-1 but not Shc have normal activation of p21ras (25). These reports suggest that the relative roles of Shc and IRS proteins for mediating insulin and IGF-I signaling depend...
Roles of IRS-2 and Shc in IGF-I Signaling

more on the nature of the signaling molecules and the individual cell types (26).

Unlike Shc, which seems to associate only with Grb2, IRS proteins can associate with a variety of other downstream signaling molecules, such as PI3K (3, 4). Among them, PI3K is implicated in insulin- and IGF-I-mediated membrane ruffling. We have demonstrated that IGF-I induces membrane ruffling and growth cone extension in SH-SY5Y cells (27). In parallel, the microinjection of a mutant p85 subunit of PI3K into cells inhibits IRS-1-PI3K association and insulin- and IGF-I-induced membrane ruffling (28). PI3K may mediate growth factor-induced membrane ruffling by acting as an upstream regulator of the small GTPase protein Rac (29–32).

In our laboratory, we are studying the pleiotrophic effects of IGF-I-IGF-IR signaling in the nervous system (21, 27, 33–37). In the current study, we examined the roles of Shc and IRS-2 in IGF-I signaling in human SH-SY5Y neuroblastoma cells. IGF-I induced distinct temporal patterns of Shc and IRS-2 tyrosine phosphorylation. The transfection of a p52 Shc tyrosine mutant, p52 Shc Y239F/Y240F/Y317F, greatly reduced the IGF-I-mediated tyrosine phosphorylation of ERK and subsequent neurite outgrowth. In contrast, IRS-2-PI3K was required for IGF-I-mediated membrane ruffling. Finally, immunoreactive Grb2 and IRS-2 were localized to membrane ruffles, whereas Shc displayed a more ubiquitous staining. Our data suggest that the IGF-I-mediated association of Shc and Grb2 plays an important role in ERK activation and neurite outgrowth, whereas the IGF-I activation of the association of IRS-2 with PI3K is necessary for IGF-I-mediated membrane ruffling and growth cone extension. These studies imply distinct roles for Shc and IRS proteins, the two most prominent downstream mediators of IGF-IR signaling.

EXPERIMENTAL PROCEDURES

Materials—Anti-phosphotyrosine antibodies were purchased from Transduction Laboratories (PY20; Lexington, KY) and Upstate Biochemicals, Inc. (4G10; Lake Placid, NY). Anti-Grb2 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Shc polyclonal antibody was obtained from Transduction Laboratories. Anti-active MAP kinase antibody was obtained from Promega (Madison, WI). Anti-IGF-IR α-subunit antibody (α-IR3) and epidermal growth factor (EGF) were obtained from Oncogene Science (Uniondale, NY). Monoclonal antibody against Myc-tag was used as described previously (21). All experiments were repeated at least twice, and results were selected by growing in media containing 0.2 mg/ml G418. Plasmid maxi kit from Qiagen (Chatsworth, CA). SH-SY5Y cells were considered neurites, as described previously (21).

Shc cDNA was subcloned into the mamma-
chemistry was performed as described previously (27). Polyclonal antibodies against IRS-2, Grb2, or Shc and a monoclonal antibody against IGF-IR (α-IR3) were used to study the cellular distribution of these proteins. Actin filaments were stained by incubating fixed, permeabilized cells with 2 units/ml rhodamine-phalloidin (Molecular Probes, Eugene, OR).

RESULTS

IGF-I Induces Transient Tyrosine Phosphorylation of IRS-2 and Prolonged Tyrosine Phosphorylation of Shc—We recently reported that in SH-SY5Y human neuroblastoma cells, IGF-I induces the tyrosine phosphorylation of several intracellular proteins including IGF-IR, IRS-2, focal adhesion kinase, paxilin, and ERKs 1 and 2 (21, 27, 37). In agreement with our previous studies (37), 10 nM IGF-I induced a rapid tyrosine phosphorylation of IRS-2, which was maintained for less than 30 min (Fig. 1A). Because it has been reported that Shc is another substrate for the IGF-IR and the insulin receptor (1), we investigated the effect of IGF-I on Shc tyrosine phosphorylation. When SH-SY5Y cells were treated with 10 nM IGF-I, anti-Shc antibody immunoprecipitated three tyrosine phosphorylated proteins ranging between 45 and 70 kDa (Fig. 1B). These correspond to the reported mobilities of three isoforms of Shc, p66, p52, and p46. Anti-Shc immunoblotting confirmed that these proteins were Shc (see Fig. 3D). Tyrosine phosphorylation of p52 and p66 Shc was detected as early as 5 min after the addition of IGF-I and, unlike IRS-2 tyrosine phosphorylation, was maintained for at least 2 h. p46 Shc showed constitutive tyrosine phosphorylation even in unstimulated cells, and IGF-I treatment had little effect on the level of tyrosine phosphorylation.

It has been reported that Grb2 can bind to both IRS-1 and Shc (14). To study the association of Grb2 with IRS-2 and Shc, we immunoprecipitated cell lysates with anti-Grb2 antibody and immunoblotted with anti-phosphotyrosine antibodies. In agreement with our previous results (37), we observe the transient association of Grb2 with IRS-2 (Fig. 1C). In the same anti-phosphotyrosine blot, we also detect all three Shc proteins, confirming the association of Grb2 with both Shc and IRS-2. Unlike the association of Grb2 with IRS-2, the Grb2-Shc association showed a prolonged time course similar to that of Shc tyrosine phosphorylation (Fig. 1C). We have reported that IGF-I induces sustained activation of ERK2 (21). The time course of ERK tyrosine phosphorylation more closely resembles the time course of the association of Shc with Grb2 than that of the IRS-2-Grb2 association.

To examine whether prolonged tyrosine phosphorylation of Shc is an intrinsic characteristic of SH-SY5Y cells, we treated the cells with 100 nM EGF. In anti-Shc immunoprecipitates, we detect strong tyrosine phosphorylation of Shc proteins after EGF stimulation (Fig. 1D). Unlike IGF-I stimulation, EGF-induced Shc tyrosine phosphorylation was transient, lasting less than 30 min. We also detect a transient EGF-mediated association between Shc and Grb2 (data not shown). In parallel with EGF-stimulated Shc tyrosine phosphorylation and Shc-Grb2 association, EGF-stimulated ERK2 tyrosine phosphorylation was also transient (Fig. 1E). EGF-stimulated ERK tyrosine phosphorylation was maximal at 5 min and rapidly decreased afterward. Unlike IGF-I (40), EGF did not mediate neurite outgrowth (data not shown).

Collectively, these results suggest that IGF-I induces distinct temporal patterns of Shc and IRS-2 tyrosine phosphorylation and their respective association with Grb2.

Transfection of the p52 Shc Mutant Blocks IGF-I-induced Shc Tyrosine Phosphorylation and Shc-Grb2 Association—Because the above results suggested the involvement of a Shc-Grb2 association in ERK tyrosine phosphorylation, we examined the role of Shc in the activation of the MAP kinase pathway.
pathway. Recent reports have identified three major tyrosine residues important for Shc tyrosine phosphorylation and Shc-Grb2 association (38, 41). We stably transfected the cells with a Myc-tagged p52 Shc construct that has mutations at the three critical tyrosine residues (p52 Shc Y239F/Y240F/Y317F). We detected an increased expression in whole cell lysates of Shc proteins after stable transfection (Fig. 2A). Efficient transfection was confirmed by immunoblotting anti-Shc immunoprecipitates with an anti-Myc antibody. The anti-Myc antibody detected the p52 band from the p52 Shc mutants but not from the cells transfected with vector alone (Fig. 2B).

Next we examined the effects of IGF-I on Shc tyrosine phosphorylation in cells transfected with mutant p52 Shc or vector alone. IGF-I treatment of vector-transfected cells resulted in the tyrosine phosphorylation of Shc proteins (Fig. 2C). In agreement with a previous report (38), transfection with mutant p52 Shc blocked the IGF-I-induced Shc tyrosine phosphorylation of SH-SY5Y cells (Fig. 2C). When the blot was stripped and reprobed with an anti-Shc antibody, the p66, p52, and p46 Shc bands were seen (Fig. 2D). In parallel with Shc tyrosine phosphorylation, IGF-I-mediated association of Shc with Grb2 was also markedly inhibited by transfection with the p52 Shc mutant (Fig. 2E). These results show the efficient transfection of the p52 Shc mutant and suggest that Shc tyrosine phosphorylation is necessary for IGF-I-mediated Shc-Grb2 association.

Transfection of the p52 Shc Mutant Reduces IGF-I-stimulated Tyrosine Phosphorylation of IRS-2—Because several studies have shown that there are distinct as well as overlapping domains of the IGF-IR and insulin receptor for Shc and IRS-1 binding (42–44), we next examined the effects of the Shc mutant on IRS-2 tyrosine phosphorylation. As expected, treatment with IGF-I induced tyrosine phosphorylation of IRS-2 in vector-transfected cells (Fig. 3A). Interestingly, transfection of the cells with the p52 Shc mutant reduced IGF-I-stimulated IRS-2 tyrosine phosphorylation by 40% (measured by densitometry; data not shown). These data suggest that an overexpression of Shc alters the normal binding of IRS-2 to IGF-IR, supporting the contention that these two proteins share IGF-IR binding domains.

We have previously reported that IRS-2 transiently binds to Grb2 and the p85 subunit of PI3K after IGF-I stimulation (37). In agreement with these results, the treatment of vector-transfected cells with IGF-I resulted in the association of IRS-2 with Grb2 and IRS-2 with p85 (Fig. 3, B and C). In parallel with the
transfected SH-SY5Y cells were treated with 10 nM IGF-I for 5 min. The Vector- or p52 Shc mutant-IRS-2 with PI3K and IRS-2 with Grb2. reduced tyrosine phosphorylation of IRS-2, and the association of A photyrosine (anti-Grb2 (C) and anti-p85\textsuperscript{pan} (B) antibodies. Results are representative of at least two different experiments.

**Fig. 3.** Transfection of the p52 Shc mutant inhibits IGF-I-induced tyrosine phosphorylation of IRS-2, and the association of IRS-2 with PI3K and IRS-2 with Grb2. Vector- or p52 Shc mutant-transfected SH-SY5Y cells were treated with 10 nM IGF-I for 5 min. The cell lysates were immunoprecipitated with anti-IRS-2 (A and B) or anti-Grb2 (C) antibodies followed by immunoblotting with anti-phosphotyrosine (A and C) or anti-p85\textsuperscript{pan} (B) antibodies. Results are representative of at least two different experiments.

**Transfection of the p52 Shc Mutant Blocks IGF-I-stimulated ERK Activation and Neurite Outgrowth**—We have reported that IGF-I mediates ERK activation in SH-SY5Y cells (21). Because the association of Shc with Grb2 can lead to activation of the MAP kinase pathway (14, 17, 18), we examined the effects of transfecting the p52 Shc mutant on ERK activation. First, we examined the effects of IGF-I treatment of the p52 Shc mutant-transfected cells by anti-phosphotyrosine immunoblotting of whole cell lysates. When vector-transfected cells were stimulated with 10 nM IGF-I, we detected prominent tyrosine phosphorylation of a 95-kDa protein (Fig. 4A) that we have confirmed as the IGF-IR (21). Transfection of the Shc mutant did not affect IGF-I-stimulated IGF-IR tyrosine phosphorylation (Fig. 4A). IGF-I also induced the tyrosine phosphorylation of a protein that migrates at 66 kDa (Fig. 4A). We have shown that this protein represents ERK2 (21). When the cells were transfected with the p52 Shc mutant, IGF-I-stimulated ERK2 tyrosine phosphorylation was markedly inhibited. We confirmed the decrease in ERK2 activation by reprobing the blot with an antibody specific for activated MAP kinase (Fig. 4B) and by observing a decrease in MAP kinase activity using the MAP kinase assay kit (New England Biolabs, Beverly, MA; data not shown).

Because we have previously demonstrated that ERK2 activation is required for IGF-I-mediated neurite outgrowth (21), we suspected that transfecting the cells with the p52 Shc mutant would reduce neurite outgrowth. In agreement with our previous results (21), treatment with IGF-I stimulated neurite outgrowth in vector-transfected cells (Fig. 4C). When we examined the effect of IGF-I on the p52 Shc mutant-transfected cells, there was a marked decrease in the number of neurite-bearing cells when compared with that of vector-transfected cells. Collectively, these results show that transfecting cells with the p52 Shc mutant blocks IGF-I-mediated ERK activation and subsequent neurite outgrowth.

**IGF-I Induces Membrane Ruffling That Is Blocked by PI3K Inhibitors**—We have shown that short-term IGF-I treatment of SH-SY5Y cells induces membrane ruffling and growth cone extension (27). We began the current study by examining the exact time course of IGF-I-mediated membrane ruffling. For these experiments, SH-SY5Y cells were serum-starved overnight, treated with 10 nM IGF-I, and examined for membrane ruffling with rhodamine-phalloidin staining. IGF-I treatment resulted in the dense staining of actin filaments at the leading membrane edges, representing membrane ruffling (Fig. 5). Membrane ruffling started as early as 2 min after the addition of IGF-I and lasted less than 30 min. This time course closely resembles the time course of IRS-2 tyrosine phosphorylation (see Fig. 1A) and the association of IRS-2 with Grb2 (see Fig. 1C) and p85 (37).

Several studies have suggested the involvement of PI3K in growth factor-mediated membrane ruffling (28, 31). Therefore, we examined the effects of PI3K inhibitors on IGF-I-induced membrane ruffling. Treating cells with 10 nM IGF-I for 5 min induced rapid membrane ruffling that was not present in untreated cells (Fig. 6, A and B). However, when the cells were preincubated with the PI3K inhibitors wortmannin or LY294002, IGF-I-mediated membrane ruffling was undetectable (Fig. 6, C and D). In contrast, an inhibitor of the MAP kinase pathway, PD98059, had no effect on IGF-I-induced membrane ruffling at concentrations of up to 100 \( \mu \text{M} \) (Fig. 6E).

In parallel, IGF-I treatment of cells transfected with the p52 Shc mutant also had no effect on the extensive membrane ruffling seen with IGF-I treatment (Fig. 6F). These results suggest that pathway(s) involving PI3K but not MAP kinase mediate the ability of IGF-I to induce membrane ruffling and growth cone extension.

**Localization of IGF-IR, IRS-2, Shc, and Grb2 in SH-SY5Y Cells**—To help elucidate the roles of Shc and IRS-2 in IGF-I signaling, we also examined the cellular distribution of each signaling molecule in IGF-I-treated SH-SY5Y cells. We found that IGF-IR is evenly distributed over the cells in a punctate pattern, with slightly more staining at the extending growth cone area (Fig. 7A). Grb2 immunoreactivity was present both diffusely over the cell body and at the leading membrane edges, where membrane ruffling is most active (Fig. 7B). IRS-2 immunoreactive protein had a very similar pattern to that of Grb2: diffuse staining throughout the cell body and a more concentrated staining at the ruffling region (Fig. 7C). We also observed diffuse Shc staining throughout the cell body (Fig. 7D). However, unlike the distribution of IRS-2 and Grb2, we could not detect a concentrated staining of Shc at the leading membrane edges. These data suggest unique roles for each protein in IGF-I signaling.

**DISCUSSION**

Our laboratory is interested in the mechanisms underlying the potent neurotrophic effects of IGF-I and IGF-IR (21, 27, 33–35, 37). In the current study, we determined the relative roles of the two major substrates of IGF-IR, Shc and IRS-2, on the neurotrophic effects of IGF-I in SH-SY5Y cells. Because we have previously reported that IGF-I mediates neurite outgrowth (21) and membrane ruffling (27) and growth factor-mediated membrane ruffling (28, 31). Therefore, we examined the effects of PI3K inhibitors on IGF-I-induced membrane ruffling. Treating cells with 10 nM IGF-I for 5 min induced rapid membrane ruffling that was not present in untreated cells (Fig. 6, A and B). However, when the cells were preincubated with the PI3K inhibitors wortmannin or LY294002, IGF-I-mediated membrane ruffling was undetectable (Fig. 6, C and D). In contrast, an inhibitor of the MAP kinase pathway, PD98059, had no effect on IGF-I-induced membrane ruffling at concentrations of up to 100 \( \mu \text{M} \) (Fig. 6E).

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**Localization of IGF-IR, IRS-2, Shc, and Grb2 in SH-SY5Y Cells**—To help elucidate the roles of Shc and IRS-2 in IGF-I signaling, we also examined the cellular distribution of each signaling molecule in IGF-I-treated SH-SY5Y cells. We found that IGF-IR is evenly distributed over the cells in a punctate pattern, with slightly more staining at the extending growth cone area (Fig. 7A). Grb2 immunoreactivity was present both diffusely over the cell body and at the leading membrane edges, where membrane ruffling is most active (Fig. 7B). IRS-2 immunoreactive protein had a very similar pattern to that of Grb2: diffuse staining throughout the cell body and a more concentrated staining at the ruffling region (Fig. 7C). We also observed diffuse Shc staining throughout the cell body (Fig. 7D). However, unlike the distribution of IRS-2 and Grb2, we could not detect a concentrated staining of Shc at the leading membrane edges. These data suggest unique roles for each protein in IGF-I signaling.
sustained ERK activation in the presence of p66 Shc tyrosine phosphorylation (21).

We found that IGF-I treatment of SH-SY5Y cells resulted in a prolonged tyrosine phosphorylation of Shc and a sustained association of Shc with Grb2. In sharp contrast, IGF-I induced a very transient tyrosine phosphorylation of IRS-2 and its association with Grb2, lasting less than 30 min. We have reported that IGF-I mediates a sustained activation of ERK2 in SH-SY5Y cells (21). The temporal pattern of IGF-I-mediated Shc tyrosine phosphorylation and its association with Grb2 in the current study correlates well with ERK2 activation.

Whereas there are conflicting reports about which pathway, Shc-Grb2 or IRS-2-Grb2, is primarily responsible for MAP kinase activation (17, 22–25), our studies suggest that the association of Shc with Grb2 is responsible for the ability of IGF-I to activate the MAP kinase pathway.

To further define the role of Shc in IGF-I signaling, we transfected SH-SY5Y cells with a p52 Shc mutant construct (p52 Shc Y239F/Y240F/Y317F), which cannot be tyrosine phosphorylated and consequently cannot bind to Grb2 (38, 48). Cellular transfection resulted in an increased expression of the p52 and p46 Shc isoforms, with little effect on the actual levels of the p66 isoform. This is an expected result; the p46 and p52 Shc isoforms are derived from alternative translation initiation sites within the same transcript (9), whereas the p66 Shc isoform is a splicing isoform, requiring the N-terminal addition of a second collagen homologous domain (49). The expression of
Roles of IRS-2 and Shc in IGF-I Signaling

mutant p52 Shc effectively blocked the tyrosine phosphorylation of p66 and p52 Shc and the association of Shc with Grb2 in SH-SY5Y cells.

The association of Grb2 with Shc has been implicated in the activation of the MAP kinase pathway (17, 22–25). In agreement with previous studies (41, 48), decreases in IGF-1-mediated Shc tyrosine phosphorylation and its association with Grb2 resulted in a loss of ERK tyrosine phosphorylation. We have reported that ERK activation is necessary for IGF-I-mediated neurite outgrowth in SH-SY5Y cells (21). In the current study, loss of IGF-I-mediated ERK tyrosine phosphorylation in the p52 Shc mutant-transfected cells paralleled the loss of IGF-I’s ability to mediate neurite outgrowth. Our results are in agreement with a previous report showing that the expression of p52 mutant Shc blocked nerve growth factor-induced neurite outgrowth in PC12 cells (48).

Interestingly, expression of the p52 Shc mutant protein also resulted in an approximately 40% decrease in IGF-I-mediated IRS-2 tyrosine phosphorylation and the association of IRS-2 with Grb2 and the p85 subunit of PI3K. It is known that both IRS-1 and Shc interact with the insulin receptor and the IGF-IR through phosphotyrosine binding domains that recognize distinct as well as overlapping sequences (42–44). It is likely that overexpression of the p52 Shc mutant construct prevented the access of endogenous Shc as well as IRS-2 to IGF-IR, causing the observed decreases in the Shc isomers and IRS-2 tyrosine phosphorylation. In agreement with our results, recent reports showed that the expression of wild type or mutant Shc caused a decrease in insulin-stimulated IRS-1 tyrosine phosphorylation (41, 50). However, even with 60% of IRS-2 tyrosine phosphorylation and IRS-2-Grb2 association remaining in the p52 Shc mutant-transfected cells, IGF-I-mediated ERK activation and neurite outgrowth were essentially blocked. These results support previous studies that contend that the association of Shc with Grb2 is the major pathway connecting signaling from either the insulin receptor or IGF-IR to activation of the MAP kinase pathway (22, 23). It is still possible that IRS-2 signaling may participate, although less prominently than Shc, in regulating the MAP kinase pathway. For example, PI3K, which binds to IRS-1 and -2, can modulate the MAP kinase pathway by interacting with p21ras (51–53).

The actin cytoskeleton plays important roles for many neuronal functions, especially the extension and guidance of growth cones (34, 54). In the current study, we observed the reorganization of the actin cytoskeleton with membrane ruffling and growth cone extension in SH-SY5Y cells after IGF-I treatment. IGF-I-mediated membrane ruffling was very transient, lasting less than 30 min. Martin et al. (55) have also shown that insulin induced transient ruffling in Rat-1 fibroblasts. We found that the time course of IGF-I-mediated membrane ruffling parallels the time course of IRS-2 tyrosine phosphorylation and IRS-2 association with Grb2 and the p85 subunit of PI3K (37). This temporal pattern implies the involvement of IRS-2 in IGF-I-mediated membrane ruffling and growth cone extension.

In the current study, the PI3K inhibitors wortmannin and LY294002 blocked IGF-I-mediated membrane ruffling. When considered in the context of the temporal pattern of IGF-IR: IRS-2 signaling in SH-SY5Y cells, these data imply that IGF-I mediates membrane ruffling via the downstream activation of IGF-IR-IRS-2-PI3K. Several reports besides our own data suggest that IRS proteins are the key links between IGF-IR and PI3K activation. For example, PI3K binds to phosphorylated IRS-1 and IRS-2, but not to Shc, after insulin and IGF-I stimulation (5, 56, 57), and the majority of PI3K activity is found to be associated with IRS-1 after insulin treatment (58–60). These interactions between IRS and PI3K are mediated by the p85 regulatory subunit, which results in activation of the p110 catalytic subunit (56, 61). In agreement with our results, Kotani et al. (28) reported that microinjection of a mutant p85 peptide that prevents PI3K binding to IRS-1 blocks insulin-and IGF-I-mediated membrane ruffling. Several recent reports in non-neuronal cells suggest that PI3K mediates growth factor-coupled membrane ruffling via downstream activation of the small GTPase protein Rac (28, 30, 31, 62, 63). Blocking PI3K activity prevents growth factor-stimulated membrane ruffling (31) that can be restored by the overexpression of wild type Rac (64).

We also found that treating SH-SY5Y cells with the ERK kinase inhibitor PD98059 had no effect on IGF-I-stimulated membrane ruffling. Furthermore, IGF-I retained the ability to enhance membrane ruffling in the p52 Shc mutant-transfected cells, a paradigm where IGF-I had clearly lost its ability to activate ERK. Similar to our findings, microinjection of anti-Shc antibody or dominant negative p21ras does not block insulin-stimulated membrane ruffling (55). Collectively, these studies and our results clearly show that signaling via PI3K, not MAP kinase, is primarily responsible for IGF-I-stimulated membrane ruffling.

The cellular distribution of each signaling molecule supported our ideas on the importance of the IRS-2-PI3K signaling cascade for membrane ruffling. We found that IGF-I is distributed evenly throughout the cell surface. This pattern is in agreement with the pleiotrophic effects of IGF-I in these cells (21, 27, 33–35, 37). IRS-2 was concentrated at the leading edge of the extending growth cones. This is the region where membrane ruffling is most active. In contrast, Shc showed even distribution over the cells and lacked concentrated staining at the ruffling region. We also found that Grb2 had a cellular distribution similar to that of IRS-2: a diffuse cellular staining with a more concentrated expression at the leading edges of the growth cones. Similar Grb2 localization in membrane ruffles was recently reported in non-neuronal cells (65). Interestingly, Nimnual et al. (66) have also shown that SOS, which binds to Grb2, can stimulate the guanine nucleotide exchange of Rac as well as p21ras (66), implying a connection between the Grb2-SOS complex and Rac activation. We have also localized Rac and the p85 subunit to membrane ruffles,2 suggesting that the association of IRS-2 with Grb2 may regulate membrane ruffling in neuronal cells by stimulating the guanine nucleotide exchange of Rac.

2 B. Kim, unpublished data.
Our current hypothesis concerning IGF-IR signaling in SH-SY5Y cells is summarized in Fig. 8. Activation of IGF-IR by IGF-1 stimulation causes the association and tyrosine phosphorylation of its major substrates, IRS-2 and Shc. Prolonged tyrosine phosphorylation of Shc and its association with Grb2 are the major signals for the downstream signaling of the MAP kinase pathway, leading to sustained ERK activity. The activation of ERK is necessary for IGF-1-mediated neurite outgrowth. In contrast, IGF-1 induces transient tyrosine phosphorylation of IRS-2 and the transient association of IRS-2 with PI3K and Grb2. IGF-I-mediated IRS-2 tyrosine phosphorylation and IRS-2-Grb2 and IRS-2-PI3K association are responsible for IGF-I-induced membrane ruffling. Our studies imply that distinct signaling cascades underlie the neurotrophic properties of IGF-I.

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