

Differential Regulation of Insulin Receptor Substrate-2 and Mitogen-Activated Protein Kinase Tyrosine Phosphorylation by Phosphatidylinositol 3-Kinase Inhibitors in SH-SY5Y Human Neuroblastoma Cells*

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ABSTRACT

Insulin-like growth factor I (IGF-I) is a potent neurotropic factor promoting the differentiation and survival of neuronal cells. SH-SY5Y human neuroblastoma cells are a well characterized *in vitro* model of nervous system growth. We report here that IGF-I stimulated the tyrosine phosphorylation of the type I IGF receptor (IGF-IR) and insulin receptor substrate-2 (IRS-2) in a time- and concentration-dependent manner. These cells lacked IRS-1. After being tyrosine phosphorylated, IRS-2 associated transiently with downstream signaling molecules, including phosphatidylinositol 3-kinase (PI 3-K) and Grb2. Treatment of the cells with PI 3-K inhibitors (wortmannin and LY294002) increased IGF-I-induced tyrosine phosphorylation of IRS-2. We also observed a concomitant increase in the mobility of

IRS-2, suggesting that PI 3-K mediates or is required for IRS-2 serine/threonine phosphorylation, and that this phosphorylation inhibits IRS-2 tyrosine phosphorylation. Treatment with PI 3-K inhibitors induced an increased association of IRS-2 with Grb2, probably as a result of the increased IRS-2 tyrosine phosphorylation. However, even though the PI 3-K inhibitors enhanced the association of Grb2 with IRS-2, these compounds suppressed IGF-I-induced mitogen-activated protein kinase activation and neurite outgrowth. Together, these results indicate that although PI 3-K participates in a negative regulation of IRS-2 tyrosine phosphorylation, its activity is required for IGF-IR-mediated mitogen-activated protein kinase activation and neurite outgrowth. (*Endocrinology* 139: 4881–4889, 1998)

INSULIN-LIKE growth factor I (IGF-I) and insulin play crucial roles in the regulation of growth and metabolism in many cell types (1). Most of their physiological effects are mediated by the type I IGF receptor (IGF-IR) and insulin receptor (IR), respectively (2, 3). Like the IR, IGF-IR is a heterodimer composed of two extracellular α -subunits linked to 2 transmembrane β -subunits. The β -subunits contain an intracellular tyrosine kinase domain that is activated by ligand binding to the α -subunits and results in autophosphorylation of the β -subunits. This kinase activity is essential for the biological actions of IGF-I and insulin, as cells expressing kinase-deficient receptors fail to mediate the effects of IGF-I or insulin (4, 5).

Autophosphorylation of the IGF-IR or IR initiates a cascade of cellular signal transduction pathways. One key event is the binding of insulin receptor substrate-1 (IRS-1) and IRS-2 to phosphotyrosine residues on the receptor β -subunits (6). Subsequent to binding by activated receptors, IRS-1 and -2 are tyrosine phosphorylated and then act as docking pro-

teins for downstream signaling molecules containing Src homology 2 (SH2) domains, such as the 85-kDa regulatory (p85) subunit of phosphatidylinositol 3-kinase (PI 3-K), and the adaptor proteins Grb2 and SHPTP2/Syp (reviewed in Refs. 6 and 7).

Binding of IRS proteins to Grb2 induces the Grb2-associated son of sevenless protein to activate p21^{ras} by stimulating GDP/GTP exchange (6). Alternatively, the Grb2/son of sevenless protein complex can activate p21^{ras} after binding to Shc, which associates with and is tyrosine phosphorylated by the IR and IGF-IR (8, 9). Stimulation of p21^{ras} leads to activation of the mitogen-activated protein (MAP) kinase pathway, which plays an important role in cellular differentiation and growth (10, 11). Tyrosine-phosphorylated IRS-1 and -2 can also bind to SH2 domains on the p85 subunit of PI 3-K, which causes activation of the p110 catalytic subunit (12). PI 3-K plays a critical role in many biological actions of insulin and IGF-I, including glucose transport, glycogen synthesis, and mitogenesis (13, 14). Several downstream targets of PI 3-K have been identified, including the Rho family of small guanosine triphosphatases, p21^{ras}, protein kinase C, and Akt/PKB (reviewed in Ref. 12).

Even though functional similarities can be suggested based on structural comparisons between IRS-1 and IRS-2, the specific role(s) and regulation(s) of IRS-2 in insulin- and IGF-I-responsive cells are still not clear. Mice made IRS-1 deficient have retarded growth and reduced glucose metabolism when stimulated by insulin or IGF-I (15, 16), suggest-

Received May 19, 1998.

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* This work was supported by NIH Grants R29-NS-32843 and R01-NS-36778, grants from the American Diabetes Association and Juvenile Diabetes Foundation (to E.L.F.), and a grant from the Millie Schembechler Adrenal Research Fund of the University of Michigan Comprehensive Cancer Center (to E.L.F. and P.S.L.).

ing the involvement of IRS-2 in insulin resistance (17). It has also been shown that IRS-1 has an important role in IGF-I-stimulated mitogenesis that cannot be replaced by IRS-2 (18). Recently, Withers and colleagues reported that disruption of IRS-2 impairs peripheral insulin signaling and pancreatic β -cell function in mice, a phenotype that resembles human type 2 diabetes (19). These results suggest unique roles of IRS-1 and IRS-2 in the signaling of insulin and IGF-I.

In our laboratory we are studying the IGF-IR signaling pathways involved in stimulation of neurite outgrowth in SH-SY5Y human neuroblastoma cells (20–24). In the current studies we focused on the role of the IRS-PI 3-K interaction in IGF-IR signaling. We found that SH-SY5Y cells lack IRS-1, but use IRS-2 as the main substrate for the IGF-IR. After IGF-I stimulation, IRS-2 is tyrosine phosphorylated and associates with Grb2 and p85. Interestingly, PI 3-K inhibitors, LY294002 and wortmannin, enhance the tyrosine phosphorylation of IRS-2, possibly by reducing its serine/threonine phosphorylation. Finally, the PI 3-K inhibitors reduced MAP kinase activation and neurite outgrowth. These results indicate that PI 3-K participates in a negative regulation of IRS-2 tyrosine phosphorylation, but at the same time its activity is required for IGF-IR-mediated MAP kinase activation and neurite outgrowth.

Materials and Methods

Materials

Antiphosphotyrosine antibodies were purchased from Transduction Laboratories, Inc. (PY20; Lexington, KY), and Upstate Biochemicals, Inc. (4G10; Lake Placid, NY). Anti-IGF-IR β -subunit and anti-Grb2 polyclonal antibodies, horseradish peroxidase-conjugated goat antimouse and antirabbit IgGs, and agarose-conjugated protein A/G-Plus were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Shc polyclonal antibody was obtained from Transduction Laboratories. IGF-IR-neutralizing antibody (α -IR3) was obtained from Oncogene Science, Inc. (Uniondale, NY). Anti-IRS-1 antiserum for immunoprecipitation (JD145), anti-IRS-2 antisera for immunoblotting (JD101) and immunoprecipitation (JD110), and, anti-p85^{pan} antiserum (JD137) were gifts from Dr. M. F. White (Joslin Diabetes Center, Harvard Medical School, Boston, MA). Anti-IRS-1 antiserum for immunoblotting was provided by Dr. A. Saliel of Parke-Davis Pharmaceutical Research (Ann Arbor, MI). Enhanced chemiluminescence reagents were obtained from Amersham Corp. (Arlington Heights, IL). LY294002 and wortmannin were purchased from Biomol (Plymouth Meeting, PA). IGF-I was a gift from Cephalon Corp. (West Chester, PA). DMEM with high glucose, L-glutamine, and sodium pyruvate was obtained from Life Technologies (Grand Island, NY). Other reagents were purchased from Sigma Chemical Co., Inc. (St. Louis, MO), or Boehringer Mannheim (Indianapolis, IN).

Cell culture

SH-SY5Y human neuroblastoma cells and 3T3-F442A cells (a gift from Dr. C. Carter-Sue, University of Michigan, Ann Arbor, MI) were grown in DMEM containing 10% calf serum and maintained at 37 C in a humidified atmosphere with 10% CO₂. Eighteen to 24 h before experiments, medium was replaced with DMEM without serum. For neurite outgrowth experiments, serum-starved cells were incubated in serum-free medium for 24 h with or without IGF-I. For the experiments using LY294002, cells were treated with the inhibitor 1 h before the addition of IGF-I. Processes longer than the cell body were considered neurites.

Immunoprecipitation and immunoblotting

Serum-starved cells were treated as indicated and harvested in lysis buffer [20 mM Tris (pH 7.2), 0.16 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.1 trypsin inhibitory

units of aprotinin/ml, 10 μ g/ml leupeptin, and 1 mM Na₃VO₄]. Equal amounts of cellular lysates (assessed by protein assay) were mixed overnight with appropriate dilutions of primary antibodies followed by incubation with agarose-conjugated protein A/G-Plus. After washing in lysis buffer, the resulting immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH), and immunoblotted with a primary antibody. Immunoreactive proteins were identified by horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence reagents. In some experiments, the nitrocellulose membranes were stripped by incubation with stripping solution (2% SDS, 0.1 M dithiothreitol, and 0.1 M Tris, pH 6.8) whereupon they were used for immunoblotting with another antibody. All experiments were repeated at least twice, and typical representative results are shown in the figures.

Assay of extracellular signal-regulated protein kinase (ERK) activity

The kinase activity of the ERKs was measured using the MAPK assay kit (New England Biolab, Beverly, MA) according to the manufacturer's protocol. Briefly, the ERKs were isolated by immunoprecipitation using the antibody specific to the phosphorylated MAP kinase. Kinase activity was assessed using an Elk-1 fusion protein as a protein substrate. The phosphorylation of Elk-1 was analyzed using an antibody that specifically recognizes phosphorylated Elk-1.

Results

IRS-2, but not IRS-1, is expressed in SH-SY5Y human neuroblastoma cells and is tyrosine phosphorylated in response to IGF-I

We recently reported that in SH-SY5Y human neuroblastoma cells, IGF-I induces the tyrosine phosphorylation of several intracellular proteins, including focal adhesion kinase, paxillin, and ERK-1 and -2 (21, 23). During these studies, we observed a protein with an electrophoretic mobility of roughly 200 kDa that is rapidly tyrosine phosphorylated in response to IGF-I. Therefore, we investigated whether this protein is IRS-1 or IRS-2. 3T3-F442A fibroblast cells that express both IRS-1 and IRS-2 (25) were used as a control.

When 3T3-F442A cells were treated with 10 nM IGF-I for 5 min, the anti-IRS-1 antibody immunoprecipitated a tyrosine-phosphorylated protein with an electrophoretic mobility of approximately 180 kDa (Fig. 1A, upper left panel). However, this protein was not detected in cell lysates from either control or IGF-I-treated SH-SY5Y cells. We confirmed that the fibroblast protein was IRS-1 by stripping and reprobing the blot with an anti-IRS-1 antibody (Fig. 1A, lower left panel). In contrast, an anti-IRS-2 antibody immunoprecipitated a protein with slightly slower mobility (Fig. 1A, upper right panel), which was tyrosine phosphorylated in response to IGF-I in both SH-SY5Y and 3T3-F442A cells. This protein was confirmed as IRS-2 by immunoblotting with the anti-IRS-2 antibody (Fig. 1A, lower right panel). These results show that SH-SY5Y cells do not express IRS-1 but do express IRS-2, which is tyrosine phosphorylated in response to IGF-I. We also observed little IRS-1 expression using RT-PCR of total SH-SY5Y RNA (data not shown).

The stimulation of IRS-2 tyrosine phosphorylation by IGF-I was concentration dependent (Fig. 2A, lower panel) and paralleled the tyrosine phosphorylation of IGF-IR (Fig. 2A, upper panel). Maximum tyrosine phosphorylation was observed at 10 nM IGF-I for both IGF-IR and IRS-2. Furthermore, as previously demonstrated (21), 10 nM IGF-I induced

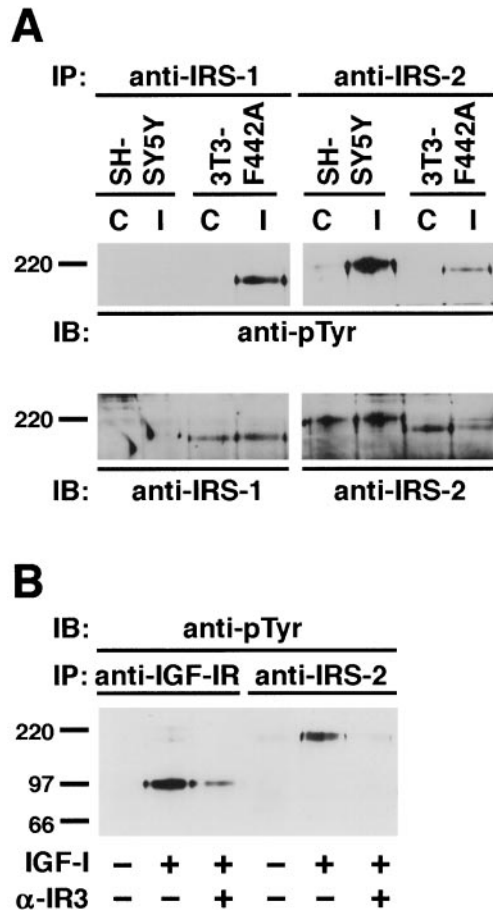


FIG. 1. SH-SY5Y cells express IRS-2, which requires IGF-IR for its tyrosine phosphorylation. A, SH-SY5Y cells and 3T3-F442A cells were serum starved overnight and either left untreated (C) or stimulated with 10 nM IGF-I for 5 min (I). Cell lysates were prepared as described in *Materials and Methods*. Equal amounts of cell lysates were immunoprecipitated with polyclonal antibodies against IRS-1 (JD145) or IRS-2 (JD110). The resulting immunoprecipitates were separated by 12.5% SDS-PAGE, transferred to nitrocellulose paper, and analyzed by antiphosphotyrosine immunoblotting (*upper panels*). The blots were stripped and reprobed for IRS-1 or IRS-2 (*lower panels*). B, Serum-starved SH-SY5Y cells were incubated for 1 h without or with 1 μ g/ml α -IR3 before a 5-min incubation with 10 nM IGF-I. Equal amounts of cell lysates were immunoprecipitated with polyclonal antibodies against IGF-IR β -subunit or IRS-2 before analysis by SDS-PAGE antiphosphotyrosine immunoblotting. IP, Immunoprecipitation; IB, immunoblotting; pTyr, phosphotyrosine. The positions of the molecular mass standards (in kDa) are shown at the *left side* of the blots. Results are representative of two independent experiments.

rapid tyrosine phosphorylation of IGF-IR, which was maintained for at least 2 h (Fig. 2A, *lower panel*). Similarly, IGF-I induced the rapid tyrosine phosphorylation of IRS-2 (Fig. 2A, *upper panel*). However, in contrast to IGF-IR tyrosine phosphorylation, the tyrosine phosphorylation of IRS-2 rapidly declined after 5 min.

In addition to IGF-IR, IGF-I can bind to IGF-binding proteins (IGFBPs) (26). We previously showed that SH-SY5Y cells express IGFBP-2, -3, -4, and -5 (27). To determine whether the effect of IGF-I on IRS-2 tyrosine phosphorylation was mediated through IGF-IR, we examined the effect of an IGF-IR neutralizing antibody, α -IR3. When SH-SY5Y cells

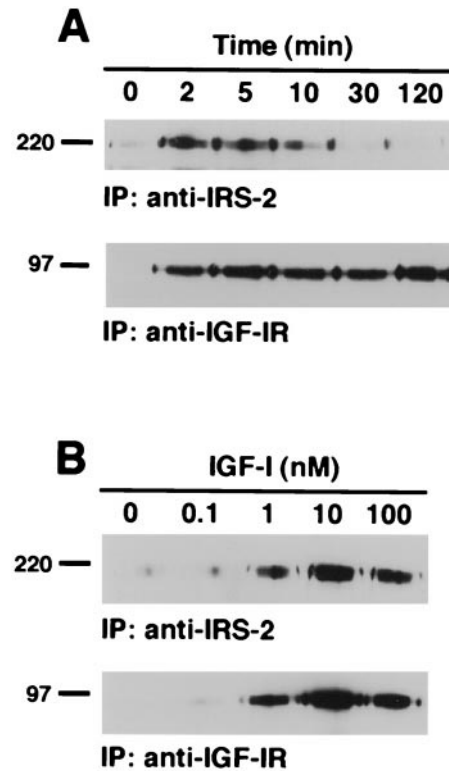


FIG. 2. Time and concentration dependence of IGF-I-stimulated IGF-IR and IRS-2 tyrosine phosphorylation. Serum-starved SH-SY5Y cells were treated with either 10 nM IGF-I (A) for the indicated times or increasing concentrations of IGF-I for 5 min (B). Equal amounts of cell lysates were immunoprecipitated with anti-IGF-IR β -subunit or anti-IRS-2 antibodies before analysis by SDS-PAGE antiphosphotyrosine immunoblotting. Results are representative of two independent experiments.

were incubated with 1 μ g/ml α -IR3 for 1 h, there was a substantial reduction in IGF-I-stimulated IGF-IR tyrosine phosphorylation (Fig. 1B, *first three lanes*). Addition of α -IR3 also caused a corresponding decrease in IRS-2 tyrosine phosphorylation (Fig. 1B, *last three lanes*). These results demonstrate that IGF-I promotion of IRS-2 tyrosine phosphorylation occurs via the IGF-IR.

IRS-2 transiently associates with p85 and Grb2 after stimulation by IGF-I

When tyrosine phosphorylated, IRS-1 and -2 bind to downstream signaling molecules containing SH2 domains, such as the p85 subunit of PI 3-K, Grb2, and SHPTP2/Syp (6, 7). Therefore, we investigated the association of IRS-2 with SH2 domain-containing proteins by coimmunoprecipitation. Figure 3A shows that the total level of immunodetectable p85 and Grb2 in SH-SY5Y cells did not change during the course of the experiments (Fig. 3A). The doublets in the anti-p85^{pan} immunoblot may represent p85 α (*lower band*) and p85 β (*upper band*) isoforms (28). When the cell lysates were immunoprecipitated with an anti-IRS-2 antibody, IGF-I stimulated the association of p85 and Grb2 with IRS-2 (Fig. 3A). Like IRS-2 tyrosine phosphorylation, the association of IRS-2 with these proteins was transient, decreasing 5 min after IGF-I treatment.

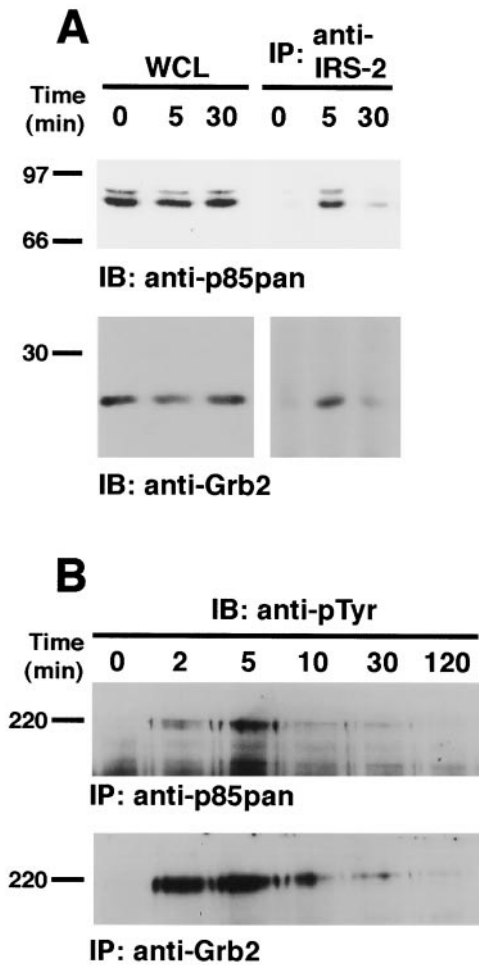


FIG. 3. IRS-2 transiently binds with PI 3-K and Grb2 after stimulation by IGF-I. Serum-starved SH-SY5Y cells were treated with 10 nM IGF-I for the indicated times. A, Whole cell lysates (WCL) or anti-IRS-2 immunoprecipitates were separated by 12.5% SDS-PAGE and immunoblotted with anti-p85^{pan} or anti-Grb2 antibodies. B, The cell lysates were immunoprecipitated with anti-p85^{pan} or anti-Grb2 antibodies before analysis by SDS-PAGE antiphosphotyrosine immunoblotting. Results are representative of three independent experiments.

To further confirm the association of IRS-2 with p85 and Grb2, we examined the ability of anti-p85^{pan} and anti-Grb2 antibodies to immunoprecipitate IRS-2. As expected, in the absence of IGF-I, anti-p85^{pan} or anti-Grb2 antibodies did not immunoprecipitate IRS-2 protein (Fig. 3B, 0 min). However, IGF-I induced the coimmunoprecipitation of an approximately 200-kDa tyrosine-phosphorylated protein with p85 and Grb2. This 200-kDa tyrosine-phosphorylated protein represents IRS-2 (Figs. 3B and 5A). Similar to the results in Fig. 3A, the association of p85 and Grb2 with IRS-2 was transient, rapidly decreasing 5 min after IGF-I treatment.

Inhibitors of PI 3-K enhance the tyrosine phosphorylation and electrophoretic mobility of IRS-2

In Fig. 2A we noticed a rapid decrease in IRS-2 tyrosine phosphorylation after 5 min even though IGF-IR tyrosine phosphorylation was maintained for at least 2 h. We were particularly interested in the mechanism(s) of IRS-2 dephos-

phorylation. It has been shown that elevated serine phosphorylation can block insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2 (29, 30) and that PI 3-K may phosphorylate IRS-1 on serine (31, 32). To further study the involvement of PI 3-K on IGF-I-mediated IRS-2 phosphorylation, we incubated the cells for 1 h with the PI 3-K inhibitor, LY294002 (50 μ M), before treatment of the cells with IGF-I (10 nM; 5 min). As expected, in the absence of the PI 3-K inhibitor, IGF-I induced transient tyrosine phosphorylation of IRS-2 (Fig. 4A, upper panels). Interestingly, treatment with LY294002 resulted in an increased and sustained tyrosine phosphorylation of IRS-2. Additionally, when the blots were stripped and reprobed with an anti-IRS-2 antibody, the lysates from the cells treated without LY294002 showed a broad band of IRS-2 after IGF-I treatment. However, treatment with LY294002 resulted in a tighter IRS-2 band with increased electrophoretic mobility (Fig. 4A, lower panels). Wortmannin, another inhibitor of PI 3-K, had a similar effect on the tyrosine phosphorylation and the electrophoretic mobility of IRS-2 (data not shown). This change in IRS-2 from a diffuse smear to a more focused band with higher mobility suggests that the PI 3-K inhibitors reduced the serine/threonine phosphorylation of IRS-2 (33). In parallel, treatment with okadaic acid, an inhibitor of serine/threonine phosphatases type 1 and type 2A, produced a concentration-dependent decrease in IGF-I-induced IRS-2 tyrosine phosphorylation (data not shown). Collectively, these results suggest that serine/threonine phosphorylation inhibits IRS-2 tyrosine phosphorylation.

This enhancement of IGF-I-stimulated IRS-2 tyrosine phosphorylation by LY294002 was concentration dependent (Fig. 4B, upper panel). The electrophoretic mobility of IRS-2 was also enhanced with increasing concentrations of LY294002 (Fig. 4B, lower panel). Finally, LY294002 had no effect on the tyrosine phosphorylation of IGF-IR (Fig. 4C). These results suggest that PI 3-K or a PI 3-K-mediated pathway can inhibit the tyrosine phosphorylation of IRS-2, possibly by promoting its serine/threonine phosphorylation.

Effects of PI 3-K inhibitors on the association of Grb2 with IRS-2 and Shc

In Fig. 3, we showed that IRS-2 associates with Grb2 as well as the p85 subunit of PI 3-K. Grb2 is one of the signaling components that mediate activation of the MAP kinase pathway (34, 35). As PI 3-K inhibitors enhanced the tyrosine phosphorylation of IRS-2, we next studied the effect of the inhibitors on the association of IRS-2 with Grb2. As predicted, Grb2 coimmunoprecipitated IRS-2 after IGF-I treatment (Fig. 5A). Treatment of the cells with LY294002 or wortmannin resulted in a concentration-dependent increase in the association of Grb2 with IRS-2. In the absence of IGF-I, the PI 3-K inhibitors had no effect on the association of Grb2 with IRS-2 (data not shown).

Shc is another substrate for the IGF-IR and the IR and also associates with Grb2 after being tyrosine phosphorylated (8, 9). However, unlike the association of IRS-2 with Grb2, PI 3-K inhibitors had no effect on the tyrosine phosphorylation of Shc or on the Shc-Grb2 association (Fig. 5, B and C).

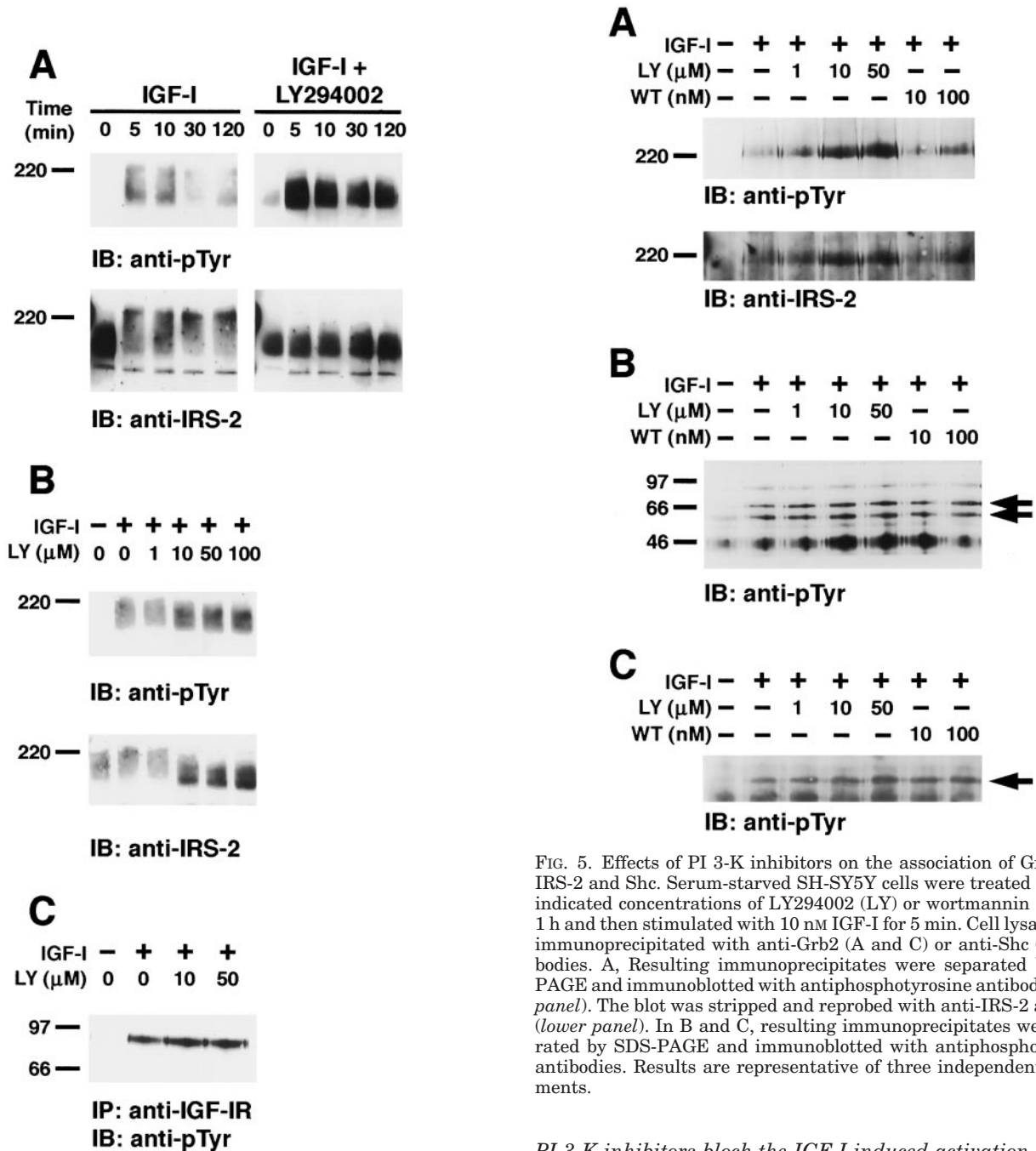


FIG. 4. LY294002 enhances IGF-I-induced tyrosine phosphorylation and electrophoretic mobility of IRS-2. A, Serum-starved SH-SY5Y cells were treated for 1 h without (*left panels*) or with (*right panels*) 50 μ M LY294002, and then incubated with 10 nM IGF-I for the indicated times in the continuous presence of LY294002. B, Cells were treated with the indicated concentrations of LY294002 (LY) for 1 h and then stimulated with 10 nM IGF-I for 5 min. Anti-IRS-2 immunoprecipitates were separated by 7.5% SDS-PAGE and immunoblotted with antiphosphotyrosine antibodies (*upper panels*). The blots were then stripped and reprobbed with an anti-IRS-2 antibody (*lower panels*). C, Cells were treated as described in B and immunoprecipitated with an anti-IGF-IR antibody before analysis by SDS-PAGE antiphosphotyrosine immunoblotting. Results are representative of two independent experiments.

FIG. 5. Effects of PI 3-K inhibitors on the association of Grb2 with IRS-2 and Shc. Serum-starved SH-SY5Y cells were treated with the indicated concentrations of LY294002 (LY) or wortmannin (WT) for 1 h and then stimulated with 10 nM IGF-I for 5 min. Cell lysates were immunoprecipitated with anti-Grb2 (A and C) or anti-Shc (B) antibodies. A, Resulting immunoprecipitates were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibody (*upper panel*). The blot was stripped and reprobbed with anti-IRS-2 antibody (*lower panel*). In B and C, resulting immunoprecipitates were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibodies. Results are representative of three independent experiments.

PI 3-K inhibitors block the IGF-I-induced activation of ERK2 and neurite outgrowth

We have previously shown that IGF-I induces MAP kinase (especially ERK2) activation and that this pathway is required for neurite outgrowth (21). As our results suggest that PI 3-K may be involved in the regulation of signaling components upstream of MAP kinase (*i.e.* Grb2-IRS-2 association), we examined the effect of PI 3-K inhibitors on IGF-I-induced ERK activation. In agreement with our previous study (21), treatment with 10 nM IGF-I for 30 min induced ERK2 tyrosine phosphorylation (Fig. 6A). Surprisingly, LY294002 and wortmannin caused a concentration-dependent inhibition of ERK2 tyrosine phosphorylation. Figure 6B shows that LY294002 and wortmannin also cause a concen-

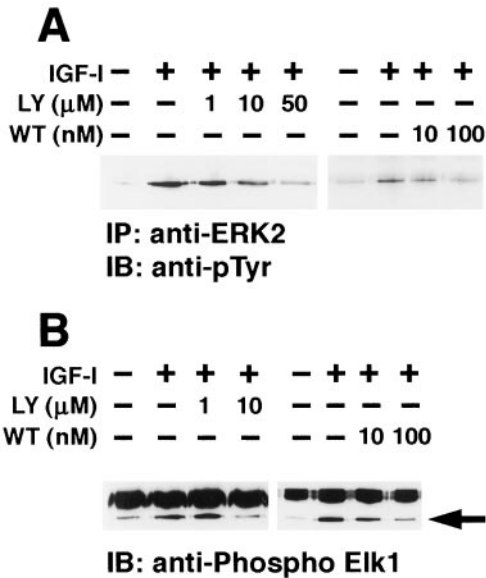


FIG. 6. LY294002 and wortmannin inhibit IGF-I-stimulated ERK tyrosine phosphorylation and activation. Cells were treated as described in Fig. 5. A, Cell lysates were immunoprecipitated with anti-ERK2 antibody and immunoblotted with antiphosphotyrosine antibodies. B, Equal amount of cell lysates were immunoprecipitated with an antibody specific to phosphorylated MAP kinase. Immunoprecipitated MAP kinase was incubated with 1 μ g Elk-1 fusion protein in kinase buffer [25 mM Tris (pH 7.5), 5 mM β -glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na_2VO_4 , and 10 mM MgCl_2] in the presence of 100 μ M ATP. The mixture was subjected to SDS-PAGE and analyzed by immunoblotting using antibody specific to phosphorylated Elk-1.

tration-dependent inhibition of ERK2-mediated phosphorylation of Elk-1. The concentration dependence of LY294002 and wortmannin inhibition of Elk-1 phosphorylation paralleled that of ERK2 tyrosine phosphorylation (Fig. 6A). These results demonstrate that PI 3-K inhibitors prevent the IGF-IR-mediated activation of ERK2 phosphorylation and kinase activation. This contrasts directly with the concomitant enhancement of IRS-2 tyrosine phosphorylation and IRS-2-Grb2 association observed in Figs. 4 and 5A.

Finally, because we previously demonstrated that ERK2 activation is required for IGF-I-induced neurite outgrowth (21), we suspected that the PI 3-K inhibitors would reduce neurite outgrowth. As wortmannin is unstable in aqueous medium (36), we assessed the effect of LY294002 only. As expected, treatment of serum-starved cells with IGF-I resulted in the promotion of neurite outgrowth (Fig. 7, A and B). When LY294002 was added along with IGF-I, there was a concentration-dependent decrease in the number of neurite-bearing cells. However, LY294002 had no effect on basal neurite outgrowth (*i.e.* in serum-free medium alone). Together, these results show that PI 3-K inhibitors produce a parallel reduction in ERK activation and neurite outgrowth, even though they enhance IRS-2 tyrosine phosphorylation and IRS-2-Grb2 association.

Discussion

Our laboratory is interested in determining the mechanisms by which IGF-I acts as a neurotropic factor (20–24). We recently reported that activation of the MAP kinase pathway

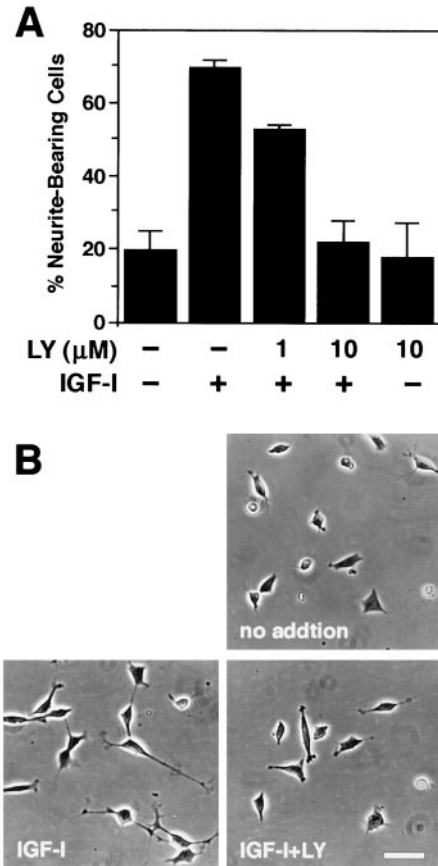


FIG. 7. LY294002 blocks IGF-I-induced neurite outgrowth. A, Serum-starved SH-SY5Y cells were treated with 10 nM IGF-I for 24 h in the presence of 0–10 μ M LY294002. Control cells were treated with 0.1% dimethylsulfoxide alone. After 24 h, the percentage of neurite-bearing cells was scored. Results are the mean \pm SEM of at least two separate observations. $P < 0.01$ (by independent Student's *t* test) compared with the cells treated with 10 nM IGF-I only. There was no statistical difference between control cells and cells treated with LY294002 alone. B, Phase contrast images of the cells from the experiment in A treated with 10 nM IGF-I in the absence or presence of 10 μ M LY294002. Bar = 50 μ m.

is essential for IGF-I-stimulated neuronal differentiation (21). IGF-I and insulin activate not only MAP kinase but also PI 3-K (2). In this report we studied the role of PI 3-K in the signaling of IGF-I in SH-SY5Y human neuroblastoma cells. For these studies we employed two distinct and well characterized inhibitors of PI 3-K, LY294002 and wortmannin (37, 38).

We found that SH-SY5Y human neuroblastoma cells lack IRS-1 and use IRS-2 as a main substrate for the IGF-IR. Recently, Welham *et al.* (39) reported that murine lymphohemopoietic cells also use IRS-2 in the absence of IRS-1. IRS-2 from both cell types binds to Grb2 as well as to the p85 subunit of PI 3-K after stimulation, suggesting that IRS-2 tyrosine phosphorylation can mediate IGF-I or IR signaling. Although IRS-1 and IRS-2 share common structural units, recent studies have shown that they may activate some unique signaling events. For example, the fact that an IRS-1 knock-out mouse showed growth retardation and reduced glucose metabolism suggests that IRS-2 cannot entirely sub-

stitute for the function of IRS-1 (15, 16). Also, using 3T3 cell lines derived from IRS-1-deficient mice, Bruning *et al.* showed that IRS-2 can substitute for IRS-1 in the activation of PI 3-K and immediate early gene induction, but that IRS-2 is not functionally interchangeable with IRS-1 for the stimulation of mitogenesis (18). Our studies show that the SH-SY5Y cells provide an additional *in vitro* model system for studying the function and regulation of IRS-2 in the absence of IRS-1.

When we examined the tyrosine phosphorylation of IGF-IR and IRS-2, we observed that both were rapidly induced by IGF-I treatment. However, in contrast to that of IGF-IR, the level of IRS-2 tyrosine phosphorylation quickly declined even in the presence of continuous IGF-IR tyrosine phosphorylation. Treatment of the cells with PI 3-K inhibitors prevented the rapid dephosphorylation of IRS-2 in a dose-dependent manner. These results as well as those of others (40, 41) indicate that PI 3-K or downstream effectors of PI 3-K regulate IRS-2 tyrosine phosphorylation.

Our data suggest that PI 3-K regulates IGF-I-mediated IRS-2 tyrosine phosphorylation by effecting serine/threonine phosphorylation. PI 3-K inhibitors caused a concomitant increase in the mobility of IRS-2 in SDS-PAGE, suggesting that these compounds prevent the serine/threonine phosphorylation of IRS-2. In agreement with our results, several previous reports confirm that serine/threonine phosphorylation of IRS proteins inhibits insulin signaling. For example, Mothe and van Obberghen (42) showed that several serine residues in a potential MAP kinase phosphorylation site are important for the modulation of IRS-1 tyrosine phosphorylation and IRS-1-p85 association. IRS-1 serine/threonine phosphorylation may also mediate the desensitization of insulin signaling by stimulating the subcellular redistribution of IRS-1 (43) or by sensitizing IRS-1 to the action of proteases (44). Furthermore, recent reports demonstrate that elevated serine/threonine phosphorylation prevents IRS-1 and IRS-2 from binding to the juxtamembrane region of IR and thereby blocks subsequent tyrosine phosphorylation by IR (29). Tumor necrosis factor- α (TNF- α) also inhibits IRS-1 tyrosine phosphorylation by elevating its serine/threonine phosphorylation (45). As TNF- α is thought to be involved in obesity-induced insulin resistance, this TNF- α enhanced IRS-1 serine/threonine phosphorylation may play a role in insulin resistance (46, 47). These studies have mostly focused on insulin-dependent phosphorylation of IRS-1, and little is known about IRS-2 regulation and IGF-IR signaling. Our studies suggest that the serine/threonine phosphorylation status of IRS-2 similarly affects IGF-IR signaling. Likewise, PI 3-K appears to be a key regulator of IRS-2 phosphorylation by increasing IRS-2 serine/threonine phosphorylation and inhibiting IRS-2 tyrosine phosphorylation. This may represent a negative feedback mechanism during the early steps of IGF-IR signaling. Candidate IRS-2 serine/threonine kinases include PI 3-K itself (31, 32) as well as protein kinases that are dependent on PI 3-K activity, such as c-Akt and possibly atypical protein kinase C isoforms (12).

We also found that in conjunction with an enhanced tyrosine phosphorylation of IRS-2, PI 3-K inhibitors increased IRS-2-Grb2 binding. As IRS-1-Grb2 association can mediate insulin-induced MAP kinase stimulation (14, 48), we inves-

tigated the effect of PI 3-K inhibitors on ERK2 activation. Interestingly, even in the presence of an increased IRS-2-Grb2 association, tyrosine phosphorylation and activity of ERK2 were inhibited by LY294002 and wortmannin. However, these inhibitors had no effect on the tyrosine phosphorylation of Shc and the formation of the Shc-Grb2 complex, which can also mediate activation of the MAP kinase pathway (14). These results suggest that PI 3-K regulates ERK activation by a mechanism distinct from the regulation of IRS-2 phosphorylation and IRS-2-Grb2 association. The point at which PI 3-K may regulate the MAP kinase pathway is unclear; some studies suggest that PI 3-K modulates the MAP kinase pathway upstream of p21^{ras} (49), whereas other results indicate regulation downstream of p21^{ras} (50). The role of PI 3-K in insulin-stimulated MAP kinase activation is also controversial; inhibition of PI 3-K by wortmannin blocked insulin-induced MAP kinase activation in one study (51) but not in another (52). Furthermore, recent studies showed that activation of MAP kinase by PI 3-K may depend on the ligand and cell type (53, 54). Our results clearly show that in SH-SY5Y human neuroblastoma cells, PI 3-K inhibitors effectively block IGF-I-induced ERK activation. We suggest that PI 3-K mediates this effect through a signaling component downstream of IRS-2 phosphorylation and IRS-2-Grb2 association.

We previously demonstrated that ERK activation is required for IGF-I-induced neurite outgrowth in SH-SY5Y cells (21). Because PI 3-K inhibitors blocked IGF-I-stimulated ERK2 phosphorylation, we studied the effect of PI 3-K inhibitors on neurite outgrowth. Here we report that LY294002 inhibits IGF-I-mediated neurite outgrowth. The effect of LY294002 on neurite outgrowth paralleled the inhibition of ERK2 tyrosine phosphorylation and activation. These results are consistent with the idea that a reduction in ERK activity participates in the prevention of neurite outgrowth by PI 3-K inhibitors. Also, our results are in agreement with previous reports that showed that PI 3-K is required for the initiation, elongation, and maintenance of nerve growth factor-stimulated neurite outgrowth in PC12 cells (36, 55). Therefore, it is possible that PI 3-K activation is a common element in neuronal differentiation.

The fact that two distinct PI 3-K inhibitors cause these same effects on IRS-2 tyrosine phosphorylation and MAP kinase activation strongly implicates PI 3-K in both responses. That these actions were all mediated by PI 3-K is further supported by the close similarity in inhibitor sensitivities. There are currently five known isoforms of the PI 3-K catalytic subunit and five of the regulatory subunit (12), so it is possible that distinct isoforms are involved in the regulation of IRS-2 phosphorylation and MAP kinase activation. However, regardless of the precise mechanism of action of these inhibitors, these results show that IRS-2-Grb2 association is not the only determinant in MAP kinase regulation and that there are additional critical regulatory steps in the MAP kinase pathway. Clearly, further studies are needed to determine the nature of MAP kinase regulation by PI 3-K and PI 3-K inhibitors.

In summary, we show that SH-SY5Y human neuroblastoma cells do not express IRS-1. In these cells IGF-I induces time- and concentration-dependent tyrosine phosphoryla-

tion of IGF-IR and IRS-2. After being tyrosine phosphorylated, IRS-2 binds to downstream signaling molecules, including Grb2 and PI 3-K. Our results also suggest that PI 3-K inhibits the tyrosine phosphorylation of IRS-2 by promoting its serine/threonine phosphorylation and thus participates in a negative feedback loop. At the same time, PI 3-K is necessary for IGF-I-induced tyrosine phosphorylation and activation of ERK2. Finally, our data support a role for PI 3-K-dependent activation of the MAP kinase pathway in IGF-I-stimulated neurite outgrowth. Collectively, these results imply that PI 3-K plays both negative and positive roles in IGF-IR signaling.

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