Insulin-like growth factor-I (IGF-I) induces neuronal differentiation in vitro. In the present study, we examined the signaling pathway underlying IGF-I-mediated neurite outgrowth. In SH-SY5Y human neuroblastoma cells, treatment with IGF-I induced concentration- and time-dependent tyrosine phosphorylation of the type I IGF receptor (IGF-IR) and extracellular signal-regulated protein kinases (ERK) 1 and 2. These effects of IGF-I were blocked by a neutralizing antibody against IGF-IR. Whereas IGF-IR phosphorylation was observed within 1 min, maximal phosphorylation of ERKs was not reached for 30 min. Both IGF-IR and ERK phosphorylation were maintained for at least 24 h. Also, the concentration dependence of IGF-I-stimulated IGF-IR and ERK tyrosine phosphorylation paralleled that of IGF-I-mediated neurite outgrowth. We further examined the role of mitogen-activated protein kinase activation in IGF-I-stimulated neuronal differentiation using the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (ERK) kinase inhibitor PD98059. Whereas PD98059 had no effect on IGF-IR phosphorylation, PD98059 reduced IGF-I-mediated ERK tyrosine phosphorylation and ERK phosphorylation of the substrate Elk-1. PD98059 also produced a parallel reduction of IGF-I-stimulated neurite outgrowth. Finally, consistent with its ability to block neuronal differentiation, PD98059 inhibited IGF-I-dependent changes of GAP-43 and c-myc gene expression. Together these results suggest that activation of ERKs is essential for IGF-I-stimulated neuronal differentiation.

Insulin-like growth factor-I (IGF-I) is a polypeptide hormone that is structurally similar to insulin and IGF-II. IGFs are mitogens for many cell types playing key roles in cell cycle progression, cell proliferation, and tumor progression (1, 2). In the nervous system, IGFs are important for embryonic and early postnatal growth and development (3). They can also act as differentiating agents promoting neurite outgrowth (4), synapse formation, and myelin synthesis (5). Most of the effects of IGF-I are mediated by binding to its specific receptor, the type I IGF receptor (IGF-IR). Like its ligand, IGF-IR is also closely related to the insulin receptor (IR) in structure and function (6).

Ligand binding by the IGF-IR and IR results in the tyrosine autophosphorylation of their β-subunits. The kinase activity intrinsic to β-subunits is essential for the biological actions of IGF-I and insulin, since cells expressing kinase deficient receptors fail to mediate the effects of these growth factors (7, 8). 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 (IRS)-1 to phosphotyrosine residues on the receptor β-subunits. Receptor IRS-1 binding is required for insulin and IGF-I-mediated signal transduction (9). Subsequent to binding by activated receptors, IRS-1 is tyrosine phosphorylated and then acts as a docking protein for the downstream signal transduction components including phosphatidylinositol 3 kinase and the Grb2-SOS complex (6, 9).

The mitogen-activated protein (MAP) kinase cascade is one of the major signaling pathways by which cells transduce extracellular stimuli into intracellular responses (reviewed in Refs. 10–12). Binding of the Grb2-SOS complex to IRS-1 leads to activation of the MAP kinase pathway by stimulating the exchange of GDP for GTP on p21ras. GTP-bound (activated) p21ras then associates with Raf kinase and contributes to its activation. Raf then activates MAP kinase/extracellular signal-regulated protein (ERK) kinase (MEK), which in turn activates MAP kinases by phosphorylation at threonine and tyrosine residues. After activation, ERKs have been shown to phosphorylate and thereby regulate many important proteins involved in diverse cellular activities (13). In mammalian cells, ERK1 and -2 are the best studied members of the MAP kinase family (14). Many growth factors, such as insulin, IGFs, nerve growth factor (NGF), epidermal growth factor (EGF), and fibroblast growth factor, stimulate cellular components that lead to the activation of ERKs (15–17).

In our laboratory, we are examining the mechanisms whereby IGF-IR activation leads to enhanced neurite outgrowth. We use SH-SY5Y human neuroblastoma cells, which are a well-characterized system for studying neuronal growth and differentiation (18–20). We have found that IGFs promote initial membrane ruffling, neurite outgrowth, and cell survival via IGF-IR (18, 20–23). We have also found that the differentiation promoting activity of IGF-I in SH-SY5Y cells is linked to increased expression of growth cone-associated protein-43 (GAP-43) and decreased expression of c-myc (24).

In this report, we investigated the role of ERKs in IGF-I-stimulated neurite outgrowth. We found that IGF-I induced...
tyrosine phosphorylation of IGF-IR, ERK2, and to a lesser extent ERK1. The tyrosine phosphorylation of these proteins correlated with IGF-I-induced neurite outgrowth. This effect of IGF-I on ERK phosphorylation and neurite outgrowth was mediated by IGF-IR and blocked by the MEK inhibitor PD98059. PD98059 also prevented IGF-I-induced changes in GAP-43 and c-myc gene expression. Together these results implicate the importance of the MAP kinase pathway in the regulation of transcriptional events leading to neuronal differentiation.

EXPERIMENTAL PROCEDURES

Materials—Anti-phosphotyrosine monoclonal antibodies PY20 and 4G10 were purchased from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. The neutralizing anti-IGF-IR antibody against the α-subunit (α-IR3) was purchased from Oncogene Science (Uniondale, NY), and another anti-IGF-IR antibody recognizing the β-subunit was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal and monoclonal anti-ERK2 antibodies, horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs, and agarose-conjugated protein A/G-PLUS were also purchased from Santa Cruz Biotechnology. Enhanced chemiluminescence reagents were from ECL Image, version 1.60.

Cell Culture and Analysis of Neurite Outgrowth—SH-SY5Y human neuroblastoma cells were grown in Dulbecco’s modified Eagle’s medium containing 10% calf serum and maintained at 37 °C in a humidified atmosphere with 10% CO2. 18–24 h before experiments, the medium was replaced with Dulbecco’s modified Eagle's medium without serum. For neurite outgrowth experiments, serum-starved cells were incubated in serum free media for 24 h with or without IGF-I. For the experiments using PD98059, cells were treated with the inhibitor 1 h before the addition of IGF-I. Processes longer than the cell body were considered as neurites.

Anti-phosphotyrosine Western Blotting and Immunoprecipitation—Immunoprecipitation was performed essentially as described previously (18, 23) using 1:100 dilution of polyclonal antibodies against ERK2 or anti-IGF-IR β-subunit per 500 μg of cell lysate. For anti-phosphotyrosine Western blotting, whole cell lysates or immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Schleicher and Schuell). Western blotting was performed as previously detailed (18, 23) using 1 μg/ml PY20 and 0.4 μg/ml 4G10. Immunoreactive proteins were identified by horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence reagents. In some experiments, nitrocellulose membranes were incubated at 70 °C for 30 min in stripping solution (2% SDS, 0.1M dithiothreitol, and 70 °C for 30 min in stripping solution). The tyrosine phosphorylation of these proteins correspond to the mobility of the 70, 44, and 42 kDa were identified by anti-phosphotyrosine monoclonal antibodies PY20 and 0.4 μg/ml 4G10. Immunoreactive proteins were identified by horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence reagents.

RESULTS

IGF-I Stimulates Tyrosine Phosphorylation of IGF-IR and ERKs in SH-SY5Y Cells—Several laboratories including ours have utilized SH-SY5Y human neuroblastoma cells to characterize the effects of IGFs on neuronal cells (18–22). These cells express IGF-IR, which mediates the biological functions of the IGFs (26). SH-SY5Y cells also survive in serum-free conditions (27), allowing analysis of the effects of IGFs in the absence of other growth factors.

In preliminary experiments, we found that IGF-I induces the tyrosine phosphorylation of several cellular proteins. Specifically, proteins with mobilities of approximately 200, 130, 95, 70, 44, and 42 kDa were identified by anti-phosphotyrosine immunoblotting (Fig. 1A). Among these, the tyrosine phosphorylation of the 95-kDa protein was most prominent, which corresponds to the mobility of the β-subunit of IGF-IR. Although SH-SY5Y cells produce IGF-II (27), which can activate IGF-IR (1), tyrosine phosphorylation of IGF-IR was not detected in untreated cells. Maximum phosphorylation of IGF-IR was observed after approximately 10 min and was maintained for at least 24 h.

IGF-I also induced strong tyrosine phosphorylation of a 42-kDa protein and to a much lesser extent a 44-kDa protein. These correspond to the reported mobilities of ERK2 and ERK1, respectively. Anti-MAPK Western blotting (Fig. 1B) supported the possibility that these proteins were the ERKs. Although tyrosine phosphorylation of the IGF-IR β-subunit was observed as early as 30 s after the addition of IGF-I (data not shown), phosphorylation of the ERKs was not detected until after 5 min but like IGF-IR was maintained for at least 24 h.

In addition to IGF-IR, IGF-I can bind to the IGF-binding proteins (2). We previously showed that SH-SY5Y cells express IGF-binding protein-2, -3, -4, and -5 (28). To determine whether the effect of IGF-I was mediated through IGF-IR, we examined the effect of an IGF-IR-neutralizing antibody, α-IR3, on IGF-I-stimulated ERK phosphorylation. When SH-SY5Y cells were incubated with 1 μg/ml α-IR3 for 1 h, there was a substantial reduction in IGF-I-stimulated IGF-IR tyrosine phosphorylation (Fig. 2, A and B). Addition of α-IR3 also caused a corresponding decrease in ERK2 tyrosine phosphorylation (Fig. 2B). These results show that promotion of ERK2 phosphorylation by IGF-I...
lysates (500 µg/ml), ERK1 (44 kDa), and ERK2 (42 kDa). Equal amounts of cell lysates (500 µg) were immunoprecipitated with polyclonal antibodies against IGF-IR β-subunit or ERK2 before analysis by SDS-PAGE anti-phosphotyrosine Western blotting (B). Blots were stripped and reprobed with anti-IGF-IR polyclonal antibody or anti-ERK2 monoclonal antibody to show that equal amounts of proteins were immunoprecipitated (C). Lane 1, no addition; lane 2, 10 nM IGF-I; lane 3, 10 nM IGF-I + 1 µg/ml α-IR3. WB, Western blotting; IP, immunoprecipitation; pTyr, phosphotyrosine. The results are representative of three independent experiments.

IGF-I Induces IGF-IR and ERK2 Tyrosine Phosphorylation and Neurite Outgrowth in a Concentration-dependent Manner—To study the effect of IGF-I more closely, we treated the cells with increasing concentrations of IGF-I. IGF-I induced a concentration-dependent increase of tyrosine phosphorylation of IGF-IR and ERK2 (Fig. 3A). Maximum tyrosine phosphorylation was observed between 10 and 100 nM IGF-I for both IGF-I and ERK2. We and others have reported that IGFs induce neurite outgrowth in neuroblastoma cells (20, 24, 29). As expected, addition of IGF-I to the serum-starved cells for 24 h induced a concentration-dependent increase in the number of neurite-bearing cells (Fig. 3B). These results show that concentration dependence of IGF-I-stimulated neurite outgrowth parallels that of IGF-IR and ERK2 tyrosine phosphorylation.

The MEK Inhibitor PD98059 Blocks IGF-I-stimulated Neurite Outgrowth—Many reports suggest the important role of ERKs in cellular differentiation (17, 30–32). Therefore, we studied the neurite-promoting effect of IGF-I in correlation with ERK activation. MEK is a dual specificity protein kinase that can activate ERKs by phosphorylating them on both threonine and tyrosine. To block the activation of ERKs, we used PD98059, a selective inhibitor of MEK. PD98059 non-competitively blocks the activation of MEK by Raf-1, without affecting other known serine/threonine and tyrosine kinases (33). Treatment of SH-SY5Y cells with PD98059 produced a concentration-dependent decrease in the IGF-I-stimulated tyrosine phosphorylation of ERK2 without affecting IGF-IR tyrosine phosphorylation (Fig. 4A). The kinase activity of ERK2 was assayed by measuring the ability of ERK2 to phosphorylate an Elk-1 fusion protein. Fig. 4B shows that PD98059 produced a concentration-dependent inhibition of IGF-I-stimulated Elk-1 phosphorylation. The concentration dependence of PD98059 inhibition paralleled that of ERK2 tyrosine phosphorylation (Fig. 4A). These results demonstrate that PD98059 prevents the IGF-I-mediated activation of ERK2 phosphorylation and kinase activity.

Our results indicated that in SH-SY5Y cells, IGF-I activates ERK2 phosphorylation and kinase activity, and moreover this can be blocked by the MEK inhibitor PD98059. Because ERKs have been previously reported to be important for cellular differentiation (17, 30–32), we examined whether they may also play a role in IGF-I-stimulated neurite outgrowth. Fig. 5, A and B shows that treatment of SH-SY5Y cells with PD98059 also resulted in a concentration-dependent decrease in the number of neurite-bearing cells. Because this effect on neurite outgrowth was observed at 24 h, we treated cells with 10 nM IGF-I alone or with increasing concentrations of PD98059 for 24 h. Continued tyrosine phosphorylation was observed for both IGF-I and ERK2 at 24 h (Fig. 6A). We again observed that treatment of SH-SY5Y cells with PD98059 produced a concentration-dependent decrease in the IGF-I-stimulated tyrosine phosphorylation of ERK2 without affecting IGF-I tyrosine phosphorylation (Fig. 6A). Being consistent with the decrease in the tyrosine phosphorylation, the kinase activity of ERKs also showed a concentration-dependent inhibition by PD98059 (Fig. 6B). Together these results show that the MEK inhibitor PD98059 produces a parallel reduction in ERK activation and neurite outgrowth.

PD98059 Blocks IGF-I-dependent Changes in the Gene Expression of GAP-43 and c-myc—The c-myc protooncogene encodes a nuclear transcription factor that is both necessary and sufficient to trigger entry of certain cell types into the S phase of the cell cycle (34). High levels of c-myc expression are often associated with cell division, whereas cellular differentiation is correlated with decreased c-myc expression (26, 35). In addition to c-myc, GAP-43, the most abundant neuron-specific protein in neuronal growth cones, is also regulated during neuronal differentiation (19, 36). In our laboratory we have shown that in SH-SY5Y cells, IGF-I induces the expression of GAP-43 and
suppresses the expression of c-myc (24). Because our present results implicated ERK activation in IGF-I-stimulated neuronal differentiation, we examined the involvement of ERK in IGF-I-induced changes of GAP-43 and c-myc gene expression.

Expression of GAP-43 and c-myc was examined by Northern blotting. Consistent with our previous results (24), incubation of SH-SY5Y cells with 10 nM IGF-I induced a significant increase in the expression of GAP-43. IGF-I also suppressed c-myc expression and similar to our results with GAP-43 increased concentrations of PD98059 prevented this change in c-myc levels (Fig. 7B). These results suggest that activation of ERKs is essential for IGF-I regulation of GAP-43 and c-myc expression.

FIG. 4. The MEK inhibitor PD98059 blocks IGF-I-induced activation of ERK2. A, anti-phosphotyrosine Western blotting. Serum-starved SH-SY5Y cells were treated with increasing concentrations of PD98059 for 1 h prior to 30 min of incubation with 10 nM IGF-I. Cell lysates (500 μg) were immunoprecipitated with anti-IGF-IR or anti-ERK2 antibodies followed by SDS-PAGE and anti-phosphotyrosine or anti-ERK2 Western blotting. Results are representative of at least three separate experiments. B, effect of PD98059 on MAP kinase activity. Equal amounts of cell lysates were immunoprecipitated with an antibody specific to phosphorylated MAP kinase. Immunoprecipitated MAP kinase was incubated with 1 μg of Elk-1 fusion protein in kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, and 10 mM MgCl2). The mixture was subjected to SDS-PAGE and analyzed by Western blotting using an antibody specific to phosphorylated Elk-1. Relative values from densitometric analyses are shown in the lower panel. Results are mean ± S.E. of three separate experiments. *, p < 0.05 and **, p < 0.01 (by independent Student’s t test) compared with the cells treated with 10 nM PD98059 only.

DISCUSSION

Our laboratory is interested in how IGF-IR signal transduction regulates neuronal differentiation. Many reports suggest that the MAP kinases, ERK1 and -2, play an essential role in growth factor signaling leading to cellular differentiation (17, 30–32, 37). However, the function of these proteins in neuronal differentiation and in the responses to IGF-IR activation have not been well characterized. In these studies we examined IGF-I activation of the ERKs in SH-SY5Y human neuroblastoma cells. We also employed the MEK inhibitor PD98059 to investigate the function of these proteins in IGF-I-stimulated neuronal differentiation.

In initial experiments we found that IGF-I induced a rapid autophosphorylation of the IGF-IR which was maintained for up to 24 h. IGF-I also stimulated tyrosine phosphorylation of ERK1 and -2. Despite the fact that there is more ERK1 protein as revealed by anti-MAPK Western blotting, ERK2 is a far better substrate for IGF-I stimulation than ERK1. Even though phosphorylation of IGF-IR is very rapid, tyrosine phosphorylation of ERKs is not apparent for 10 min. This long lag between receptor and ERK phosphorylation is unique in this cell compared with the effects of IGF-I in other cell types (38). This pattern is not an intrinsic characteristic of the MAP kinase pathway in SH-SY5Y cells because both EGF and NGF can stimulate very rapid phosphorylation of ERKs (data not shown). Besides IGF-IR and ERKs, IGF-I also induced tyrosine phosphorylation of proteins with mobilities of approximately 200, 130, and 70 kDa. In the work by Leventhal et al. (23), we...
proved that SH-SY5Y cells express both paxillin and focal adhesion kinase, and both proteins are tyrosine phosphorylated by IGF-I. Based on a previous study, it is likely that the 200-kDa protein is IRS-2, which SH-SY5Y cells express and use in the absence of IRS-1.²

We believe that prolonged ERK activation is characteristic of growth factor-mediated differentiation. In the current study, IGF-1-mediated ERK activation was maintained for extended periods of time, i.e. up to 24 h. This time course paralleled IGF-1-mediated neurite outgrowth and supports previous reports from us (24) and others (19, 29, 39) that IGF-I promotes neuronal differentiation. In PC12 cells, NGF-induced differentiation is also associated with prolonged activation of ERK, whereas EGF induces transient activation (32). Prolonged ERK activation is accompanied by translocation of ERK into the nucleus (17) where it can regulate gene expression of proteins important in differentiation. Collectively, these data suggest that sustained ERK activation may be instrumental in growth factor-mediated differentiation.

Inhibition of ERK tyrosine phosphorylation by α-IR3 indicates that IGF-I acted through IGF-IR and not by binding to IGF-binding proteins. Kato et al. (7) have reported IGF-I mimetic effects of α-IR3 for cellular tyrosine phosphorylation. In our laboratory, however, we have consistently found an inhibitory effect of α-IR3 on the various effects of IGF-I (22, 24). In the previous report showing the IGF-I mimetic effect of α-IR3, IGF-IR-overexpressing fibroblasts were used as an experimental model. Therefore we suspect that the IGF-I mimetic effect of α-IR3 is a feature of receptor overexpression. Alternatively this may be due to differences in the cell types used (fibroblast versus neuroblastoma cells).

The tyrosine phosphorylation of ERK correlated well with neurite outgrowth in SH-SY5Y cells. The role of the MAP kinase pathway in SH-SY5Y cell differentiation was confirmed by the use of an inhibitor of MEK, PD98059. This compound selectively inhibits the activity of MEK and subsequent activation of ERKs (33, 37). We found that PD98059 effectively blocks IGF-I-induced activation of ERK without affecting the phosphorylation of IGF-IR. Inhibition of ERK paralleled the reduction in IGF-I-induced neurite outgrowth, suggesting a critical role of ERK in the differentiation of SH-SY5Y cells. Our results are similar to those of Pang et al. (37) who showed that PD98059 blocked NGF-induced PC12 cell differentiation. In both our work and that of Pang et al. (37), PD98059 treatment did not completely inhibit growth factor-mediated differentiation, suggesting that other signaling cascades may also be operative. In sharp contrast, Coolican et al. (40) recently reported that PD98059 greatly enhanced IGF-I-induced differentiation of L6A1 myoblasts. Therefore, it is possible that the requirement of MAP kinase activation for cellular differentiation is a neuron-specific feature.

Our results suggest that ERKs can regulate neuronal differentiation by modulating gene expression. IGF-I enhanced GAP-43 expression and depressed c-myc expression. In each instance, PD98059 blocked the effect of IGF-I. Recent evidence indicates that in PC12 cells the MAP kinases translocate from

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² B. Kim, unpublished observations.
cytoplasm to nucleus after differentiation signals such as NGF or overexpression of the EGF receptor (17, 41, 42). These results suggest a direct role of MAP kinases in the regulation of gene expression inside the nucleus. Consistent with our study, several previous reports have shown that ERKs can regulate transcription and translation of Myc (43, 44). Oliver et al. (43) suggested that MAP kinase can increase the synthesis of c-Myc as well as the message level of c-myc. Furthermore, Chuang and Ng (45) reported that ERK2 but not ERK1 activated Myc. The preferential tyrosine phosphorylation of ERK2 over ERK1 in SH-SY5Y cells after differentiation signals such as NGF suggests a direct role of MAP kinases in the regulation of or overexpression of the EGF receptor (17, 41, 42). These results suggest that MAP kinase can increase the synthesis of c-Myc in SH-SY5Y cells is in agreement with their results. Thus, it is likely that, in SH-SY5Y cells, ERK2 plays a key role in IGF-I activation with the MEK inhibitor PD98059 blocks these effects and GAP-43 gene expression. Moreover, inhibition of ERK activation with the MEK inhibitor PD98059 blocks these effects and GAP-43 gene expression. However, we cannot exclude the possibility that the observed changes in gene expression are merely temporally associated with SH-SY5Y differentiation and not directly regulated by ERK activation.

In summary, we show that IGF-I induces a time- and a concentration-dependent phosphorylation of IGF-IR and ERK2 in SH-SY5Y cells. The phosphorylation of these proteins parallels IGF-I-induced neurite outgrowth and changes in c-myc and GAP-43 gene expression. Moreover, inhibition of ERK activation with the MEK inhibitor PD98059 blocks these effects of IGF-I. Collectively, these results indicate that IGF-IR-mediated activation of MAP kinases is essential for IGF-I-induced neuronal differentiation.

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