Insulin-like Growth Factor I Receptor Signaling in Differentiation of Neuronal H19-7 Cells

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ABSTRACT

The type I insulin-like growth factor receptor (IGF-IR) is known to send two seemingly contradictory signals inducing either cell proliferation or cell differentiation, depending on cell type and/or conditions. H19-7 cells are rat hippocampal neuronal cells immortalized by a temperature-sensitive SV40 large T antigen that grow at 34°C in epidermal growth factor or serum but differentiate at 39°C when induced by basic fibroblast growth factor. At 39°C, expression of the human IGF-IR in H19-7 cells induces an insulin-like growth factor (IGF) 1-dependent differentiation. We have investigated the domains of the IGF-IR required for differentiation of H19-7 cells. The tyrosine 950 residue and serines 1280–1283 in the COOH terminus of the receptor are required for IGF-I-induced differentiation at 39°C, although they are dispensable for IGF-I-mediated growth at 34°C. Both domains have to be mutated to inactivate the differentiating function. The inability of these mutant receptors to induce differentiation correlates with mitogen-activated protein kinase activation. In contrast, inhibitors of phosphatidylinositol 3'-kinase have no effect on IGF-I-mediated differentiation of H19-7 cells, although they do inhibit the mitogenic response.

INTRODUCTION

The role of the IGF-IR in cell growth, transformation, and protection from apoptosis has been well established in our laboratory and several other laboratories (reviewed in Refs. 1 and 2). The IGF-IR also promotes differentiation in different cell types, such as myoblasts, osteoblasts, hemopoietic cells (reviewed in Ref. 3), and macrophages (4). There is also substantial evidence that the IGF-IR plays a role in neural differentiation, beginning with the previous observations on oligodendrocytes (5) and neuronal cells, where the IGFs promoted neurite outgrowth and tubulin mRNA production (6–8). Additional references on the participation of the IGF-IR in neuronal differentiation can be found in two recent reviews by Leventhal et al. (9) and Anlar et al. (10). Even more interesting is a recent study by Arsenijevic and Weiss (11), in which the authors state that IGF-I is a differentiation factor for central nervous system stem cell-derived neuronal precursors. These findings should not be construed as meaning that the IGF-IR is the sole or even the most important receptor in neuronal differentiation. However, they indicate a role of the IGF-IR in this process, in association with other growth factor receptors.

Given a role of the IGF-IR in neuronal differentiation, it is reasonable to ask how the IGF-IR participates in the differentiation of neuronal cells. One approach is to determine the domains of the IGF-IR required for neuronal differentiation because the identification of these domains could give important clues on the mechanisms involved. For this purpose, we infected H19-7 cells with retroviral vectors expressing a WT or several mutants of the human IGF-IR. H19-7 cells are rat hippocampal cells that have been conditionally immortalized by transducing a retroviral vector expressing a temperature-sensitive SV40 large T antigen (12). This cell line proliferates at the permissive temperature (34°C) in response to epidermal growth factor or serum and differentiates to a neuronal phenotype in N2 medium supplemented with bFGF at the nonpermissive temperature (39°C; Refs. 12 and 13). Differentiated H19-7 cells do not respond to serum, extend neurites, and express neuronal markers, such as neurofilament proteins and brain type II sodium channels, and display action potentials (12–14). Cells similarly immortalized by a temperature-sensitive SV40 large T antigen show region-specific neuronal differentiation on transplantation into rat brains (15, 16).

In the present experiments, we wished first to establish whether the activated IGF-IR could induce differentiation of H19-7 cells at 39°C and then to determine the domains in the IGF-IR required for the induction of differentiation. As a control, we examined the WT and mutant IGF-IRs for their ability to respond to IGF-I with mitogenesis at 34°C. We show here that tyrosine 950 and the serines 1280–1283 in the COOH terminus of the IGF-IR are necessary for differentiation of H19-7 cells but are dispensable for IGF-I-mediated mitogenesis. This finding clearly separates the mitogenicity of the IGF-IR from its ability to modulate differentiation in neuronal cells. We also carried out preliminary experiments on IGF-IR signaling in these cells at the two temperatures. The inability of certain mutant receptors to promote differentiation correlates with their inability to give a sustained activation of ERK1/2, and differentiation is inhibited by MEK inhibitors, thus confirming previous results by other investigators on the role of MAPK in the differentiation of neuroblastoma cells (8, 17, 18). However, inhibitors of the PI3K pathway have no effect on the differentiation of H19-7/IGF-IR cells, although they completely inhibit the mitogenic response at 34°C.

MATERIALS AND METHODS

Cell Lines. H19-7 are rat hippocampal cells immortalized by transduction of a retroviral vector expressing a temperature-sensitive SV40 large T antigen (12). H19-7 cells are maintained in DMEM supplemented with 10% fetal bovine serum and 200 μg/ml G418 (selection for the T antigen plasmid) in flasks coated with 15 μg/ml poly-L-lysine (Sigma).

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R12, R508, R600, and p6 cells are mouse embryo fibroblasts expressing different numbers of human IGF receptor as described previously (19, 20).

Plasmids and Retroviral Vectors. pHIT60 and pHIT123 were kindly provided by Dr. Alan Kingsman (Oxford University, Oxford, United Kingdom) and are described elsewhere (21). pHIT60 contains the murine leukemia virus gag/pol cassette under the control of the human cytomegalovirus immediate early promoter, whereas pHIT123 contains the human cytomegalovirus, i.e., driven murine leukemia virus ecotropic envelope. pMSCV vectors were kindly provided by Dr. Robert G. Hawley (University of Toronto, Toronto, Canada). The human IGF-IR receptor cDNA was excised from the CVN plasmid (20, 22) by HindIII and HpaI digestion, filled in with Klenow charges. This article must therefore be hereby marked advertisement.
IGF-IR AND DIFFERENTIATION OF H19-7 NEURONAL CELLS

A polyclonal antibody against Shc (Transduction Laboratories). Phosphotyrosine blots were performed with an anti-phosphotyrosine horseradish peroxidase-conjugated antibody (PY20; Transduction Laboratories). Endogenous Shc proteins were detected using an anti-Shc monoclonal antibody from Santa Cruz Biotechnology.

Phosphorylated ERK1 and ERK2 were detected using antibodies from Promega. Akt (Ser-473), was detected using PhosphoPlus kits (New England Biolabs) according to the manufacturer’s instructions. The level of endogenous ERK1/2 was detected using polyclonal antibodies from Santa Cruz Biotechnology, whereas Akt was detected with antibodies also included in the PhosphoPlus kits.

**Kinase Inhibitors.** The PI3K inhibitor LY294002 (Biomed Research Laboratories) was dissolved in DMSO. For cell growth and differentiation experiments, LY294002 was added to the cells at the time of stimulation at a concentration of 10 or 30 μM. For Akt phosphorylation, cells were preincubated for 15 min with 30 μM LY294002 before stimulating with IGF-I. For inhibition of MAPK, we used the MEK inhibitor PD98059 (New England Biolabs). For cell growth and differentiation experiments, PD98059 was added to the cells at the time of stimulation at a concentration of 25 or 50 μM. For ERK1/2 phosphorylation, cells were preincubated for 30 min with 50 μM PD98059 before stimulation with IGF-I. U0126 (Calbiochem) was used at a concentration of 1, 2.5, 5, and 10 μM at 39°C and was added at the time of stimulation.

**RESULTS**

**Establishment of H19-7/IGF-IR Cells.** H19-7 rat hippocampal cells proliferate at the permissive temperature (34°C) in response to epidermal growth factor or serum and differentiate to a neuronal phenotype in N2 medium supplemented with bFGF at the nonpermissive temperature of 39°C (12, 13). We infected H19-7 cells with a retrovector expressing the human IGF-IR, and after selection, the mixed population was tested by Western blot for the expression of the receptor. Fig. 1C shows the levels of expression in the parental (Lane 1) and transduced cell lines, either with an empty vector (Lane 2) or with the WT IGF-IR vector (Lane 3). This last cell line expresses a considerable amount of the IGF-IR protein (arrow) compared with parental or vector-transduced H19-7 cells. The other lanes in Fig. 1C are lysates from different cell lines (mouse embryo fibroblasts) with a known number of IGF-IRs (19, 20). For instance, R600 cells (Lane 6) are known to express 3 × 10^4 receptors/cell, whereas p6 cells (Lane 7) express roughly 5 × 10^5 receptors/cell. On this basis, the level of IGF-IR in transduced H19-7 cells is estimated to be about 100,000 receptors/cell.

**The IGF-IR Promotes Differentiation of H19-7 Cells.** H19-7/IGF-IR cells were tested for differentiation at 39°C or growth at 34°C in NT2 SFM alone or supplemented with either IGF-I or insulin (see “Materials and Methods”). The extent of differentiation was determined by the number of cells with neurite formation (see “Materials and Methods” and the text below), although other markers were occasionally used for confirmation. Fig. 1A shows that H19-7/IGFIR cells differentiate in the presence of IGF-I or insulin at supraphysiological concentration. This concentration of insulin is known to activate the IGF-IR (32). In NT2 SFM medium, there is a small background level of differentiation, which is only slightly above the level of differentiation of the parental cell line (or H19-7 cells transduced with the empty vector). Fig. 1B shows that all cell lines fail to grow and actually decrease in number in N2 SFM. Only H19-7/ IGF-IR cells grow at 34°C, when the medium contains either IGF-I or insulin. The other two cell lines still die under these conditions. Interestingly, insulin at 5 μg/ml is as good as IGF-I in inducing differentiation but is less effective in inducing cell growth. We did not pursue this observation, which may be caused by differences in insulin binding to the IGF-IR at the two temperatures. Another interesting feature of Fig. 1 is that the parental H19-7 cells are incapable of...
growing at 34°C in IGF-I, despite the presence of an active SV40 T antigen and a modest number of IGF-IRs.

H19-7/IGFIR cells at 39°C show a clear concentration dependence on IGF-I for differentiation (Fig. 2A). The cells extend neurites (Fig. 2B, right) and show increased expression of NF68 (Fig. 2C, right), which was used as a marker of neuronal differentiation (27). H19-7/V cells were used as a control (left). These experiments indicate that increased expression of the human IGF-IR induces either IGF-I-mediated growth at 34°C or IGF-I-mediated differentiation at 39°C.

Mutational Analysis of the IGF-IR. To identify the specific domains or residues in the receptor required for the differentiation signal, we transduced H19-7 cells with several mutants of the IGF-IR. These mutants are listed in Table 1 and have been described previously (21, 24, 25, 33–36). After selection, mixed populations were analyzed for the expression of the IGF-IR protein, using an antibody

Fig. 1. The IGF-IR induces differentiation of H19-7 cells. Three different cell lines were used: the parental H19-7 cells and the same cells stably infected with an empty retroviral vector (H19-7/V) or with a retroviral vector expressing the WT human IGF-IR. The last two cell lines were mixed populations selected with puromycin. Three conditions were used: N2 SFM only or N2 SFM supplemented with either insulin (5 μg/ml) or IGF-I (50 ng/ml). A, differentiation at 39°C as determined by counting cells expressing neurites after 48 h. B, cell proliferation, expressed as the percentage increase in cell number over the number of cells plated after 72 h. C, Western blot of the IGF-IR in lysates from various cell lines, using an antibody to the β subunit. Lane 1, H19-7 parental cells; Lane 2, H19-7/V cells; Lane 3, H19/7/IGFIR cells; Lane 4, R12 cells; Lane 5, R508 cells; Lane 6, R600 cells; Lane 7, p6 cells (See “Materials and Methods”).

Fig. 2. IGF-I-mediated differentiation of H19-7/IGFIR cells. A, extent of differentiation of H19-7/IGFIR cells as a function of IGF-I concentration. B, neurite formation in H19-7/IGFIR cells 48 h after IGF-I stimulation (right). H19-7/V cells were used as a control (left). C, increased expression of NF68 in differentiating H19-7/IGFIR cells (right). H19-7/V cells were used as a control (left).

Table 1  IGF-IR mutants used in these experiments

<table>
<thead>
<tr>
<th>H19-7 code</th>
<th>IGF-IR mutants</th>
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<tbody>
<tr>
<td>61245</td>
<td>Truncation at residue 1245</td>
</tr>
<tr>
<td>Y950F-81245</td>
<td>Tyrosine 950 to phenylalanine and truncation at residue 1245</td>
</tr>
<tr>
<td>Y950F</td>
<td>Tyrosine 950 to phenylalanine</td>
</tr>
<tr>
<td>1289, 90, 93, and 94A</td>
<td>Residues 1289, 90, 93, and 94 to alanine</td>
</tr>
<tr>
<td>Y1316F</td>
<td>Tyrosine 1316 to phenylalanine</td>
</tr>
<tr>
<td>Y1250 and 51F</td>
<td>Tyrosines 1250 and 1251 to phenylalanine</td>
</tr>
<tr>
<td>4S</td>
<td>Serines 1280–1283 to alanine</td>
</tr>
<tr>
<td>6S</td>
<td>Serines 1272, 1278, and 1280–1283 to alanine</td>
</tr>
<tr>
<td>Y950F-4S</td>
<td>Tyrosine 950 to phenylalanine and serines 1280–1283 to alanine</td>
</tr>
<tr>
<td>Y950F-3YF</td>
<td>Tyrosines 950, 1131, 1135, and 1136 to phenylalanine</td>
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to the α subunit. Fig. 3C shows that all mutant receptors were expressed at a substantial level. Note that the proreceptors of the δ1245 and Y950F-δ1245 cells are, as expected, slightly shorter than the other proreceptors. Although there is some variability, all mutant receptors are clearly overexpressed and much in excess of the $3 \times 10^4$ receptors/cell that are sufficient for mitogenicity and transforming activity of the IGF-IR (Ref. 19; see also Fig. 1). These cell lines were tested at 39°C for differentiation. As shown in Fig. 3A, all but four mutant receptors induce differentiation at a level that is not significantly different from that of the WT receptor. The four exceptions are: (a) the Y950F-δ1245 IGF-IR mutant; (b) the Y950-4S mutant; and (c) the Y950F-3YF mutant, which have completely lost the ability to induce differentiation; and (d) the δ1245 mutant, which is defective in inducing differentiation. The difference from the WT receptor is statistically significant by the Student’s t test, where values of $P < 0.05$ were considered to be significant.

The loss of the differentiating function could simply be due to the fact that a mutant receptor is a disabled receptor, incapable of transmitting an IGF-I-mediated signal. The same cells were then tested for their ability to grow at 34°C in N2 SFM supplemented solely with IGF-I. The results are shown in Fig. 3B. All mutant receptors can induce the growth of the transduced H19-7 cells, with the exception of the Y950F-3YF mutant. This receptor is indeed a disabled receptor, as also shown in mouse embryo fibroblasts (21). However, the other three mutant receptors that were defective in differentiation are fully capable of responding to IGF-I at 34°C with a growth response. For simplicity, we have omitted the values of cell growth in N2 SFM only. As shown in Fig. 1B, even the cell line with the WT receptor is not proliferating without IGF-I. We can therefore say that a functional IGF-IR is an absolute requirement for either mitogenesis or differentiation of H19-7 cells in N2 SFM supplemented solely with IGF-I. A mutation at tyrosine 950 in combination with a deletion of the COOH terminus or a mutation at serines 1280–1283 results in receptors that are normal for IGF-I-mediated mitogenesis (21, 35, 37) but incapable of inducing differentiation.

It could be objected that the H19-7/Y950F-δ1245 and H19-7/Y950–4S cell lines may have simply lost the capacity to differentiate. They were therefore tested at 39°C for differentiation induced by a combination of IGF-I and bFGF, a combination that is known to promote differentiation of H19-7 parental cells (12, 13). Under these conditions, these cells were fully differentiating, ruling out the possibility that they might have lost the ability to differentiate (data not shown).

**Fate of Differentiating Cells.** Two important questions at this point concern the ability of parental cells to respond to IGF-I and the possibility that the lack of differentiation by some of the mutant receptors may be due to their inability to sustain survival. To answer the first question, we tested the levels of DNA synthesis at 34°C by BrdUrd incorporation on selected cell lines. All of the cell lines tested showed a clear increase in DNA synthesis after stimulation with IGF-I (data not shown). The most relevant experiments are summarized in Table 2. Interestingly, even the vector-transduced H19-7 cells used as a control showed increased BrdUrd incorporation. This suggests that the level of endogenous IGF-IR in H19-7 cells is sufficient to sustain DNA synthesis (Table 2) but is not sufficient to induce cell division (Fig. 1B; Fig. 3B). The dissociation between DNA synthesis and mitosis is not surprising, and it has been reported for the IGF-IR in previous studies (23, 28, 38, 39). However, this result shows that even the parental cell line is sensitive to the action of IGF-I.

<table>
<thead>
<tr>
<th>H19-7 cell lines</th>
<th>% BrdUrd-positive cells (34°C)</th>
<th>No. of cells (39°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>13.7</td>
<td>12</td>
</tr>
<tr>
<td>WT</td>
<td>20.4</td>
<td>13</td>
</tr>
<tr>
<td>Y950F-δ1245</td>
<td>18.7</td>
<td>13</td>
</tr>
<tr>
<td>SFM</td>
<td>42</td>
<td>T = 0</td>
</tr>
<tr>
<td>IGF-I</td>
<td>60</td>
<td>T = 24 h</td>
</tr>
<tr>
<td>% decrease</td>
<td>30</td>
<td>55</td>
</tr>
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</table>

Table 2. DNA synthesis at 34°C and survival at 39°C of various H19-7 cell lines

For DNA synthesis, $10^5$ cells were starved for 24 h in N2 SFM at 34°C and then stimulated with IGF-I (50 ng/ml). BrdUrd was added 2 h after stimulation, and the cells were fixed after 24 h. For survival, $10^5$ cells were plated at 34°C in serum-containing medium. After 24 h, the cells were shifted to 39°C in N2 SFM supplemented with IGF-I (50 ng/ml; $T = 0$) and counted after 24 h. The number of cells is expressed in $10^5$ and represents averages from two separate experiments. V, vector; WT, wild type receptor; Y950F-δ1245, double mutant.
To answer the second question, we determined the number of viable cells for various cell lines at 39°C. There were no significant differences in the number of surviving cells after induction of differentiation between differentiating and nondifferentiating cell lines. If anything, the nondifferentiating cells survived slightly better (Table 2). We also tried to correlate differentiation with DNA synthesis by counting the cells that incorporated BrdUrd and displayed neurite outgrowth. We could not find any correlation between these two processes (data not shown).

**Autophosphorylation of the IGF-IR.** We have compared the autophosphorylation of the WT IGF-IR with selected mutant receptors: (a) the Y950F-Δ1245 mutant, which is mitogenic but does not induce differentiation; and (b) the Δ1245 single mutant, which is defective in differentiation. Fig. 4A shows that all receptors were autophosphorylated on IGF-I stimulation at either temperature (compare with vector-transduced cells). The decrease in receptor autophosphorylation in the double mutant at 39°C is due in part to a slight decrease in the amount of the IGF-IR protein immunoprecipitated (Fig. 4B). However, the truncated receptors are expected to show a decreased level of phosphorylation because they lack three tyrosine residues (tyrosine 1250, tyrosine 1251, and tyrosine 1316) that are known to be phosphorylated on IGF-I stimulation. As expected, the β subunit of the IGF-IR is detected as a faster migrating band by phosphotyrosine antibodies in the two cell lines expressing the truncated receptors (Fig. 4A). This difference is not evident in the total protein blot (Fig. 4B), where we used antibodies against the α subunit of the IGF-IR.

The endogenous IGF-IR in H19-7/V cells is not detectable (Fig. 4B) under these conditions; increasing the exposure time can visualize it, although the overexpressed receptors would be grossly overexposed.

**Phosphorylation of IRS-1 and Shc.** Although we could not detect significant differences in the autophosphorylation of the IGF-IR, it is reasonable to ascertain whether these receptors may differ in the activation of IRS-1 (40) and Shc proteins (41), the two major substrates of the IGF-IR. We determined tyrosyl phosphorylation of these two substrates at both temperatures. The results for Shc are shown in Fig. 4C (tyrosyl phosphorylation) and Fig. 4D (amounts of Shc protein immunoprecipitated), and the results for IRS-1 are shown in Fig. 4E (tyrosyl phosphorylation). The most important comparison is between the cells with the WT receptor and the cells with the double mutant Y950F-Δ1245 receptor.

The M, 52,000 Shc was tyrosyl phosphorylated in cells with either the WT receptor or the Δ1245 receptor, but not in cells with the Y950F mutation or the double mutant Y950F-Δ1245 (Fig. 4C). This was expected because tyrosine 950 is a major binding site for Shc proteins (42) and is required for their activation. However, the results were the same at both temperatures. When we tested the level of phosphorylation of IRS-1, we could not detect any significant difference in tyrosyl phosphorylation of IRS-1 between the cells expressing the WT IGF-IR and the double mutant on stimulation with IGF-I at either temperature (Fig. 4E). In fact, IRS-1 is phosphorylated in all four cell lines tested. We also determined the level of tyrosyl phosphorylation of IRS-2 (43) at 39°C, and we did not detect any difference in tyrosyl phosphorylation of IRS-2 between cells expressing the WT and cells expressing the Y950F-Δ1245 mutant receptor (data not shown).

These results show that these receptors, whether capable of inducing differentiation or not, are signaling to one or the other of their major substrates. The results with Shc proteins will be discussed below.

**The PI3K Pathway Is Dispensable for Differentiation of H19-7/IGF-IR Cells.** According to a number of investigators (44), there are two major signaling pathways for the IGF-IR. The first is through IRS-1, the activation of PI3K (45) and Akt/PKB kinase (46–50), whereas the second major pathway is through MAPK (51, 52). We investigated the role of PI3K in differentiation of H19-7/IGFIR cells by incubating them with an inhibitor of PI3K, LY294002. Fig. 5A shows that the inhibitor LY294002 does not inhibit IGF-I-mediated differentiation of H19-7/IGF-IR cells, even at a concentration of 30 μM. The inhibitor is effective on these cells because when the experiment is conducted at 34°C, it markedly inhibits IGF-I-mediated growth (Fig. 5B). Thus, the activation of PI3K is necessary for the mitogenic response of H19-7/IGFIR cells, but not for their differentiation. This was confirmed by examining Akt/PKB activation in these same cells. Fig. 5C shows that Akt is activated by IGF-I in H19-7/IGFIR cells (Lanes 2 and 5). The addition of LY294002 causes a complete inhibition of Akt/PKB activation at both 34°C and 39°C (Lanes 3 and 6). However, despite the complete inhibition of Akt/PKB activation, H19-7/IGFIR cells still differentiate at 39°C. Incidentally, this last experiment also rules out the possibility that LY294002 may be inactivated at 39°C because its effect on Akt/PKB activation is just as dramatic as it was at 34°C.

Because the PI3K Akt/PKB pathway activates p70s6k (44, 53), we investigated the activation of p70s6k (44, 53) in the cell lines mentioned above (Fig. 4, C–E) at either temperature. p70s6k was activated in all four cell lines at both temperatures (data not shown). The activation of p70s6k confirms that IGF-I activates this pathway in H19-7/IGFIR cells, although the pathway is not crucial for differentiation.

**MAPK Activation.** In these experiments, we examined activation of ERK1 and ERK2 at various times (up to 2 h) after stimulation with IGF-I. For convenience, we show only five cell lines (those expressing the WT receptor, the 61245 receptor, the Y950F receptor, and the two double mutants). The other receptors listed in Table 1 are already known to activate MAPK (21, 32). A representative experiment is shown in Fig. 6, but these experiments were repeated several times with essentially the same results. In all cell lines, stimulation with IGF-I at 34°C causes a strong and prolonged activation of ERK1 and ERK2, as illustrated for two of the cell lines tested in Fig. 6G. However, at 39°C (Fig. 6, A, C, and E), the cells with the double...
mutant receptors show an activation that is not sustained but returns to basal levels after 10 min. In the other cell lines (WT receptor and Y950F), activation of ERK1 and ERK2 is sustained for at least 2 h, even at 39°C. This result indicates a correlation between sustained MAPK activation and IGF-I-mediated differentiation of H19-7/IGFIR cells. Because the cells with the Δ1245 receptor are slightly defective in differentiation, one would have expected a stronger impairment in MAPK activation in these cells. Although there is a clear decrease between 10 and 30 min, the level of MAPK activation in the Δ1245 mutant is still above the basal level at 2 h (Fig. 6A). We will return to this observation in the “Discussion.”

**Effect of a MAPK Inhibitor.** To confirm the importance of MAPK signaling in differentiation of H19-7/IGFIR cells, we incubated these cells with the MEK inhibitor PD98059. Fig. 7A shows that PD98059 inhibits the differentiation of H19-7/IGFIR cells in a concentration-dependent manner. The inhibition is not complete; nevertheless, it is both significant and reproducible. Fig. 7B shows that PD98059 markedly inhibits the activation of MAPK at a concentration of 50 μM. The MEK inhibitor was also tested at 34°C, and Fig. 7D shows that it also inhibits IGF-I-mediated mitogenesis. We also tried another inhibitor of MEK, UO126 (54), which has a higher affinity for MEK than PD98059. At 2.5 μM, UO126 inhibited IGF-I-mediated differentiation in H19-7/IGFIR cells by 50%, and the inhibition was complete at 10 μM (data not shown). Therefore, using the inhibitors, one can say that in these cells MAPK activation is required for both mitogenesis and differentiation, whereas the PI3K pathway is required only for mitogenesis.

**DISCUSSION**

The primary conclusions of these experiments are summarized as follows. (a) The expression of a WT human IGF-IR promotes IGF-I-mediated differentiation of H19-7 rat hippocampal cells at 39°C. The ability of the IGF-IR to induce differentiation of H19-7 cells is dependent on the concentration of IGF-I. (b) The WT IGF-IR is also mitogenic at 34°C. A functional IGF-IR is necessary for IGF-I-mediated mitogenesis at 34°C or differentiation at 39°C. (c) We have identified two domains in the IGF-IR that are necessary to induce differentiation of H19-7 cells. The first domain is tyrosine 950, and the second domain localizes in the COOH terminus at a serine quartet in residues 1280–1283. Both domains have to be mutated for differentiation to be inhibited. The double mutant receptors that fail to induce differentiation are still capable of responding to IGF-I at 34°C with mitogenesis. Secondary conclusions of these experiments include: (a) sustained activation of MAPKs correlates with the ability of the various receptors to induce differentiation of H19-7 cells. Inhibitors of the MAPK pathway inhibit both mitogenesis and differentiation; and (b) in contrast, the PI3K pathway is necessary for IGF-I-induced mitogenesis but not for differentiation of H19-7/IGF-IR cells. These points will be discussed separately.
The role of the IGFs and the IGF-IR in the central nervous system has been well established (see “Introduction” and Refs. 55 and 56). This study therefore deals not with the role of the IGF-IR in neuronal differentiation but with the mechanism(s) by which this receptor participates in the differentiation process. The domains of the IGF-IR necessary for mitogenesis, transformation, and survival have been well characterized (for reviews, see Refs. 2 and 57). There is one report on the domains of the IGF-IR required for the granulocytic differentiation of murine hemopoietic cells (58), but no data are available at the moment on the domain(s) of the IGF-IR necessary for the differentiation signal in neuronal cells.

Several mutants of the IGF-IR have been examined. A mutant receptor truncated at residue 1245 (therefore lacking the last 92 amino acids) was defective in differentiation. A double mutant (mutation at tyrosine 950 in combination with a truncation at the COOH terminus, Y950F-51245 mutant) completely lost the ability to induce differentiation while maintaining its ability to induce growth at 34°C. Another double mutant, Y950F-serines 1280–1283A (Y950F-45), has also lost the ability to differentiate H19-7 cells while still being capable of giving a mitogenic response. We can therefore say that we have identified two domains in the IGF-IR required for IGF-I-induced differentiation of H19-7 rat hippocampal cells: (a) tyrosine 950 (which is located in the juxta-membrane region); and (b) the serines 1280–1283 in the COOH terminus. The receptor with these two mutations is not a disabled receptor that cannot transmit an IGF-I-induced signal because it can induce mitogenesis in H19-7 cells at 34°C. Interestingly, similar results have been reported recently for FGF receptor 1, where both the juxtamembrane and the COOH-terminal regions of the receptor were identified as necessary for induction of FGF-stimulated neurite outgrowth of PC12 cells (59).

It is important to establish that the receptors defective in differentiating ability are not disabled receptors. Using the double mutant receptors, we have shown that these receptors are autophosphorylated at 39°C and can activate some of the transducing molecules downstream of the IGF-IR, including tyrosyl phosphorylation of IRS-1 and IRS-2 and activation of the p70^s6k protein. The tyrosine 950 mutants are defective in Shc phosphorylation, and this may be related to the defect in MAPK activation that will be discussed below. A truly disabled IGF-IR is the Y950F-3YF mutant. H19-7 cells expressing this mutant do not differentiate, but they are also totally insensitive to IGF-I-mediated mitogenesis. This receptor has also been found to be incapable of stimulating growth in mouse embryo fibroblasts, where it cannot induce tyrosyl phosphorylation of either IRS-1 or Shc (21).

Of the two domains we have identified, the tyrosine 950 residue is not required for mitogenesis but is required for transformation of mouse embryo fibroblasts (21). Its importance in apoptosis is ambiguous because it provides only partial protection (21, 60). A single mutation at tyrosine 950 does not seem to affect IGF-I-mediated differentiation of H19-7 cells. This is in sharp contrast to the results in the differentiation of hemopoietic 32D cells, where a single mutation at tyrosine 950 did inhibit IGF-I-mediated differentiation (58).

The second domain we have identified in these experiments is constituted by the serine quartet at 1280–1283. It is important to establish that the receptors defective in differentiation and protection from apoptosis but is perfectly normal for monolayer growth induced by IGF-I (21, 24). The fact that these two mutations do not affect the mitogenicity of the IGF-IR (Refs. 21 and 24 and this study) but do affect differentiation clearly separates these two functions of the IGF-IR to different domains.

The next question is how these mutations may influence IGF-I signaling. Tyrosine 950 is the main binding site for one of the major substrates of the IGF-IR, the Shc proteins (42). Because Shc proteins seem to play a role in IGF-I-mediated differentiation of 32D cells (58), we examined the behavior of Shc proteins during the differentiation of H19-7 cells. The phosphorylation of the M_i 52,000 Shc protein (61) was severely impaired in cells expressing the Y950F-Δ1245 receptor. Unfortunately, it is even less phosphorylated in cells expressing the Y950F mutant, which differentiate normally. Kim et al. (17) reported that a dominant negative mutant of Shc inhibited differentiation of neuroblastoma cells. We tested a dominant negative mutant of Shc in H19-7/IGF-IR cells, but it had no effect on differ-
entiation (data not shown). The same mutant was quite effective in one of our laboratories in inhibiting IGF-I-mediated differentiation of 32D cells (58). We also overexpressed Shc p46 and p52 proteins by retroviral infection in parental H19-7 cells, but we could not detect any differentiation induced by IGF-I in H19-7/Shc cells at 39°C (data not shown). It seems that in these cells, the role of Shc proteins in differentiation is ambiguous. They may play a role, but only in combination with other signal(s).

As to the other domain, the serines at 1280–1283 are a binding site for 14-3-3 adapter proteins (62, 63), and this interaction is valid only for the IGF-IR and is not shared by the IR (62, 63). 14-3-3 proteins have been shown to stabilize and activate Raf kinases (64–68). A mutation at serine 1280–1283 has also been shown to interfere with the mitochondrial translocation of Raf, which occurs when cells expressing the WT receptor are stimulated with IGF-I (69). The interaction of 14-3-3 proteins with the IGF-IR at the serines in the COOH terminus could therefore serve as an alternative pathway to activate Raf kinases in a Ras-independent way, a pathway that would not be shared by the IR. Indeed, although it may be coincidental, we have shown that the IR, even when overexpressed, cannot induce differentiation of H19-7 cells (70).

It could be argued that the COOH terminus signal may be more important than the tyrosine 950 signal because there is a small but significant decrease in differentiation with the 61245 receptor but not with the Y950F receptor. It seems that an intact tyrosine 950 can partially replace the need for a COOH terminus signal. On the other hand, the presence of a COOH terminus signal seems to completely obviate the need for tyrosine 950. It is difficult, at this point, to explain this difference. One could speculate that the COOH terminus sends two separate signals, one that is specific to the COOH terminus (the four serines?) and one that is redundant with the signal from tyrosine 950 (Shc proteins?). In an attempt to gain some information on this point, we have explored IGF-IR signaling in these cells.

One pathway of the IGF-IR that does not seem to be required for differentiation of H19-7/IGFIR cells is the class I PI3K pathway (53). This statement is supported by the following findings: (a) inhibitors of PI3K activity have no effect on IGF-I-mediated differentiation, although they inhibit mitogenesis; (b) these inhibitors completely inhibit Akt/PKB activation at both temperatures, but only mitogenesis is affected; and (c) the p70s6k kinase (53) is normally activated in WT and relevant mutant receptors at both temperatures.

In contrast, it is clear that MAPK activation is required for differentiation of H19-7/IGF-IR cells. The importance of MAPKs in differentiation of neuronal cells has already been reported in different cellular models, and, in this respect, our experiments simply confirm and extend the results previously reported from other laboratories with different cells of neuronal origin. Sustained activation of MAPKs (71–73) has been shown to promote either FGF- or nerve growth factor-mediated differentiation of PC12 cells (74, 75). A role of MAPKs in differentiation of PC12 cells has been also reported by Nguyen et al. (76). Activation of MAPKs is also necessary for IGF-I-mediated neurite outgrowth of SH-SY5Y neuroblastoma cells (8, 17, 18), but the signaling leading to MAPK activation is still controversial. One report shows a role of the Shc-Grb2 complex in mediating ERK activation (17), whereas in another report, PK3K seems to be required for ERK activation and differentiation (18). Another important difference between SH-SY5Y neuroblastoma cells and H19-7 cells is that the former cells do not have IRS-1 (18). IRS-1 is known to have a profound effect on the differentiation of murine hemopoietic cells (58), and the presence of IRS-1 in H19-7/IGF-IR cells could also explain the lack of effect on differentiation, as we mentioned previously, of a dominant negative of Shc. The difference at the two temperatures in the duration of MAPK activation in the double mutant receptors can be explained. The SV40 T antigen, active at 34°C is sending an additional signal to activate MAPK, a signal that is lost at 39°C, where the T antigen is inactive. We have shown previously that the interaction between the T antigen and IRS-1 promotes transformation of mouse embryo fibroblast (77) and protects 32D cells from apoptosis (78). The activation of IRS-1 by the SV40 T antigen is sending a strong mitogenic signal that is prevailing over the differentiation program at 34°C, a signal that is missing at 39°C, where the differentiation program prevails.

As mentioned above, PI3K activation seems dispensable for differentiation of H19-7/IGF-IR cells. Clearly, IGF-IR signaling and functions vary from one cell type to another, and this variability has been vigorously demonstrated in a recent review by Petley et al. (3). The variability in signals probably depends on the availability of substrates and transducing molecules, as demonstrated in the granulocytic differentiation of 32D cells (58).

In conclusion, our experiments have identified two domains in the IGF-IR required for IGF-I-mediated differentiation of H19-7 neuronal cells. When both domains are mutated, the IGF-IR no longer induces H19-7 cell differentiation. Our experiments also point out how little certain mutations of the IGF-IR affect its mitogenicity. Unless the receptor is simply inactivated (mutations at the ATP-binding site or at the tyrosine kinase domain), other mutations have little effect on the ability of the IGF-IR to transmit a mitogenic signal, as we had observed repeatedly in other cell lines (2, 57). The two domains we have identified send signals that apparently converge on the activation of ERKs. Additional studies will be necessary to prove our hypothesis that one of these signals is ras dependent (through Shc signaling), whereas the other one is ras independent, perhaps by the activation of Raf kinases through their interaction with the 14-3-3 proteins.

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