Insulin and insulin-like growth factor-I (IGF-I) receptors are highly homologous tyrosine kinase receptors that share many common steps in their signaling pathways and have ligands that can bind to either receptor with differing affinities. To define precisely the signaling specific to the insulin receptor (IR) or the IGF-I receptor, we have generated brown preadipocyte cell lines that lack either receptor (insulin receptor knockout (IRKO) or insulin-like growth factor receptor knockout (IGFRKO)). Control preadipocytes expressed fewer insulin receptors than IGF-I receptors (20,000 versus 60,000), but during differentiation, insulin receptor levels increased so that mature adipocytes expressed slightly more insulin receptors than IGF-I receptors (120,000 versus 100,000). In these cells, insulin stimulated IR homodimer phosphorylation, whereas IGF-I activated both IGF-I receptor homodimers and hybrid receptors. Insulin-stimulated IRS-1 phosphorylation was significantly impaired in IRKO cells but was surprisingly elevated in IGFRKO cells. IRS-2 phosphorylation was unchanged in either cell line upon insulin stimulation. IGF-I-dependent phosphorylation of IRS-1 and IRS-2 was ablated in IGFRKO cells but not in IRKO cells. In control cells, both insulin and IGF-I produced a dose-dependent increase in phosphorylated Akt and MAPK, whereas IGF-I activated common intracellular pathways. Both receptors phosphorylate insulin receptor substrate (IRS) proteins on the insulin receptor (IR) and IGF-I receptor (IGFR), which have similar heterodimeric αβ2 structures and belong to the family of receptor tyrosine kinases (1). The analysis of the metabolic and mitogenic effects elicited by IR and IGFR in vivo is complicated by many factors. The two receptors are expressed on the surface of most cells, but their relative proportions vary in different tissues. Insulin and IGF-I are capable of binding to each other’s receptors, although with a 100-fold lower affinity than that of its own cognate receptor (2, 3). In addition, individual αβ heterodimers from IR and IGF-IR can also combine to form disulfide-linked hybrid receptors, which can bind both insulin and IGF-I (4–6). To complicate matters further, using similar mechanisms, the two receptors activate common intracellular pathways. Both receptors phosphorylate insulin receptor substrate (IRS) proteins on the same tyrosine residues (7–11). These IRS proteins then act as adaptor molecules to recruit and activate downstream signaling cascades such as the phosphatidylinositol 3-kinase and mitogen-activated protein kinase (MAPK) pathways (12–15).

Despite this apparent overlap in receptor function, IR and IGF-I are not functionally redundant molecules, as illustrated by the distinct phenotypes of IR- and IGFR-deficient mice. Mice lacking IR are born with very slight growth retardation, rapidly develop diabetic ketoacidosis, and die within a few days after birth (16, 17). On the other hand, IGFR-deficient mice are severely growth-retarded, exhibit multiple growth- and differentiation-dependent abnormalities, and die shortly after birth, probably due to respiratory failure (18, 19). These experiments suggest that IR is more important in fuel metabolism, whereas IGFR mediates growth and that one receptor cannot functionally compensate when the other receptor is absent.

Previous studies performed to distinguish the signaling properties of the receptors have focused on the overexpression of wild type or chimeric receptors in transfected cells (2, 21–23). The aberrant level of expression may distort the specificity of the receptors that would be seen with more physiological levels of receptor expression. Additionally, the cultured fibroblasts used in these studies normally do not express high levels of insulin or IGF-I receptors and, therefore, display a limited range of responses to insulin or IGF-I compared with physiologically relevant target tissues. Brown fat is a specialized form of adipose tissue involved in thermogenic energy regulation. Similar to white adipose tissue, brown fat is an insulin-sensitive endocrine organ that expresses both insulin and IGF-I receptors (24, 25). Recently, a novel technique has evolved to generate brown adipose cell lines from genetically modified mice, allowing us to examine the role of insulin/IGF-I signaling molecules in adipocyte biology. In this study we have generated receptor knockout; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase.
brown preadipocyte cell lines that lack either IR or IGFR and have compared insulin- versus IGF-I-induced adipogenesis and signaling in these receptor-deficient cells. We find that IR is required for brown adipocyte differentiation whereas IGFR is not, and each receptor activates downstream signaling molecules in distinct ways.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies used for immunoprecipitation and immunoblotting included anti-insulin receptor β subunit (C-19), anti-IGF β subunit (C-20), anti-IGF α subunit (N-20), anti-IRS-1 (C-20), and anti-peroxisome proliferator-activated receptor γ (E-8) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-IRS-2 and anti-phosphoryrosine 4G10 (kindly provided by Morris White, Joslin Diabetes Center, Boston, MA); anti-insulin receptor C terminus (Joslin Diabetes Center, Boston, MA); anti-phospho-Akt and anti-phospho-MAPK (New England Biolabs, Beverly, MA); and anti-Glut4 (Chemicon International Inc., Temecula, CA). Protein A-Sepharose, 125I-insulin, and 125I-IGF-I were purchased from Sigma. HEPES binding buffer (HBB: 100 mM HEPES, pH 7.4, 118 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 8.8 mM dextrose, 1% bovine serum albumin) containing 0.01 mM 125I-insulin or 125I-IGF-I with increasing concentrations (0.1–1000 nM) of the respective unlabeled ligand for 2 h at 22°C. Binding was performed in triplicate for each dose. Cells were washed once with phosphate-buffered saline containing 1% bovine serum albumin, followed by two washes of phosphate-buffered saline. Cells were solubilized with 0.1 M NaOH containing 0.1% SDS, and radioactivity was measured using a γ-counter.

**Cell Isolation and Culture**—Cells that were homozygous for a floxed allele of exon 3 of the IGF-I receptor (IGFRlox) served as controls for all studies. Cells were also derived from mice homozygous for a floxed allele of exon 3 of the IGF-I receptor (IGFRlox). Brown preadipocytes were isolated from newborn control IRlox and IGFRlox mice by collagen-saline treatment (25). All cell lines in this study were used prior to passage 25 and were shown to be modified virus for 48 h and then placed in selection medium containing 2 mg/ml puromycin. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.1% SDS, and radioactivity was measured using a γ-counter.

**RESULTS**

**Insulin and IGF-I Receptors Are Required for Brown Adipocyte Differentiation**—To determine the role of IR and IGFR in adipogenesis, we have examined the differentiation capacity of brown preadipocyte cell lines that lack or overexpress each of these receptors created by homologous recombinant gene targeting (27, 28). Cell lines containing the floxed allele of IR (IRlox) or IGFR (IGFRlox) served as controls. IR-deficient (IRKO) and IGFR-deficient (IGFRKO) cell lines were created by cre recombinase for each of the floxed lines as described under “Experimental Procedures.” Receptors were re-introduced into control and receptor-deficient cell lines by retroviral infection to create cell lines that overexpress either IR (IRlox + IR; IRKO + IR) or IGFR (IGFRlox + IGFR; IGFRKO + IGFR). We have shown that the insulin receptor that lacks exon 11 (IRα) is the predominant isoform expressed in brown preadipocytes (29), and we have used this isoform for the following overexpression studies. Cells were induced to differentiate by the addition of isobutylmethylxanthine, dexamethasone, and indomethacin and were identified as mature adipocytes on day 6 of the differentiation program. As shown in Fig. 1, both control cell lines differentiated into adipocytes as indicated by oil red O staining of accumulated lipid. All 10 clonal populations of IR-deficient cells failed to differentiate indicating that this receptor was required for adipogenesis. Of the three clonal cell lines deficient for IGFR, one clone failed to differentiate, whereas the remaining two clones accumulated lipids normally (Fig. 2). We confirmed the absence of IGFR in the clones by immunoblotting with an IGFR-specific antibody on protein extracted during the days of differentiation. During adipogenesis, IGFR levels increased slightly in control cells at day 6 (Fig. 2, top panel). As expected IGFR expression was not detectable in any of the IGFRKO clones. Similar to control cells, expression of peroxi-
some proliferator-activated receptor γ and Glut4, two markers of adipocyte differentiation, were induced in IGFRKO clones 2 and 3 (Fig. 2, middle panels). The fact that two IGFR-deficient clonal cells were capable of differentiation indicates that IGFR was not essential for this process. As we have shown previously (29), overexpression of IR failed to restore differentiation of IRKO cells, and overexpression of IR in control cells inhibited differentiation. In contrast, overexpression of IGFR in control cells did not inhibit adipogenesis (Fig. 1). This suggests that both the exact content and balance between these two receptors may be important in the adipogenic program.

**Insulin and IGF-I Receptors Are Differentially Expressed in Brown Preadipocytes and Adipocytes**—To understand the discordant effects of receptor overexpression, we examined the levels of IR and IGFR in differentiating adipocytes and in the various established cell lines. In control cells at the onset of differentiation (day 0), preadipocytes expressed very low levels of insulin receptor as determined by immunoblotting with an antibody specific to the β subunit (Fig. 3A). During the course of differentiation, total IR level was increased and attained its highest level at day 6. Changes in IGFR expression, assessed by immunoblotting for its β subunit, were less dramatic, although there was a slight increase at the later days of differentiation (Figs. 2 and 3A). Fig. 3B shows the relative protein levels of IR and IGFR in the genetically modified cell lines. Because the knockout cell lines cannot differentiate, we have focused on cells in the preadipocyte stage for further analyses. As expected, IR expression was absent in IRKO cells and easily detected in cells overexpressing IR. Similarly, IGFR was absent in IGFRKO cells but was highly elevated in cells overexpressing IGFR. There was no change in insulin receptor levels in IGFRKO cells, and IGFR levels in IRKO cells were similar to that seen in control cells. Thus, there was no receptor compensation when either receptor was absent.

To examine whether overexpression of either receptor would alter hybrid receptor formation, protein extracts from preadipocyte cell lines were immunoprecipitated with antibodies directed against the β subunit of IR or IGFR, and immune complexes were immunoblotted with antibodies directed against IR or IGFR as described under “Experimental Procedures.”

**Fig. 1. Differentiation of receptor-modified cell lines.** Control (IRlox and IGFRlox), receptor-deficient (IRKO and IGFRKO), and receptor-overexpressing (+IR/IGFR) brown adipose precursor cells were grown to confluence and induced to differentiate as described under “Experimental Procedures.” At day 6 of differentiation, cells were fixed and stained with oil red O.

**Fig. 2. Differentiation of IGFR-deficient clones.** Control (IRlox) and three clones of IGFR-deficient cell lines were induced to differentiate as described under “Experimental Procedures.” At the indicated days of differentiation, protein was extracted and immunoblotted with the designated antibodies. At day 6, parallel plates were fixed and stained with oil red O.

**Fig. 3. Insulin and IGF-I receptor expression in brown adipocyte cell lines.** Protein was harvested from differentiating brown adipocytes on the indicated days (A) or from genetically modified preadipocyte cell lines (B). For hybrid receptor formation (C), cell lysates were subjected to immunoprecipitation (IP) with antibodies for the insulin receptor (upper panels) or the IGF-I receptor (lower panels). Total lysates and immune complexes were immunoblotted with antibodies directed against IR or IGFR as described under “Experimental Procedures.”
To determine more precisely the number of receptors on the cell surface, we performed insulin and IGF-I ligand binding assays on control preadipocytes and mature adipocytes, as well as on cells overexpressing IR or IGFR. Scatchard plots of ligand binding showed that insulin binding was low in preadipocytes (Fig. 4A), which parallels the low expression of IR seen in immunoblots (see Fig. 3A). By extrapolation of the curve, we estimated that preadipocytes express about 20,000 insulin receptors on their cell surface (Table I). Following differentiation, adipocytes showed a dramatic increase in insulin binding (Fig. 4A), and this correlates with an approximate 6-fold increase in IR number to 120,000 receptors as estimated by Scatchard plot (Table I). In IRKO preadipocytes overexpressing insulin receptors (IRKO + IR), insulin binding was similar to that seen in control adipocytes with a calculated receptor number of 100,000.

IGF-I binding was higher than insulin binding in control preadipocytes (compare the scale in Fig. 4B with Fig. 4A). This was reflected in a higher number of IGF receptors, 60,000 as estimated by Scatchard analysis, expressed on these cells (Table I). Following differentiation, IGF-I binding was slightly increased (Fig. 4B) with an increase in receptor number to 100,000 receptors per cell (Table I). This modest increase in IGF receptor number reflected the changes in expression observed by immunoblotting (see Fig. 3A). IGFRKO preadipocytes expressed 120,000 receptors, which was slightly greater than levels seen in control differentiated adipocytes. In both preadipocytes and adipocytes the average affinity of the IGF-I receptor was higher than that of the insulin receptor (1.26 ± 0.20 versus 6.69 ± 1.25 nM; Table I).

We next performed competition binding assays to assess the ability of each ligand to interact with the alternate receptor. In binding to the insulin receptor, the competition for binding of 125I-insulin by insulin and IGF-I was similar, although IGF-I competition was displaced by an order of magnitude to the right (ED50 = 43.7 ± 2.6 versus 3.4 ± 0.1 nM; Fig. 5A). IGF-I was able to inhibit 125I-IGF-I binding with an ED50 of 0.55 ± 0.11 nM, indicating high affinity receptors (Fig. 5B, open circles). In contrast, only high concentrations of insulin were able to displace IGF-I from IGFR (Fig. 5B, closed circles). However, there was a low, but detectable, level of binding of 125I-insulin to IRKO cells; this represents the ability of insulin to bind IGF-I receptors (supplemental Fig. 1). Based on the low affinity of insulin for the IGFR, the activity of insulin to stimulate signaling was greater than one would predict based on binding (see below).

**Insulin Stimulates IR Homodimer Phosphorylation, whereas IGF-I Stimulates IGFR Homodimers and Hybrid Receptors—**To analyze receptor activation by the different ligands, cells were stimulated with varying concentrations of insulin or IGF-I for 10 min, and protein extracts were immunoprecipitated with antibodies directed against the β subunit of IR or IGFR and immunoblotted with a phosphotyrosine antibody. In control IRloxp cells, insulin stimulated phosphorylation of IR at 10 and 100 nM (Fig. 6A). IGF-I induced phosphorylation of two bands that precipitated with anti-IR antisera, one corresponding to the IR β subunit (the faster migrating species) and the other corresponding to the IGFR β subunit (the slower migrating species). The intensities of the two bands were equivalent, reflecting an equal molar ratio of receptor β subunits, which indicates that this doublet results from the presence of hybrid receptors. As expected, neither insulin nor IGF-I could induce tyrosine phosphorylation of protein precipitated by an anti-IR antibody from IRKO cells. On the other hand, insulin-stimulated phosphorylation of IR was slightly increased in IGFRKO cells, and in these cells, 100 nM IGF-I was able to weakly phosphorylate IR. In cells that overexpress IR, insulin greatly enhanced phosphotyrosine levels of IR because of the increased IR levels. In these same cells, IGF-I was also able to induce phosphorylation of IR and hybrid receptors as indicated by the doublet. In IGFRKO cells re-expressing IGFR, insulin- and IGF-I-stimulated phosphorylation of IR or hybrids, respectively, were similar to that seen in control cells.

In control cells, insulin was only able to stimulate tyrosine phosphorylation of IGFR present in anti-IGFR precipitates at 100 nM (Fig. 6B), reflecting that some binding of insulin to the IGFR receptors occurred at this high concentration of ligand. IGF-I was able to induce phosphorylation of both IGFR and IR in the complex; however, phosphorylation of IGF-I was more intense than phosphorylation of IR, which indicates the presence of IGFR homoreceptors as well as the presence of hybrid receptors. In the absence of insulin receptors, only the high concentration of insulin was able to induce IGFR phosphorylation. IGF-I was only able to phosphorylate IGFR in IRKO cells, and the level of phosphorylation was similar to that seen in control cells for IGFR homodimers. Insulin and IGF-I failed to stimulate phosphorylation of IGFR immune complexes in IGFRKO cells. In cells overexpressing IR, insulin was able to

![Fig. 4. Insulin and IGF-I binding in preadipocytes and adipocytes.](image)

**Table I**

| Ligand      | Cell line | Cell type | Receptor no. | Average affinity (nM) |
|-------------|-----------|-----------|--------------|-----------------------|
| Insulin     | LOX       | Preadipocyte | 20,000       | 9.47                  |
| Insulin     | LOX       | Adipocyte  | 120,000      | 6.40                  |
| Insulin     | IRKO + IR| Overexpression | 100,000     | 4.20                  |
| IGF-I       | LOX       | Preadipocyte | 60,000       | 1.10                  |
| IGF-I       | LOX       | Adipocyte  | 100,000      | 1.73                  |
| IGF-I       | IGFRKO + IGFR| Overexpression | 120,000     | 0.94                  |
weakly phosphorylate hybrid receptors. IGF-I was also able to stimulate phosphorylation of hybrids and IGFR homodimers in IRKO/H11001IR cells, although to a lesser extent than in control cells. In IGFRKO cells re-expressing IGFR, only 100 nM insulin was able to induce IGFR phosphorylation, and no phosphorylation of IR was detected. Because of the abundance of IGF receptors in these cells, IGF-I greatly enhanced phosphorylation of IGFR homodimers at all concentrations.

Insulin- and IGF-I-stimulated Phosphorylation of IRS-1 and IRS-2 Is Partially Dependent on Their Respective Receptors—Insulin and IGF-I both phosphorylate and activate IRS proteins. To determine whether either ligand preferentially phosphorylates specific IRS proteins, we stimulated the receptor-deficient and receptor overexpressing cell lines with insulin or IGF-I, and no phosphorylation of IR was detected. Because of the abundance of IGF receptors in these cells, IGF-1 greatly enhanced phosphorylation of IGFR homodimers at all concentrations.

In control cells, both insulin and IGF-I elicited a dose-dependent increase in tyrosine phosphorylation of IRS-1, although IGF-I was more potent than insulin in this effect (Fig. 7A). This may reflect the relative levels of IR and IGFR expressed on these cells (see Fig. 4), as well as the higher affinity of IGF-I as compared with insulin for their cognate receptors (see Table I). The tyrosine phosphorylation of IRS-1 at 1 nM IGF-I was similar to that seen with 10 nM insulin, reflecting these differences. Despite elevated IRS-1 protein levels, insulin-stimulated IRS-1 phosphorylation was reduced in IRKO cells, although the highest concentration was able to induce some phosphorylation of IRS-1, presumably by activating IGFR (see Fig. 6B). IGF-I-stimulated phosphorylation of IRS-1 was unaltered in IRKO cells.

In IGFRKO cells, insulin-induced phosphorylation of IRS-1 was enhanced, suggesting that IGFR may actually inhibit IR signaling to some extent. As expected, phosphorylation of IRS-1 by IGF-I was impaired in IGFRKO cells, although 100 nM IGF-I induced modest phosphorylation of IRS-1, possibly through cross-reactivity with IR. In cells overexpressing either IR or IGFR, both insulin and IGF-I were able to enhance phosphorylation of IRS-1. Increased IRS-1 phosphorylation in IRKO + IR cells may be partially explained by the elevated IRS-1 levels. In control cells, equivalent concentrations of insulin and IGF-I induced similar levels of IRS-2 phosphorylation (Fig. 7C). This ligand-induced phosphorylation was unaltered in IRKO cells, suggesting that insulin can signal fully through the IGFR to stimulate IRS-2. In IGFRKO cells, although insulin-stimulated phosphorylation of IRS-2 was not altered, IGF-I-induced phosphorylation of IRS-2 was dramatically reduced, indicating that IGFR was required for this response. Insulin-stimulated IRS-2 phosphorylation was elevated in cells overexpressing IR. However, the response to IGF-I was similar to control cells. This lends further evidence that IGF-I-dependent phosphorylation of IRS-2 required IGFR. Most surprisingly, phosphorylation of IRS-2 by insulin or IGF-I was inhibited in cells overexpressing IGFR, again suggesting some negative effect created by expression of IGFR.
Akt phosphorylation was enhanced, although this did not reach statistical significance. However, these data suggest that IGFR may impair IR activation of Akt. Overexpression of IR greatly elevates insulin-induced Akt phosphorylation with a maximal stimulation at 10 nM, which was 2-fold higher than that seen at 100 nM in control cells. Insulin-stimulated phosphorylation of Akt was also increased in cells overexpressing IGFR, although the dose response was shifted to the right compared with cells overexpressing IR.

IGF-I also induced a dose-dependent increase in Akt phosphorylation in control cells, with a maximal response at 10 nM (Fig. 8B). Note that IGF-I induced a greater phosphorylation of Akt than insulin, and this may reflect the fact that there are more IGF-I receptors in these cells compared with insulin receptors (see Table I). Most surprisingly IGF-I-stimulated Akt phosphorylation was impaired in IRKO cells at 1 and 10 nM, although at the latter concentration this was not statistically significant. This occurred despite the fact that IGFR levels are unaltered in these cells. In IGFRKO cells, phosphorylation of Akt by IGF-I was reduced, although this was only significant at 1 nM. Because IGF-I-dependent phosphorylation of Akt at 1 nM required both IGF-I and insulin receptors, we presume that this response occurred through a hybrid receptor. IGF-I induced Akt phosphorylation was unaltered in cells overexpressing IR. However, in cells overexpressing IGFR, Akt phosphorylation was increased with 1 nM IGF-I but unaltered at 10–100 nM, presumably because the response at 10 nM had already reached maximal stimulation.

Phosphorylation of MAPK by insulin or IGF-I followed a different pattern than Akt. In control cells insulin induced a dose-dependent increase in MAPK phosphorylation (Fig. 8C). In IRKO cells, this response was slightly, although not significantly, blunted, indicating that insulin could signal through IGFR to stimulate MAPK. The dose response in IGFRKO cells was unaltered. As would be expected, insulin-stimulated MAPK phosphorylation was elevated in cells overexpressing IR with all doses eliciting an ~2-fold higher response. Most surprisingly, overexpression of IGF-I impaired insulin-stimulated phosphorylation of MAPK, providing additional evidence that IGFR may inhibit IR signaling.

IGF-I also elicited MAPK phosphorylation in a dose-dependent manner in control cells (Fig. 8D). This response was only slightly elevated compared with insulin stimulation. IGF-I-stimulated MAPK phosphorylation was unaltered in either IRKO or IGFRKO cells, suggesting that the presence of either receptor was adequate for this signal. Similarly, overexpression of either IR or IGFR did not effect MAPK phosphorylation by IGF-I.

**DISCUSSION**

Insulin and IGF-I receptors are structurally similar and activate many of the same signaling cascades. When IR and IGFR are expressed in the same cells, it is difficult to separate the actions of insulin and IGF-I because these ligands can bind either receptor, although with a lower affinity than their cognate receptor. To complicate matters further, individual αβ heterodimers from IR and IGFR can combine to form hybrid receptors, which mostly act as IGF-I receptors but can also bind insulin (4, 6). To isolate the individual contributions of IR and IGFR to signaling and eliminate hybrid receptor formation, we have established brown preadipocyte cell lines lacking either receptor, IRKO or IGFRKO. Previous reports have focused on the actions of one ligand on one receptor-deficient cell line, i.e. IGF-I on IR-deficient fibroblasts (33) or insulin on IGFR-deficient cells (34, 35). This current study has examined the actions of both insulin and IGF-I on IR- and IGFR-deficient cell lines that were established by similar methods.

Previously, our laboratory has developed brown preadipocyte...
cell lines from mice with targeted disruptions of genes involved in the insulin/IGF-I signaling system to help define functional differences among structurally similar molecules. For example, brown preadipocytes lacking IRS-1 failed to differentiate into mature adipocytes and had impaired insulin-stimulated lipid synthesis (36, 37). IRS-2-deficient adipocytes showed only modest defects in adipogenesis, but Glut4 translocation and glucose uptake were reduced (38). Recently (29), we have shown that the insulin receptor was required for differentiation of brown preadipocytes in vitro. In this current study, we have shown that IGFR is not required for adipogenesis of brown preadipocytes, which is unexpected considering that preadipocytes express more IGFR than IR. However, insulin-stimulated expression of various adipogenic markers is reduced in brown adipocytes derived from IGFR-null mice (35). These data suggest that both IR and IGFR have independent roles in adipogenesis that cannot be compensated for by signaling through the other receptor. Most surprisingly, when overexpressed to similar levels in control preadipocytes (~100,000 receptors/cell), the IR inhibits differentiation whereas IGFR does not, providing additional evidence that different signals may emanate from the receptors to effect adipogenesis.

A similar example of the individual requirements for IR and IGFR in differentiation can also be seen in skin development. Primary keratinocytes normally express both insulin and IGF-I receptors and respond to either ligand during differentiation (39). However, keratinocytes from IRKO mice fail to differentiate as measured by the absence of keratin markers K1 and K10 (40). This occurs despite elevated autophosphorylation of IGFR. Additionally, there appear to be defects in keratinocyte development in IGFR-null mice as evidenced by increased transparency and reduced cellularity of the skin (18).

In preadipocytes, there are roughly 3-fold more IGFR than IR, making IGFR the quantitatively predominant receptor in these cells. Therefore, it is not surprising that IGF-I is more potent than insulin in stimulating downstream targets such as IRS-1 and Akt. Even so, IGF-I receptors alone are not sufficient for brown fat differentiation or the activation of some biological targets. Because the interactions of these receptors with their ligands differ with the concentration of the ligand due to cross-receptor binding at high doses, we have designed a simplified model of IR and IGFR signaling by focusing on events elicited by physiological levels of ligand (i.e., 1–10 nM). One caveat to the interpretation of our results is that other factors not assessed here could effect signaling. Such possible differences include the time course of stimulation in different cell types or with different ligands, differences in the association or dissociation rates of the ligands, or the internalization kinetics of the receptor. Because of these variables, we have focused on the ability of insulin or IGF-I to activate signals in the receptor-deficient cell lines instead of the intensity of the signals evoked.

In these cells, insulin primarily binds to and activates the insulin receptor but does not appear to activate hybrid receptors (Fig. 9A, thick arrows). The phosphorylation of both IRS-1

**FIG. 8.** Phosphorylation of Akt and MAPK in response to insulin or IGF-I stimulation. Preadipocyte cell lines were serum-starved overnight and stimulated with increasing concentrations of insulin (A and C) or IGF-I (B and D) for 10 min. Protein lysates were directly immunoblotted with antibodies specific for the phosphorylated (P) forms of Akt (A and B) or MAPK (C and D). Results are expressed as the percentage of stimulation at 100 nM insulin of control cells (LOX) and are the means of four experiments ± S.E. (*, p < 0.05; **, p < 0.01).
and Akt is dependent on the interaction of insulin with its cognate receptor. On the other hand, insulin-stimulated phosphorylation of IRS-2 and MAPK occurs in the absence of insulin receptors, indicating that insulin binding to IGFR can support the generation of these signals (Fig. 9A, dashed arrows). IGF-I binds to and activates IGFR homodimers (Fig. 9B, thick arrows), as well as hybrid receptors (thin arrows). Unlike insulin, IGF-I stimulation of both IRS-1 and IRS-2 requires the presence of IGFR, although our data do not preclude the possibility that hybrid receptors may also be involved in these events. However, it is clear that hybrid receptors are essential for IGF-I activation of Akt because this phosphorylation does not occur when either IGFR or IR is absent (Fig. 9B, thin arrows). IGF-I-stimulated MAPK phosphorylation in either receptor-deficient cell line is equivalent to that seen in wild type preadipocytes, suggesting that IGF-I may use either receptor with equal efficacy to activate this pathway.

It is difficult to ascertain if Akt or MAPK is directly linked to one specific IRS protein because both insulin and IGF-I are capable of phosphorylating IRS-1 and IRS-2. One inference that can be drawn, however, is that in IRKO cells physiological concentrations of insulin are capable of stimulating phosphorylation of IRS-2 and MAPK but not IRS-1 or Akt. Additionally, IGF-I, even at high concentrations, is not capable of stimulating phosphorylation of IRS-2 in IGFRKO cells, although stimulation of Akt phosphorylation does occur. These data suggest that stimulation of Akt by either ligand is more tightly linked to IRS-1 phosphorylation, whereas MAPK is downstream of either IRS protein or perhaps another adaptor molecule not examined here.

By dissecting signaling patterns in the receptor-deficient cell lines, we can assess the level of cross-receptor activation that occurs in these biologically responsive cells. At high concentrations, IGF-I is able to act through the IR in IGFRKO preadipocytes, and these signaling responses parallel the competitive binding capacity of IGF-I for the insulin receptor. By contrast, when insulin acts through the IGFR to stimulate downstream signals in IRKO cells, the responses are greater than one would predict based on its low binding affinity. Thus, insulin acting through the IGFR has a higher intrinsic activity than IGF-I for the insulin receptor. By contrast, IGF-I, even at high concentrations, is not capable of stimulating phosphorylation of IRS-2 in IGFRKO cells, although stimulation of Akt phosphorylation does occur. These data suggest that stimulation of Akt by either ligand is more tightly linked to IRS-1 phosphorylation, whereas MAPK is downstream of either IRS protein or perhaps another adaptor molecule not examined here.

In summary, although insulin and IGF-I have been shown to activate similar signaling cascades, IR- and IGFR-mediated signalings are not functionally redundant. In the current example of brown adipocyte differentiation, deficiency of IR,
which is expressed at low levels, limits adipogenesis, whereas deletion of the IGFR, which is relatively abundant, does not, suggesting that these homologous receptors have different roles in adipocyte biology. Similarly overexpressing one receptor would presumably increase the overall level of tyrosine kinase activity, which in turn would lead to an elevated signaling capacity. It appears that total receptor number is not as important as the ratio of IR and IGFR levels in a given cell. In the fat cell, mechanisms have evolved to regulate the number and ratio of insulin and IGF-I receptors and to facilitate proper signal output.

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