Insulin Receptor Substrate (IRS) Proteins IRS-1 and IRS-2 Differential Signaling in the Insulin/Insulin-Like Growth Factor-I Pathways in Fetal Brown Adipocytes

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In the present study we have investigated the contribution of the insulin receptor substrate proteins (IRS-1 and IRS-2) to the insulin/insulin-like growth factor I (IGF-I)-signaling pathways in fetal rat brown adipocytes, a model that expresses both insulin and IGF-I receptors. Insulin/IGF-I rapidly stimulated IRS-1 and IRS-2 tyrosine phosphorylation, their association with p85α, and IRS-1- and IRS-2-associated phosphatidylinositol (PI) 3-kinase activation to the same extent, the effect of insulin being stronger than the effect of IGF-I at the same physiological dose (10 nM). Furthermore, insulin/IGF-I stimulated IRS-1-associated Grb-2 phosphorylation. However, IRS-2-associated Grb-2 phosphorylation was barely detected. Pull-down experiments with glutathione-S-transferase-fusion proteins containing SH2-domains of p85α revealed a strong association between IRS-1 and IRS-2 with p85α in response to insulin/IGF-I, the insulin effect being stronger than IGF-I. However, the Grb-2-SH2 domain showed functional differences. While a strong association between IRS-1/Grb-2 was found, IRS-2/Grb-2 association was virtually absent in response to insulin/IGF-I, as also demonstrated in competition studies with a phosphopeptide containing the phosphotyrosine 895 residue within the putative Grb-2-binding domain. Finally, insulin/IGF-I stimulated tyrosine phosphorylation of the three SHC proteins (46, 52, and 66 kDa). Moreover, insulin/IGF-I markedly increased the amount of Grb-2-associated SHC proteins by the same extent. Our results suggest that both IRS-1 and IRS-2 are required for phosphatidylinositol 3-kinase activation that leads to adipogenic and thermogenic differentiation of fetal brown adipose tissue; meanwhile, IRS-1 and SHC, but not IRS-2, associate with Grb-2 leading to the ras-mitogen-activated protein kinase-signaling pathway required for fetal brown adipocyte proliferation.

INTRODUCTION

The pleiotropic effects of insulin and insulin-like growth factor-I (IGF-I) on metabolic and mitogenic processes are mediated by a complex network of intracellular signaling pathways (reviewed in Refs. 1 and 2). The biological effects of both molecules are mediated by the activation of their cognate cell surface receptors, consisting of two extracellular α-subunits and two membrane-spanning β-subunits that possess tyrosine kinase activity (3, 4). These receptors are believed to transduce signals, at least in part, by phosphorylation of cellular proteins. It is well known that phosphorylation of the insulin receptor substrate-1 (IRS-1) upon multiple tyrosine residues results in the
interaction with SH2 domain-containing proteins including the growth factor receptor-bound-2 (Grb-2/ Sem5), the protein tyrosine phosphatase SHPTP2, and the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) that leads to activation of various signaling pathways, including the stimulation of the p21 ras-MAPK cascade (5–8).

To elucidate the role of IRS-1 in insulin/IGF-I action, two laboratories (9, 10) created IRS-1-deficient mice by targeted gene mutation. These mice showed an alternative high molecular weight substrate of the insulin receptor named IRS-2, which has been recently purified and cloned (11, 12). IRS-2 has been found to be the major substrate of tyrosine kinases activated by interleukin (IL)-4 in murine lymphohemopoietic cells (13). Furthermore, the expression of IRS-2 was detected in a variety of cell types, including fibroblasts, liver, skeletal muscle, and brain (12). However, the exact role of both IRS proteins in mediating physiological functions in the different tissues has not yet been clarified.

Fetal brown adipocyte primary cultures offer a non-fibroblastic mesenchymal cell model that has proven to be an excellent system by which to study the role of IGF-I/insulin in the proliferation (14–16) and differentiation processes (15, 17–20), as well as their balance in the insulin/IGF-I signal transduction pathways (18, 21–22). These cells bear a large number of high-affinity insulin and IGF-I receptors per cell (15, 20), allowing a variety of studies to be performed with no overexpression of signaling molecules. We have recently shown that IRS-1 is tyrosine phosphorylated in fetal brown adipocytes upon insulin/IGF-I stimulation, resulting in the activation of IRS-1-associated PI 3-kinase (18, 21, 22). Importantly, this enzyme is a requirement for IGF-I-induced brown adipocyte adipogenic and thermogenic differentiation, but not for mitogenesis (18). However, the presence of IRS-2 in brown adipose tissue and its potential role in the insulin/IGF-I signaling cascade throughout late fetal development has not yet been established.

In the present study, we demonstrate that IRS-1 and IRS-2 proteins equally contribute to the association and subsequent activation of PI 3-kinase by insulin/IGF-I that lead to adipogenic and thermogenic differentiation of primary fetal brown adipocytes. However, the association of IRS-2 with the adapter protein Grb-2 is very poor, IRS-1 and SHC being the main docking proteins involved in the activation of the ras-MAPK pathway in brown adipose tissue before birth.

RESULTS

Both IRS-1 and IRS-2 Are Tyrosine Phosphorylated by Insulin/IGF-I in Fetal Brown Adipocytes

Insulin and IGF-I stimulate tyrosine phosphorylation of 180- to 190-kDa proteins that are composed of IRS-1 and the recently reported IRS-2. Our first purpose was to establish the balance of tyrosine phosphorylation of the IRS proteins in fetal brown adipocyte primary cultures. Quiescent cells (20 h serum-deprived) were incubated with 10 nm insulin or 10 nm IGF-I, which has been shown previously to give maximal phosphorylation (21, 22), for 5 min at 37 C. After incubation, cell lysates were prepared, and equal amounts of protein (600–800 μg) were immunoprecipitated either with the anti-IRS-1 or with the anti-IRS-2 glutathione-S-transferase (GST)-fusion protein antibodies. The immune complexes were analyzed by SDS-PAGE, followed by Western blotting with the anti-Tyr(P) antibody (Py20). Figure 1 shows the tyrosine phosphorylation of the IRS proteins induced by insulin or IGF-I treatment. As shown in Fig. 1, both insulin/IGF-I induce a significant increase in tyrosine phosphorylation of IRS-1, as compared with control cells, the insulin effect being stronger than the effect of IGF-I at the same dose (10 nm). When the anti-IRS-2 immunoprecipitates were analyzed by anti-Tyr(P) Western blotting, we found a strong phosphorylation band showing a slightly higher molecular mass (approximately 190 kDa) in insulin/IGF-I-treated cells. However, the level of tyrosine phosphorylation of this band was similar to the level that we previously found in the IRS-1 immunoprecipitates, the effect of insulin being also stronger than the effect of IGF-I at the same dose. These results suggest that both IRS-1 and IRS-2 are involved in the insulin/IGF-I signaling in fetal brown adipocytes.

![Figure 1. Effect of Insulin/IGF-I on IRS-1 and IRS-2 Tyrosine Phosphorylation in Fetal Brown Adipocytes](https://example.com/figure1.png)

Western antibody: Py20

Fetal brown adipocytes serum starved for 20 h were incubated with 10 nm insulin or 10 nm IGF-I, Control cells were cultured in serum-free medium. At the end of the culture time, cells were lysed and immunoprecipitated with the anti-IRS-1 and anti-IRS-2 polyclonal antibodies. The immune complexes were washed and analyzed by Western blotting with the anti-Tyr(P) antibody (Py20). The positions of IRS-1 and IRS-2 are indicated by an arrowhead. The positions of mol wt markers (×10^-3) are shown on the left. The results shown are representative of at least three independent experiments.
Association of IRS Proteins with the p85α Subunit of Phosphatidylinositol 3-Kinase by Insulin/IGF-I

The next step was to investigate whether tyrosine-phosphorylated IRS-2 also associates with the p85α subunit of PI 3-kinase as compared with IRS-1. Serum-deprived cells were incubated with 10 nM insulin or 10 nM IGF-I for 5 min at 37 C. After incubation, cell lysates were prepared, and equal amounts of protein were immunoprecipitated either with the anti-IRS-1 or anti-IRS-2 GST-fusion protein antibodies. The immune complexes were analyzed by Western blotting with the anti-αp85 polyclonal antibody. The results shown are representative of at least three independent experiments.

To further demonstrate the balance of the association IRS-1/2-p85α subunit of PI 3-kinase in fetal brown adipocytes, quiescent cells were stimulated with 10 nM insulin or 10 nM IGF-I for 5 min at 37 C. The cells were lysed, and 600 μg of protein were immunoprecipitated with the anti-αp85 polyclonal antibody as described in Materials and Methods. The immune complexes were subjected to Western blotting analysis with the anti-IRS-1 and anti-IRS-2 polyclonal antibodies. As shown in Fig. 2B, we found a huge increase in the amount of both IRS-1 and IRS-2 in the anti-αp85 immunoprecipitates upon treatment with insulin/IGF-I, insulin stimulating more binding of either IRS-1 or IRS-2 to p85α than IGF-I did (Fig. 2A).

Both IRS Proteins Induce PI 3-Kinase Activity in Fetal Brown Adipocytes

We have recently demonstrated the stimulation of IRS-1-associated PI 3-kinase enzymatic activity in fetal brown adipocyte primary cultures treated with insulin or IGF-I (21, 22). Our next step was to investigate whether PI 3-kinase activity was also associated with IRS-2 as it was to IRS-1, under the same experimental conditions. Upon stimulation with 10 nM insulin or 10 nM IGF-I, cell lysates were subjected to immunoprecipitation with the anti-IRS-1 or IRS-2 antibodies and assayed for PI 3-kinase activity as described in Materials and Methods. As shown in Fig. 3, insulin/IGF-I markedly increased IRS-1 and also IRS-2-associated PI 3-kinase activity, the effect of insulin on both activities being stronger than that induced by IGF-I. Similar PI 3-kinase activity associated with IRS-1/IRS-2 induced by insulin was found after two successive immunoprecipitations with anti-IRS-1 and anti-IRS-2 antibodies of the same lysate (results not shown).

Association of IRS Proteins with the Adapter Protein Grb-2 in Brown Adipocytes

Alignment of the sequences of IRS-2 and IRS-1 revealed a poorly conserved carboxyl terminus containing several tyrosine phosphorylation motifs (11). To further explore possible signaling diversity downstream IRS-1/2 in brown adipocytes, we proceeded to investigate the balance of the association of both IRS proteins with the adapter protein Grb-2. To study this, quiescent cells were incubated with 10 nM insulin or 10 nM IGF-I for 5 min at 37 C. After incubation, cell lysates were prepared, and equal amounts of protein were

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**Fig. 2.** Effect of Insulin/IGF-I on the Association of IRS-1 and IRS-2 with p85α Subunit of PI 3-Kinase in Fetal Brown Adipocytes

A, Quiescent fetal brown adipocytes were incubated for 5 min at 37 C with 10 nM insulin or 10 nM IGF-I. Control cells were cultured in serum-free medium. At the end of the culture time, cells were lysed and immunoprecipitated with the anti-IRS-1 and anti-IRS-2 polyclonal antibodies. Immunoprecipitates were analyzed by SDS-PAGE followed by transfer of proteins to Immobilon and Western blotting with the anti-αp85 antibody. The position of p85α is indicated by an arrowhead. The positions of mol wt markers (× 10^3) are shown on the left. The results shown are representative of at least three independent experiments. B, Cells were stimulated as described in panel A, immunoprecipitated with the anti-αp85 antibody, and analyzed by Western blotting with the anti-IRS-1 and anti-IRS-2 antibodies. The positions of IRS-1 and IRS-2 are indicated by arrowheads. The results shown are representative of at least three independent experiments.
immunoprecipitated either with the anti-IRS-1 or anti-IRS-2 GST-fusion protein polyclonal antibodies. The resulting immunoprecipitates were analyzed by Western blotting with the anti-Grb-2 polyclonal antibody. As shown in Fig. 4A, control cells showed a considerable amount of IRS-1-associated Grb-2. However, IRS-2-associated Grb-2 was barely detected. Insulin/IGF-I induced a marked increase in the amount of IRS-1-associated Grb-2 but a very poor increase in the amount of IRS-2-associated Grb-2. To confirm these data, we analyzed the presence of IRS-1 and IRS-2 in anti-Grb-2 immunoprecipitates. Cells were stimulated with 10 nM insulin or 10 nM IGF-I for 5 min at 37 C, and lysates were immunoprecipitated with the anti-Grb-2 polyclonal antibody as described in Materials and Methods. The immune complexes were subjected to Western blotting analysis with the anti-IRS-1 and anti-IRS-2 GST-fusion protein polyclonal antibodies. As shown in Fig. 4B, insulin/IGF-I induced a marked increase in the amount of Grb-2-associated IRS-1. However, the amount of Grb-2-associated IRS-2 in response to insulin/IGF-I was virtually undetectable.

Association of IRS-1 and IRS-2 with SH2-Containing Proteins

To substantiate the results shown above, we investigated the ability of GST-fusion proteins containing SH2-domains of the p85 subunit of PI 3-kinase, or full-length Grb-2, to bind tyrosine-phosphorylated IRS-1 and IRS-2 in fetal brown adipocytes. Cell lysates were incubated with 10 μg GST fusion proteins containing SH2 domains as indicated in Materials and Methods and precipitated with glutathione Sepharose beads. Samples were subjected to SDS-PAGE followed by Western blotting with the anti-IRS-1 and anti-IRS-2 polyclonal antibodies.

As shown in Fig. 5, the NH2-terminal SH2 domain of p85a bound strongly to both IRS-1 and IRS-2 upon stimulation with 10 nM insulin or 10 nM IGF-I, insulin stimulating more binding than IGF-I did. However, the SH2 domains of Grb-2 revealed functional differences. Thus, control cells showed a basal amount of Grb-2-associated IRS-1, but not Grb-2-associated IRS-2. Insulin/IGF-I markedly increased the amount of Grb-2-associated IRS-1. However, the amount of Grb-2-associated IRS-2 in response to insulin/IGF-I was virtually undetectable (Fig. 5).

IRS-1 and IRS-2 Displayed Differential Affinity for Grb-2 in Fetal Brown Adipocytes

The functional difference between IRS-1 and IRS-2 with respect to the ability to bind Grb-2 and subsequently activate the ras-MAPK pathway could be explained by differential affinities of both IRS proteins for the Grb-2 adapter protein. To examine this possibility, a synthetic phosphopeptide containing a single tyrosine-phosphorylated motif, based on the established Grb-2-binding site in IRS-1 and IRS-2 (11), was tested for the ability to displace IRS-1 or IRS-2 from Grb-2 immune complexes. Serum-deprived brown adipocytes were stimulated for 5 min with 10 nM insulin, and immune complexes, prepared with the anti-Grb-2 antibody, were incubated with increasing doses of phosphopeptide as described in Materials and Methods. The remaining endogenous IRS-1/IRS-2 bound to Grb-2 was analyzed by Western blotting with the anti-IRS-1 and anti-IRS-2 antibodies. Represent-
tive phosphopeptide competition experiments are shown in Fig. 6, A and B. Dissociation of IRS-2 from Grb-2 was much more sensitive (ED_{50} = 0.01 \mu M) (Fig. 6D) to the phosphopeptide than dissociation of IRS-1 from Grb-2 (ED_{50} > 1 \mu M) (Fig. 6C), suggesting that the Grb-2-binding site of IRS-1 displays a much higher affinity for Grb-2 than that of IRS-2.

**Insulin/IGF-I-Induced SHC Tyrosine Phosphorylation and Its Association with Grb-2**

Finally, we have investigated the contribution of SHC proteins to the Grb-2-association, which could be relevant to the proliferation induced by IGF-I in fetal brown adipocyte primary cultures. Serum-detk; 2prived cells were stimulated either with 10 nm insulin or 10 nm IGF-I for 5 min at 37 C. Cell lysates were prepared, and equal amounts of protein were immunoprecipitated with the Py72 anti-Tyr(P) antibody. The resulting immunoprecipitates were analyzed by Western blotting with the anti-SHC polyclonal antibody. As shown in Fig. 7A, control cells showed basal phosphorylation bands of the three SHC proteins. However, 10 nm insulin/IGF-I increased the 46- and 52-kDa SHC phosphotyrosine bands by 2-fold and the 66-kDa SHC phosphotyrosine band by 3-fold.

**Fig. 4.** Effect of Insulin and IGF-I on IRS-1 and IRS-2 Association with the Adapter Protein Grb-2 in Fetal Brown Adipocytes

A, Quiescent fetal brown adipocytes were cultured as described in Fig. 2. At the end of the culture time, cells were lysed and immunoprecipitated with the anti-IRS-1 and anti-IRS-2 polyclonal antibodies. Immunoprecipitates were analyzed by SDS-PAGE followed by transfer of proteins to Immobilon and Western blotting with the anti-Grb-2 antibody. The position of Grb-2 is indicated by an *arrowhead*. The positions of mol wt markers (x10^{-3}) are shown on the left. The results shown are representative of at least three independent experiments. B, Cells were stimulated as described in panel A, immunoprecipitated with the anti-Grb-2 antibody, and analyzed by Western blotting with the anti-IRS-1 and anti-IRS-2 antibodies. The positions of IRS-1 and IRS-2 are indicated by *arrowheads*. The results shown are representative of at least three independent experiments.

**Fig. 5.** Differential Binding of p85α and Grb-2 SH2 Domains to IRS-1 and IRS-2 in Insulin/IGF-I-Stimulated Fetal Brown Adipocytes

Quiescent brown adipocytes were incubated in the absence or presence of insulin (10 nm) (ins) or IGF-I (10 nm) (I) for 5 min at 37 C. Cell lysates were precipitated with the indicated GST fusion proteins as described in *Materials and Methods*, separated by SDS-PAGE, and immunoblotted with the anti-IRS-1 and anti-IRS-2 antibodies. The positions of IRS-1 and IRS-2 are indicated on the right. The data are representative of two similar experiments.
To further investigate the importance of the association of SHC-Grb-2 in fetal brown adipocytes, we determined the ability of the SH2Grb-2 GST-fusion protein to bind tyrosine-phosphorylated SHC following insulin/IGF-I treatment in brown adipocytes. Cell lysates were incubated with 10 ng GST fusion protein as indicated in Materials and Methods and precipitated with glutathione Sepharose beads. Samples were subjected to SDS-PAGE followed by Western blotting with the anti-SHC antibody. As shown in Fig. 7B, there is an important association of SHC with Grb-2 before stimulation of brown adipocytes with insulin/IGF-I. However, insulin/IGF-I markedly increased by the same extent the amount of Grb-2-associated SHC proteins.

**DISCUSSION**

The recent identification and cloning of IRS-2 as an IRS-1-like molecule indicate that a family of docking proteins may be used for insulin/IGF-I and cytokine signaling. Our laboratory has established that quiescent fetal brown adipocytes in primary culture constitute a suitable cell model in which to study the insulin/IGF-I signaling network, under physiological conditions (21, 22). These cells bear a large number of high-affinity insulin- and IGF-I-binding sites per cell (15, 20), which allow the unhampered investigation of the early events in the insulin/IGF-I action. In previous reports we have shown that IRS-1 tyrosine phosphorylation is maximally induced at physiological concentrations of both molecules (21, 22). In the present paper, we have shown that in fetal brown adipocytes IRS-2 is tyrosine phosphorylated, to the same extent as IRS-1, in insulin/IGF-I-treated cells. These results prompted us to study, in our cell model, the contribution of both IRS-1 and IRS-2 docking proteins to the association with two molecules that propagate two distinct pathways downstream of IRSs: the association with the lipid/serine-threonine kinase PI 3-kinase and the association with the adapter protein Grb-2.

Previous studies performed in IRS-1-deficient mice showed that the residual insulin/IGF-I action of these...
between IRS-2/p85<sup>a</sup>-deficient fibroblasts, although a strong association between IRS-1 and IRS-2 to the adipogenic and thermogenic differentiation of brown adipocytes before birth, in a p21<sup>r</sup>Kras-dependent manner (16). Furthermore, IRS-2 has been proposed to account for the differential signaling of IRS-1 and IRS-2 underlies the differential affinities for Grb-2 of their conserved Grb-2 binding domains, probably due to the different environment surrounding this domain in both molecules. These data are consistent with the severe inhibition of the DNA synthesis found in IRS-1-deficient fibroblasts, in which the presence of IRS-2 could not compensate for the absence of IRS-1 in maintaining the mitogenic response of IGF-I (26).

SC has recently been established as another early phosphotyrosine substrate, which has been proposed as the predominant signaling molecule coupling insulin receptors to p21 ras.GTP formation in Rat-1 fibroblasts (29, 30). More recently, SHC signaling, rather than IRS-1, has been proposed to account for the proliferation of Rat-1 fibroblasts in response to insulin (31). In fetal brown adipocyte primary cultures, the three SHC proteins are tyrosine phosphorylated in response to insulin/IGF-I. Furthermore, we have found a marked increase in the amount of SHC-Grb-2 fusion protein associated with SHC in response to insulin/IGF-I (26). The results presented here indicate that in fetal brown adipocytes IRS-1 and SHC, but not IRS-2, are the docking proteins that lead to the ras-MAPK cascade after insulin and IGF-I stimulation. The fact that IGF-I is as potent as insulin in inducing Grb-2 binding site (11, 12, 28), our data predict a tyrosine phosphorylation motif that could serve as a Grb-2 binding site (11, 12, 28), our data show that the association between IRS-2/Grb-2 in response to insulin/IGF-I is virtually absent. In addition, the phosphopeptide competition assay revealed that IRS-2 binds to Grb-2 with 2 orders of magnitude lower affinity than IRS-1. Therefore, a molecular mechanism that may account for the differential signaling between IRS-1 and IRS-2 underlies the differential affinities for Grb-2 of their conserved Grb-2 binding domains, probably due to the different environment surrounding this domain in both molecules. These data are consistent with the severe inhibition of the DNA synthesis found in IRS-1-deficient fibroblasts, in which the presence of IRS-2 could not compensate for the absence of IRS-1 in maintaining the mitogenic response of IGF-I (26).

In summary, the findings described in this paper strongly suggest that IRS-1 and IRS-2 contribute and IRS-2-associated PI 3-kinase activity, as compared with IGF-I, further supports our previous data showing that insulin was a more potent signal than IGF-I in adipogenic and thermogenic differentiation (20).

The adapter protein Grb-2 has been shown to link IRS-1 to the activation of ras and the MAPK cascade (6, 27). Activation of p21 ras to its GTP active form has been shown to occur in fetal brown adipocytes upon insulin or IGF-I stimulation (21, 22). Importantly, serum-starved brown adipocytes showed a considerable percentage of ras in its active GTP form (21), as a consequence of their intrinsic mitogenic competence (15). Results presented in this paper show that there is a significant IRS-1/Grb-2 association in control cells in the absence of external stimuli, which could explain the intrinsic ras-GTP activation that we found in fetal brown adipocyte primary cultures. Furthermore, there is a marked increase in the amount of Grb-2-associated IRS-1 in response to insulin/IGF-I. However, despite the fact that the amino acid sequence of IRS-2 predicts a tyrosine phosphorylation motif that could serve as a Grb-2 binding site (11, 12, 28), our data show that the association between IRS-2/Grb-2 in response to insulin/IGF-I is virtually absent. In addition, the phosphopeptide competition assay revealed that IRS-2 binds to Grb-2 with 2 orders of magnitude lower affinity than IRS-1. Therefore, a molecular mechanism that may account for the differential signaling between IRS-1 and IRS-2 underlies the differential affinities for Grb-2 of their conserved Grb-2 binding domains, probably due to the different environment surrounding this domain in both molecules. These data are consistent with the severe inhibition of the DNA synthesis found in IRS-1-deficient fibroblasts, in which the presence of IRS-2 could not compensate for the absence of IRS-1 in maintaining the mitogenic response of IGF-I (26).

In summary, the findings described in this paper strongly suggest that IRS-1 and IRS-2 contribute
equal to the association with the p85α that leads to the PI 3-kinase activation in response to insulin/IGF-I, a signaling required for the onset of brown adipose tissue adipogenic and thermogenic differentiation before birth. Moreover, IRS-1 and SHC, but not IRS-2, associate with the adapter protein Grb-2 that leads to the ras-MAPK cascade, a signaling required for fetal brown adipocyte proliferation.

**MATERIALS AND METHODS**

**Materials**

FCS and culture media were from Imperial Laboratories (Hampshire, U.K.). IGF-I was purchased from Calbiochem (Calbiochem-Novabiochem, La Jolla, CA). Insulin, l-α-phosphatidylinositol, and l-α-phosphatidyl-L-serine were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase and Protein A-agarose were purchased from Boehringer Mannheim (Mannheim, Germany). Glutathione Sepharose beads were purchased from Pharmacia (Uppsala, Sweden). [γ-32P]ATP (3000 Ci/mmol) was purchased from Amersham (Aylesbury, U.K.). All other reagents used were of the purest grade available.

**Cell Culture**

Brown adipocytes were obtained from interscapular brown adipose tissue of 20-day Wistar rat fetuses and isolated by collagenase dispersion as previously described (15). Cells were plated at 5 \times 10^6 cells/100 mm or 1–2 \times 10^6 cells/60-mm tissue culture plates in MEM supplemented with 10% FCS to allow cell attachment to the plastic surface of the plates. After 4–6 h of culture at 37°C, cells were rinsed twice with PBS, and 80% of the initial cells were attached. Cells were maintained for 20 h in a serum-free medium supplemented with 0.2% (wt/vol) BSA. At this time cells were harvested, centrifuged, and resuspended in PBS, and 80% of the initial cells were reattached. Cells were then reseeded at 5 \times 10^5 cells/ml in primary culture HEPES (pH 7.4, 10 mM Na2VO4, and 0.5 mM EGTA). After 15 min at room temperature, the reaction was stopped by the addition of 500 μl CHCl3-methanol (1:2) in a 1% concentration HCl plus 125 μl chloroform and 125 μl HCI (10 mM). The samples were centrifuged, and the lower organic phase was removed and washed once with 480 μl methanol-100 mM HCl plus 2 mM EDTA (1:1). The organic phase was extracted, dried in vacuo, and resuspended in chloroform. Samples were applied to a silica gel TLC plate. TLC plates were developed in 2% propanol-1-acetic acid (65:35, vol/vol), dried, visualized by autoradiography, and quantitated by scanning laser densitometry (Molecular Dynamics personal densitometer, Sunnyvale, CA).

**Immunoprecipitations**

Quiescent fetal brown adipocytes (after 20 h of serum deprivation) were treated with insulin or IGF-I for 5 min and subsequently lysed at 4°C in 1 ml of a solution containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na2VO4, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, pH 7.6 (lysis buffer). Lysates were clarified by centrifugation at 15,000 \times g for 10 min, and the supernatants were transferred to a fresh tube. After protein content determination, equal amounts of protein were immunoprecipitated at 4°C with the corresponding antibodies, and the immune complexes were collected on Protein A-agarose beads. Immunoprecipitates were washed three times with lysis buffer and extracted for 10 min at 95°C in 2× SDS-PAGE sample buffer (200 mM Tris-HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and analyzed by SDS-PAGE and as described in Results and in the figure legends. Anti-p85α and anti-Grb-2 polyclonal antibodies were from Santa Cruz (Santa Cruz Biotechnology, Palo Alto, CA). Anti-SHC polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY). For IRS-1/IRS-2 immunoprecipitations, the Py72 monoclonal antibody was the generous gift of Dr. E. Rozengurt and J. Sinnett-Smith (Imperial Cancer Research Foundation, London). The monoclonal anti-Tyr(P) antibody used for Western blotting (Py20) was purchased from Santa Cruz.

**Western Blotting**

After SDS-PAGE, proteins were transferred to Immobilon membranes and were blocked using 5% nonfat dried milk in 10 mM Tris-HCl and 150 mM NaCl, pH 7.5, and incubated overnight with several antibodies as indicated in 0.05% Tween-20, 1% nonfat dried milk in 10 mM Tris-HCl and 150 mM NaCl, pH 7.5. Immunoreactive bands were visualized using the enhanced chemiluminescence Western blotting protocol (Amersham, Arlington Heights, IL).

**PI 3-Kinase Activity**

PI 3-kinase activity was measured by in vitro phosphorylation of phosphatidylinositol as described (32). Fetal brown adipocytes were incubated with IGF-I in the absence or presence of PI 3-kinase inhibitors as indicated in the figure legends. After washing, with ice-cold PBS, cells were solubilized in lysis buffer containing leupeptin (10 μg/ml), aprotonin (10 μg/ml), and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 15,000 \times g for 10 min at 4°C, and proteins were immunoprecipitated with the anti-IRS-1/IRS-2 polyclonal antibodies. The immunoprecipitates were washed successively in PBS containing 1% Triton X-100 and 100 μM Na3VO4 (twice), 100 μM Tris (pH 7.5) containing 0.5 mM LiCl, 1 mM EDTA and 100 μM Na2VO4 (two times), and 25 mM Tris (pH 7.5) containing 100 mM NaCl and 1 mM EDTA (twice). To each pellet were added 25 μl of 1 mg/ml l-α-phosphatidylinositol/l-α-phosphatidyl-L-serine sonicated in 25 mM HEPES (pH 7.5) and 1 mM EDTA.

The PI 3-kinase reaction was started by the addition of 100 μM [γ-32P]ATP (10 μCi) and 300 μM ATP in 25 μl of 25 mM HEPES, pH 7.4, 10 mM MgCl2, and 0.5 mM EGTA. After 15 min at room temperature, the reaction was stopped by the addition of 500 μl CHCl3-methanol (1:2) in a 1% concentration HCl plus 125 μl chloroform and 125 μl HCI (10 mM). The samples were centrifuged, and the lower organic phase was removed and washed once with 480 μl methanol-100 mM HCl plus 2 mM EDTA (1:1). The organic phase was extracted, dried in vacuo, and resuspended in chloroform. Samples were applied to a silica gel TLC plate. TLC plates were developed in 2% propanol-1-acetic acid (65:35, vol/vol), dried, visualized by autoradiography, and quantitated by scanning laser densitometry (Molecular Dynamics personal densitometer, Sunnyvale, CA).

**Differential Binding of SH2 Domains from p85α and Grb-2 with the IRS-Proteins**

GST-fusion proteins containing nSH2-dig8 and SH2Grb-2 were prepared as described (7). Cell lysates were prepared from unstimulated, insulin-stimulated, or IGF-I-stimulated fetal brown adipocytes in lysis buffer. The extracts were clarified by centrifugation at 12,000 \times g for 15 min at 4°C. The supernatants were incubated with 10 μg of the GST fusion proteins containing SH2 domains as indicated at 4°C for 1 h and precipitated with glutathione Sepharose at 4°C for 1 h, washed twice in 50 mM Tris/HCl (pH 7.4) containing 100 mM NaCl, 250 μg/ml BSA, 0.2 mM Na2VO4, and 0.4 mM phenylmethylsulfonyl fluoride, and boiled for 5 min in Laemmli sample buffer. Samples were separated on 7% SDS-PAGE and analyzed by immunoblotting.

**Phosphopeptide Competition Studies**

Phosphopeptide binding competition assay was performed as described (33). Lysates from insulin-stimulated cells (600–
800 μg of protein) prepared as described above were incubated with the anti-Grb-2 antibody for 30 min at 4°C, divided into 1-ml aliquots, and then incubated at 4°C for 14 h without or with various doses (0.001–100 μM) of a synthetic phosphopeptide (PEP[(PK)SPGEY(P)NVIEFG]) corresponding to the IRS-1/IRS-2 consensus Grb-2 binding domain (11). The immune complexes were collected on 40 μl protein A-agarose, resolved on a 7% gel, and analyzed by immunoblotting with the anti-IRS-1 and anti-IRS-2 antibodies.

**Protein Determination**

Protein determination was performed by the Bradford dye method (34), using the Bio-Rad (Richmond, CA) reagent and BSA as the standard.

**Experimental Animals**

The animals used for the required experiments in this report were treated in accord with the “Guidelines for Care and Use of Experimental Animals.”

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