We recently cloned IRS-4, a new member of the insulin receptor substrate (IRS) family. In this study we have characterized IRS-4 in human embryonic kidney 293 cells, where it was originally discovered. IRS-4 was the predominant insulin-elicted phosphotyrosine protein in these cells. Subcellular fractionation revealed that about 50% of IRS-4 was located in cellular membranes, and immunofluorescence indicated that IRS-4 was concentrated at the plasma membrane. Immunoelectron microscopy conclusively established that a large portion of the IRS-4 was located at the cytoplasmic surface of the plasma membrane in both the unstimulated and insulin-treated states. IRS-4 was found to be associated with two src homology 2 (SH2) domain-containing proteins, phosphatidylinositol 3-kinase and Grb2, the adaptor to the guanine nucleotide exchange factor for Ras. On the other hand, no significant association was detected with two other SH2 domain proteins, the SH2-containing protein tyrosine phosphatase 2 and phospholipase Cγ. Insulin-like growth factor I acting through its receptor was as effective as insulin in eliciting tyrosine phosphorylation of IRS-4, but interleukin 4 and epidermal growth factor were ineffective.

Signal transduction from the insulin and IGF-I receptors is initiated by the activated receptors phosphorylating insulin receptor substrates (IRSs) on multiple tyrosine residues. The IRSs in turn bind SH2 domain-containing signaling proteins. The binding of these SH2 domains proteins activates them in various ways and thereby initiates signaling cascades that regulate cell metabolism and proliferation (reviewed in Refs. 1–3). Until recently, there appeared to be only two IRSs, IRS-1 and IRS-2. However, in the past year we have isolated and cloned two more members of this family, IRS-3 and IRS-4 (4, 5). All four members have a similar architecture, consisting of an NH2-terminal pleckstrin homology domain followed by a phosphotyrosine binding domain and COOH-terminal portion with tyrosine phosphorylation sites in short motifs that can bind to specific SH2 domains.

We discovered IRS-4 in human embryonic kidney 293 (HEK) cells as a 160-kDa protein that undergoes tyrosine phosphorylation in response to insulin (5). Other than cloning, there has been no characterization of IRS-4 and comparison of its properties with the other IRSs. The present study describes its subcellular location, association with SH2 domain proteins, and tyrosine phosphorylation in response to other growth factors in HEK cells.

**EXPERIMENTAL PROCEDURES**

Antibodies—Antibodies against Grb2 (SC255), SHP-2 (SC280), and phospholipase Cγ (SC426) were purchased from Santa Cruz Biotechnology. Antiserum against the 85-kDa subunit of PI 3-kinase (6D19-5) and antibodies against IRS-2 (6D506) were from Upstate Biotechnology. An antiserum against the carboxyl-terminal peptide of IRS-2, which was kindly provided by Dr. Jacalyn Pierce, National Institutes of Health, was also used for some initial experiments. The preparations of affinity-purified antibodies against Tyr(P) and the carboxyl terminus of IRS-1 were those described previously (6). An antiserum against a truncated form of human SHP-2 lacking the SH2 domains (7) was the generous gift of Dr. Zhizhuang Zhao, University of Washington; it was used throughout for immunoblotting, whereas the Santa Cruz antibody was used for immunoprecipitation.

Two types of rabbit antibodies were generated against IRS-4. One was raised against the carboxy-terminal 16 amino acids of human IRS-4, coupled to hemocyanin via a cysteine at the amino terminus of the synthetic peptide. The antibodies were affinity-purified on the immobilized peptide as described by Lamphere and Lienhard (6). This preparation is referred to as IRS-4C. The other antibodies were raised against a GST fusion protein with amino acids 984–1197 of mouse IRS-4. This region of the mouse IRS-4 genomic DNA was amplified by PCR using a 5′ primer with an appended BamH I restriction site and a 3′ primer with a Xba I restriction site. The product was ligated into the BamH I/Xba I sites of the pGEX-5X-3 plasmid (Amersham Pharmacia Biotech). The GST fusion protein was expressed in Escherichia coli strain HB101 and then purified according to the method described by Amersham Pharmacia Biotech. The antibodies against the GST fusion protein were affinity-purified from the serum by adsorption of the serum with immobilized GST followed by chromatography on the immobilized GST fusion protein, as described by Keller et al. (8). This preparation is referred to as anti-IRS-4GST. Both types of antibodies immunoprecipitated and immuno bloted IRS-4 from lysates of HEK cells. Both types of antibodies are against regions of IRS-4 that show little or no homology with IRS-1 or IRS-2 (5), and consequently there is no reason to expect any cross-reactivity.

**Cell Culture**—HEK cells were cultured as described previously (9) and used upon reaching confluence. Before use cells were put into serum-free Dulbecco’s modified Eagle’s medium for 2 h. Unless stated otherwise, insulin treatment was with 1 μM insulin for 10 min. All experiments with HEK cells, except for those in Fig. 5, were performed with a line of HEK cells provided by Dr. Zhizhuang Zhao at the University of Washington. Those in Fig. 5 were performed with a line provided by Dr. Derek LeRoith at the National Institutes of Health. This latter line showed slightly greater insulin stimulation of IRS-4 tyrosine phosphorylation, and its content of insulin and IGF-1 receptors has previously been determined (10). In this latter set of experiments,
60-nm plates of cells in medium with 1 mg/ml bovine serum albumin were treated for 10 min with various concentrations of porcine insulin, human IGF-1 (Boehringer Mannheim), human IL-4 (R&D Systems), or human EGF (Upstate Biotechnology). The medium was aspirated, and the cells were lysed in 1.5 ml of SDS sample buffer with 20 mM dithiothreitol, 1 mM sodium vanadate, and a mixture of protease inhibitors (10 μM leupeptin, 1 mM pepstatin, 10 μM EP475, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotones). The SDS samples were held at 100 °C for 4 min, and the DNA in them sheared by passage through a syringe needle.

**Cell Fractionation—** Plates (10-cm) of untreated or insulin-treated HEK cells were washed with ice-cold 150 mM NaCl, 10 mM sodium phosphate, pH 7.4, and scraped into 2 ml of homogenization buffer containing 1% C₁₂E₉. Insoluble material was removed by centrifugation at 140,000 × g for 1 h to yield solubilized membranes and a pellet of organelles. The latter was resuspended in 6 ml of homogenization buffer containing 1% C₁₂E₉. The resuspended material was centrifuged at 140,000 × g for 1 h, to yield solubilized membranes and a pellet. The pellet was dissolved in SDS sample buffer.

**Immunoprecipitations—** Plates (10-cm) of untreated or insulin-treated HEK cells were lysed in 6 ml of homogenization buffer containing 1% C₁₂E₉. Insoluble material was removed by centrifugation at 140,000 × g for 1 h. Aliquots of the cleared lysates (0.8 ml containing about 1 mg of protein) were immunoprecipitated with 10 μg of antibodies against IRS-4, SHP-2, Grb2, or irrelevant IgG, or with 5 μl of antisera against PI 3-kinase at 4 °C for 2 h. The immune complexes were then collected on 20 μl of protein A-Sepharose for 2 h. Tyr(P) proteins were isolated by incubation of 0.8 ml of the cleared lysate with 20 μl of the monoclonal antibody against Tyr(P) 4G10 linked to agarose (Upstate Biotechnology).

**Immunoblotting—** Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore) for 2.5 h at 400 mA in 25 mM Tris, 190 mM glycine, 20% methanol, 0.005% SDS. Tyr(P) immunoblots were blocked with 3% bovine serum albumin in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4 (TBS), and then treated with anti-Tyr(P) at 4 μg/ml in TBS containing 0.2% albumin followed by the secondary antibody in the same buffer. Immunoblots with the other antibodies were blocked in 5% nonfat dry milk in TBS, and then treated with antibodies against IRS-4 at 4 μg/ml or against Grb2 or phospholipase Cγ at 0.5 μg/ml, or antisera against PI 3-kinase or SHP-2 at 1/1000 dilution in 1% milk in TBS and then with the secondary antibody in the same buffer. Membranes were washed with 0.3% Tween 20 in TBS. Blots were developed with goat anti-rabbit antibodies conjugated to horseradish peroxidase (Bio-Rad), followed by chemiluminescence detection (Fierce reagent).

**Immunofluorescence—** Immunofluorescence was carried out with subconfluent HEK cells grown on glass coverslips according to the method described previously (12). In this method cells were fixed with formaldehyde, permeabilized with saponin, and treated first with 5 μg/ml anti-IRS-4GST or, as a control, irrelevant rabbit immunoglobulin, and then with goat antibodies against rabbit immunoglobulin conjugated to fluorescein, as well as the DNA stain 4',6-diamidino-2-phenylindole. Anti-IRS-4GST, rather than anti-IRS-4C, was used for both immunofluorescence and immunoelectron microscopy (see below) because it was the more specific reagent. On an immunoblot of a total HEK cell lysate anti-IRS-4GST gave only a single band at 160 kDa, whereas anti-IRS-4C gave both this band and several bands of smaller sizes.

**Immunoelectron Microscopy—** Plates (10 cm) of basal and insulin-treated HEK cells were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 1 h. The cells were washed with this buffer and scraped into 1% paraformaldehyde in the same buffer. Ultrathin cryosectioning and immunogold labeling were carried out as described previously (13). Four different labeling experiments were performed with both untreated and insulin-treated cells, and approximately 500 gold particles were assigned to various organelles and counted with a section from each labeling experiment. The percentages of gold particles in the various locations from the four counts agreed with each other within ±3% (S.E.). Labeling associated with the plasma membranes, intracellular vesicles (mainly near the cell surface), and the cytoplasm was observed. In addition, some labeling of the mitochondria and nuclei, which amounted to 15 and 21% of the total, respectively, was found. However, the latter was probably nonspecific, since labeling of human HeLa cells with anti-IRS-3 gave about the same density of gold particles over the mitochondria and nuclei even though HeLa cells had no IRS-4 as assessed by immunoblotting. By contrast, the HeLa cells showed a lower density of labeling over the cytosol and virtually none over the plasma membrane. Consequently, in calculating the percentages of IRS-4 labeling of the plasma membranes, vesicles, and cytosol, we have not included the gold particles over the mitochondria and nuclei.

**Results**

**Subcellular Distribution of IRS-4—** Untreated (−) and insulin-treated (+) HEK cells were fractionated as described under “Experimental Procedures.” The total homogenate, cytosol (Cyt), membranes (Mem), and residual pellet (Pel) were immunoblotted for IRS-4 with anti-IRS-4C and for Tyr(P), IRS-1, IRS-2, and the 85-kDa subunit of PI3K. The Tyr(P) blot shows the signal at the mobility of IRS-4 (160 kDa). The loads per lane were as shown, where load 1 was derived from 0.3% of a 10-cm plate, except for the IRS-2 blot where load 1 was derived from 2.4% of a plate. The fractionation and analyses for IRS-4 and Tyr(P) were performed three times with similar results.

**RNA Samples for Human Tissue Distribution—** A human RNA master blot containing mRNA samples from 50 human tissues (7770-1) and a human multiple tissue Northern blot (7760-1) were purchased from CLONTECH.

**FIG. 1. Subcellular distribution of IRS-4 in HEK cells.** Untreated (−) and insulin-treated (+) HEK cells were fractionated as described under “Experimental Procedures.” The total homogenate, cytosol (Cyt), membranes (Mem), and residual pellet (Pel) were immunoblotted for IRS-4 with anti-IRS-4C and for Tyr(P), IRS-1, IRS-2, and the 85-kDa subunit of PI3K. The Tyr(P) blot shows the signal at the mobility of IRS-4 (160 kDa). The loads per lane were as shown, where load 1 was derived from 0.3% of a 10-cm plate, except for the IRS-2 blot where load 1 was derived from 2.4% of a plate. The fractionation and analyses for IRS-4 and Tyr(P) were performed three times with similar results.
primarily cytosolic, whereas the Tyr(P) protein was primarily in the membranes and pellet (Fig. 1), is further evidence for this conclusion.

IRS-4 in untreated cells exhibited significant Tyr(P) content, which increased approximately 3-fold overall in response to insulin (Fig. 1, Tyr(P) blot, total). This increase occurred mainly in the IRS-4 that fractionated with the membranes and pellet; IRS-4 in the cytosol showed little or no increase in Tyr(P) content.

Further information about the subcellular distribution of IRS-4 was obtained by immunofluorescence and immunoelectron microscopy. Immunofluorescence showed distinct staining at the boundary of the cell, weaker diffuse staining in the interior, and little or no staining of the nucleus (Fig. 2). Thus, a significant portion of the membrane-bound IRS-4 appeared to be at the plasma membrane. Insulin treatment did not alter the distribution of IRS-4. These results were confirmed by immunoelectron microscopy. Considerable labeling at the inner surface of the plasma membrane was observed (Fig. 3). The proportions of the IRS-4 at the plasma membrane, on the surface of vesicles, and in the cytosol of untreated and insulin-treated HEK cells were estimated by counting gold particles and found to be 39, 16, and 45%, respectively, for cells in both states (see “Experimental Procedures”). This distribution agrees reasonably well with that found by subcellular fractionation (Fig. 1).

Association of IRS-4 with SH2 Domain Proteins—The amino acid sequence of IRS-4 shows seven potential sites for tyrosine phosphorylation in motifs that should bind to the two SH2 domains on the 85-kDa regulatory subunit of PI 3-kinase, as well as one site in a motif expected to bind the SH2 domain of Grb2 and another site in a motif likely to bind to the NH2-terminal SH2 domain of the tyrosine phosphatase SHP-2 and of phospholipase Cγ (5). In order to determine whether these associations occurred, we immunoprecipitated IRS-4 from C12E9 lysates of untreated and insulin-treated HEK cells and then immunoblotted for PI 3-kinase, SHP-2, Grb2, and phospholipase Cγ, as well as for IRS-4 and Tyr(P) (Fig. 4A). For comparison, the Tyr(P) proteins were also immunoprecipitated and analyzed in the same way. Immunoprecipitation with irrelevant rabbit IgG served as the negative control (Fig. 4A). PI 3-kinase was associated with IRS-4 in lysates from both untreated and insulin-treated cells (Fig. 4A, lanes 8 and 9); Grb2 was associated with IRS-4 but only in lysates from insulin-treated cells. There was no detectable association of SHP-2 or phospholipase Cγ with IRS-4. The Tyr(P) immunoprecipitates showed the same pattern of associations as IRS-4 (Fig. 4A, lanes 12 and 13). This outcome is consistent with the observation that IRS-4 is the predominant Tyr(P) protein in HEK cells (see Ref. 9 and above). As expected, the immunoprecipitates with the irrelevant IgG showed none of the proteins (lanes 16 and 17).

To confirm the associations found by immunoprecipitation of IRS-4, we immunoprecipitated PI 3-kinase, Grb2, and SHP-2 and then immunoblotted the immunoprecipitates for IRS-4, as well as PI 3-kinase, Grb2, and SHP-2 (Fig. 4B). Under the conditions used, 75% or more of the PI 3-kinase, Grb2, and SHP-2 was immunoprecipitated (Fig. 4B, compare Unb with Total). In agreement with the results from the IRS-4 immuno-

**Fig. 2.** Subcellular localization of IRS-4 in HEK cells by immunofluorescence. Untreated and insulin-treated cells were stained with anti-IRS-4GST or irrelevant rabbit IgG (left side) and simultaneously for DNA (right side), as described under “Experimental Procedures.”
precipitation, IRS-4 was found associated with PI 3-kinase and Grb2, but there was little or no association with SHP-2 (Fig. 4B, lanes 8 and 9, 12 and 13, and 16 and 17, respectively). Approximately the same amount of IRS-4 was associated with PI 3-kinase in the lysates of untreated and insulin-treated cells (lanes 8 and 9), whereas more IRS-4 was associated with Grb2 in the lysate from insulin-treated cells than that from untreated cells (lanes 12 and 13). Finally, some Grb2 was present in the PI 3-kinase immunoprecipitates (lanes 8 and 9), and some PI 3-kinase was present in the Grb2 immunoprecipitates (lanes 12 and 13); in both cases more was present in the immunoprecipitates from the lysate of insulin-treated than untreated cells. Most likely the co-precipitations of Grb2 and PI 3-kinase are due to their simultaneous association with IRS-4 rather than to direct binding of Grb2 to PI 3-kinase.

Tyrosine Phosphorylation of IRS-4 in Response to Growth Factors—A number of growth factors besides insulin, including IGF-I and IL-4, but not EGF, elicit tyrosine phosphorylation of IRS-1 (3). We examined whether the same was true for IRS-4. In order to allow a direct comparison of IGF-I and insulin, these experiments were performed with a HEK line that was reported to contain about the same number of IGF-I and insulin receptors per cell (approximately 10,000 of each) (10). Fig. 5 indicates that IGF-I and insulin stimulated tyrosine phosphorylation of IRS-4 by about 4-fold at maximal concentrations, and that the half-maximal effect of each occurred at about 1 nM. Since there is no significant binding of insulin to the IGF-I receptor or of IGF-I to the insulin receptor at concentrations less than 10 nM (14), these results suggest that the two receptors are approximately equally active in tyrosine-phosphorylating IRS-4. The data in Fig. 5 also provide another demonstration that IRS-4 is the predominant Tyr(P) protein elicited by insulin and IGF-I in HEK cells.

Since Fig. 5 presents Tyr(P) immunoblots of whole cell lysates, it was remotely possible that the 160-kDa protein, which is tyrosine phosphorylated in response to IGF-I, was not IRS-4. However, we have demonstrated by immunoprecipitation that the 160-kDa Tyr(P) elicited by IGF-I as well as by insulin in this experiment was IRS-4. The SDS lysates of the untreated cells and cells treated with 10 nM IGF-I or insulin were made 0.3% in SDS and 1.5% in C12E9, and the IRS-4 was then immunoprecipitated with anti-IRS-4C. For all the samples approximately 75% of the IRS-4 and the same proportion of the 160-kDa Tyr(P) protein were immunoprecipitated, as assessed by immunoblotting the total lysates, the lysates after immunoprecipitation, and the immunoprecipitates with anti-IRS-4C and anti-Tyr(P) (data not shown).

Fig. 5 also shows that neither IL-4 nor EGF at 10 nM concentration for 10 min caused detectable tyrosine phosphorylation of IRS-4. Previous studies have shown that at this concentration and for this time period IL-4 treatment of HEK cells activates the transcription factor Stat6 (15), and EGF treatment activates the kinase ERK-1 (16). These studies thus indicate that HEK cells contain functional IL-4 and EGF receptors. However, it is uncertain whether the amounts of these receptors would be sufficient to cause detectable enhanced tyrosine phosphorylation of IRS-4. On the basis of Tyr(P) blotting, there appeared to be more insulin and IGF-I receptors than IL-4 and EGF receptors in the HEK cells. The tyrosine-phosphorylated β subunits of the activated insulin and IGF-I

Fig. 3. Subcellular localization of IRS-4 in HEK cells by immunoelectron microscopy. Ultrathin cryosections of HEK cells were labeled with anti-IRS-4GST followed by protein A 10-nm gold particles. A, labeling at the plasma membranes between cells (arrows indicate gold particles); B, labeling at ruffled portions of the cell borders, which was often relatively high; C, labeling associated with coated vesicles or pits at the cell surface (arrows), which was not uncommon. Bars: 200 nm.
receptors, which migrate at about 100 kDa, were very evident upon longer exposures of the Tyr(P) blot in Fig. 5. However, neither the activated tyrosine-phosphorylated forms of the JAK1 and JAK3 kinases, which are elicited by and complexed with the activated IL-4 receptor and migrate at about 130 kDa (17, 18), nor the activated tyrosine-phosphorylated EGF receptor, which migrates at about 180 kDa (19), were evident (data not shown).

Tissue Distribution of IRS-4—As an approach to the human tissue distribution of IRS-4, we attempted to determine the expression of its mRNA. A significant amount of mRNA was not detected in any tissue, and because of this outcome, only a brief description of these experiments will be presented. First, a human mRNA Dot Blot containing mRNA from 50 different human tissues including seven major fetal tissues (100–500 ng/spot) was probed with single-stranded digoxigenin-labeled cDNA corresponding to nucleotides 104–369 (5), as described by Keller et al. (8). No significant signal was given by any tissue, even though a positive control of 100 ng of HEK mRNA was analyzed. The human Dot Blot was reprobed with a 32P-riboprobe. No mRNA was detected with the digoxigenin-labeled probe. The riboprobe yielded some faint bands of sizes less than that of the IRS-4 mRNA in HEK cells (see below) after 4 days of exposure of the autoradiogram. In contrast, a Northern blot of the same amount (2 μg) of HEK mRNA probed at the same time with the same preparations of each probe yielded two strong bands, at 6 kilobases and greater than 9.5 kilobases (reported in Ref. 5).

**DISCUSSION**

Our results indicate that in HEK cells IRS-4 is the major tyrosine-phosphorylated protein elicited by the activated insulin and IGF-I receptors. Although the data does not demonstrate direct phosphorylation by the activated receptors, this is almost certainly the case. IRS-4 has a phosphotyrosine binding domain that is highly homologous to the one in IRS-1 that binds to these receptors and accounts in part for its direct phosphorylation (5, 21).

Despite the fact that HEK cells contain IRS-1 and IRS-2 as well as IRS-4, the former showed no tyrosine phosphorylation in response to insulin. We have previously estimated that HEK cells have about 25 times more IRS-4 mRNA than IRS-1 (9); and from the relative intensities of the IRS-4 and IRS-2 immunoblots (see Fig. 1), it seems likely that there is also a large excess of IRS-4 over IRS-2. In addition, from the nanogram amount of IRS-4 purified from an SDS lysate of HEK cells and its yield from the lysate as assessed by immunoblotting for Tyr(P), we have estimated that 1 mg of HEK cell protein contains about 0.3 μg of IRS-4 (Ref. 9 and data not shown). Since a 10-cm plate of HEK cells contains about 10 mg of protein and 5 × 10^6 cells, a single HEK cell contains about 2 × 10^6 IRS-4 molecules, an amount that is 100-fold greater than approximately 2 × 10^4 insulin and IGF-I receptors. These considerations thus suggest that no tyrosine phosphorylation of IRS-1 or IRS-2 occurred because the receptors were saturated with IRS-4. Studies in which a cell expresses roughly equal numbers of two or more IRSs will be needed to determine the relative efficiencies of the four IRSs as substrates for the insulin and IGF-I receptors.

Immunoelectron microscopy revealed that approximately 40% of the IRS-4 was located at the cytoplasmic surface of the plasma membrane, both in the unstimulated and insulin-treated state. The main basis for this association is almost certainly not a direct interaction with the insulin and IGF-I receptors, since as estimated above, there is not enough of these receptors to account for more than a few percent of IRS-4 bound to the plasma membrane. Moreover, if the association were largely due to the binding of IRS-4 via its phosphotyrosine binding domain to the receptors, then the amount at the plasma membrane should have increased in response to insulin, since tyrosine phosphorylation of a motif in the receptors is required for binding to the phosphotyrosine binding domain (22). More likely the basis for the association is the binding of the pleckstrin homology domain of IRS-4 to one or more of the PI phosphates concentrated in the inner leaflet of the plasma membrane. Pleckstrin homology domains in various proteins are known to bind to PI 4,5-bisphosphate, PI 3,4-bisphosphate, and PI 3,4,5-trisphosphate (23, 24). Alternatively, there may be a protein in the plasma membrane that binds specifically to IRS-4 via the pleckstrin homology domain or some other region of the latter. The observation that that the IRS-4 in the membrane and pellet fractions, but not that in the cytosol fraction, became more highly tyrosine phosphorylated in response to insulin suggests that the activated insulin receptor in the plasma membrane preferentially phosphorylates the IRS-4 associated with the membrane.

To our knowledge no earlier study has examined the subcellular distribution of an IRS by immunoelectron microscopy,
and thus direct comparison of the distribution of IRS-4 with those of other IRSs is not possible. The location of IRS-1 in 3T3-L1 adipocytes has previously been examined by subcellular fractionation (25). In 3T3-L1 adipocytes, IRS-1 is the predominant IRS present (26), so that the case is similar to IRS-4 in HEK cells. Only 3% was present in the plasma membranes of both untreated or insulin-treated adipocytes. Most (62%) was in the low density microsomes, with the remainder in the cytosol (35%). Thus, it may be that IRS-4 and IRS-1 are targeted to different locations in the cell, although it should be recognized that the distribution of IRS-1 upon subcellular fractionation may not necessarily coincide with its distribution in vivo. Our finding from subcellular fractionation that most of the IRS-1 is in the cytosol in HEK cells contrasts with the distribution in 3T3-L1 adipocytes. One possible explanation is that microsomal membranes of HEK cells lack the binding sites for IRS-1 that are present in the adipocytes. Alternatively, it may be that microsomal binding sites are present in HEK cells but are not available for IRS-1 because they are occupied by the more abundant IRS-4 (see above).

The associations of IRS-4 with PI 3-kinase and Grb2 directly demonstrates that like the other IRSs (1–3) IRS-4 is a docking and effector protein for specific SH2 domain-containing proteins. As is the case for IRS-1 (27), it is likely that the association of IRS-4 with PI 3-kinase results in the activation of this enzyme and the generation of PI 3,4-bisphosphates and 3,4,5-trisphosphates. These lipids in turn lead to activation of protein kinase B, and the latter in its activated form regulates a number of cellular processes, including protection of cells from apoptosis (28). Similarly, as in the case for IRS-1 (29), it is likely that the association of IRS-4 with Grb2, which is a linker to the guanine nucleotide exchange factor for Ras, leads to the elevation of the GTP form of Ras, which in turn stimulates the downstream mitogen-activated protein kinase cascade. In the case of both PI 3-kinase and the Grb2 complex, the localization of IRS-4 at the cytoplasmic surface of the plasma membrane will facilitate signaling, since PI 4,5-bisphosphate and the GDP form of Ras, the respective substrates, are located in the same place. In agreement with these expected roles for IRS-4 in signal transduction, treatment of HEK cells with IGF-I has been found to activate both protein kinase B and the mitogen-activated protein kinase ERK2 (30, 31). No association of IRS-4 was detected with SHP-2 or phospholipase C3, even though there is a tyrosine-phosphorylation motif in IRS-4 to which these would be expected to bind. It is possible that this site is not phosphorylated, but it could also be that the interaction occurs in the cell but not significantly in the lysate due to the large dilution of the proteins in the lysate.

Approximately the same amount of PI 3-kinase was found associated with IRS-4 from untreated and insulin-treated HEK cells, whereas the amount of associated Grb2 was increased by insulin treatment. The most likely explanation lies in the finding that IRS-4 from unstimulated cells contained some Tyr(P). If this Tyr(P) is mainly located in the YXXM motifs that bind to the SH2 of IRS-4, then association of IRS-4 with PI 3-kinase and Grb2 would prompt phosphorylation of Tyr in the single YVNM motif predicted to bind to the SH2 domain of Grb2. IRS-4 cells are transformed through integration of adenovirus genes (32). It is possible that the transformation in some way causes the tyrosine phosphorylation of IRS-4 in unstimulated cells, and that as a consequence there is partial continuous activation of PI 3-kinase, which may also contribute the transformed phenotype (28).

IRS-1 binds PI 3-kinase, Grb2, and SHP-2 (1 –3). IRS-2 binds PI 3-kinase, and has been shown to bind to the isolated SH2 domain of Grb2 but not to that from SHP-2 (26). IRS-3 binds PI 3-kinase but has not yet been examined for association with Grb2 or SHP-2 (4). Thus, the signaling properties of IRS-4 overlap with those of the other IRSs, but there are differences among the IRSs.

In various cell types IL-4, acting through the complex of its receptor with the JAK tyrosine kinases, elicits tyrosine phosphorylation of IRS-1 and IRS-2 (33, 34). On the other hand, abundant activated EGF receptor does not cause tyrosine phosphorylation of IRS-1 in adipocytes, whereas insulin does (19). Although no detectable tyrosine phosphorylation of IRS-4 in response to IL-4 or EGF treatment of HEK cells was observed, this outcome could simply be due to insufficient amounts of the IL-4 or EGF receptors. Definitive determination of the effect of IL-4 will require examination of the tyrosine phosphorylation of IRS-4 in cell lines where IL-4 has been shown to cause tyrosine phosphorylation of IRS-1 and IRS-2, whereas the effect of EGF should be assessed in a cell line with equal amounts of insulin and EGF receptors.

In our initial effort at determining the tissue distribution of IRS-4, we have been unable to detect the mRNA in human tissues, even though it is easily detected in HEK cells. This result suggests that IRS-4 is not abundant and/or is expressed only in certain specific cell types. By contrast, IRS-1 and IRS-2 have widespread tissue distributions (26, 35), and IRS-3 is...
present in a number of tissues (36). In the future it should be possible to determine the tissue distribution of IRS-4 mRNA and protein in a species more amenable to experimentation, such as mouse, by sensitive methods such as reverse transcription PCR, immunoprecipitation followed by immunoblotting, and immunofluorescence. We are currently generating mice with targeted disruption of the IRS-4 gene. Comparison of these with normal mice should allow unambiguous determination of the IRS-4 tissue distribution, as well as provide information about the physiological role of IRS-4.

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