Insulin Receptor Substrate 4 Associates with the Protein IRAS*

Received for publication, December 12, 2001, and in revised form, March 12, 2002 Published, JBC Papers in Press, March 23, 2002, DOI 10.1074/jbc.M111838200

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The insulin receptor substrates (IRSs) are key components in signaling from the insulin receptor, and consequently any proteins that interact with them are expected to participate in insulin signaling. In this study we have searched for proteins that interact with IRS-4 by identifying the proteins that coimmunoprecipitated with IRS-4 from human embryonic kidney 293 cells by microsequencing through mass spectrometry. A group of proteins was found. These included phosphatidylinositol 3-kinase, a protein previously identified as an IRS-4 interactor, and several proteins for which there was no previous evidence of IRS-4 association. One of these proteins, named IRAS, that had been found earlier in another context was examined in detail. The results from the overexpression of IRAS, where its amount was about the same as that of IRS-4, indicated that IRAS associated directly with IRS-4 and showed that the increased complexation of IRS-4 with IRAS did not alter the insulin-stimulated tyrosine phosphorylation of IRS-4 or the association of IRS-4 with phosphatidylinositol 3-kinase or Grb2. On the other hand, overexpression of IRAS enhanced IRS-4-dependent insulin stimulation of the extracellularly regulated kinase. The domains of IRAS and IRS-4 responsible for the association of these two proteins were identified, and it was shown that IRAS also associates with IRS-1, IRS-2, and IRS-3.

The insulin receptor substrates (IRS)-1, IRS-2, IRS-3, and IRS-4 are a family of four similar proteins that play a key role in signaling from the insulin receptor (reviewed in Ref. 1). The activated insulin receptor phosphorylates each IRS on multiple tyrosine residues. The tyrosine-phosphorylated form of the IRS then binds to various SH2 domain-containing signaling proteins. These include the lipid kinase PI 3-kinase and the linker protein Grb2, which is complexed with Sos, the guanine nucleotide exchanger for Ras. Association of the IRS with PI 3-kinase stimulates its activity, and the resulting elevation of the lipid PI 3,4,5-trisphosphate leads to activation of Akt kinases. Association of the IRS with the Grb2-Sos complex enhances guanine nucleotide exchange on Ras, and the resulting elevation of the GTP form of Ras leads to activation of ERK kinases. The stimulation of PI 3-kinase is a required part of the signal transduction pathway to many of the cellular effects of insulin (2). The architecture of each IRS consists of an amino-terminal PH and PTB domain and a large carboxyl-terminal region with the sites of tyrosine phosphorylation. The PTB domain binds directly to the activated insulin receptor, and both the PH and PTB domains are required for efficient tyrosine phosphorylation of the IRS.

In an effort to identify additional proteins involved in insulin signaling, we and others have searched for proteins that associate with the IRSs. To date three approaches have been taken. First, proteins that were expected to interact with an IRS for various reasons have been selected, and their association with the IRS has been examined, generally by means of immunoprecipitation and immunoblotting (1, 3–7). Second, expression libraries have been screened with recombinant IRS (8–11). Third, yeast two-hybrid screens have been performed with portions of the IRS as bait (12, 13). These approaches have been mainly applied to IRS-1 and IRS-2 and have yielded a number of IRS-interacting proteins (1, 3–13).

In the present study, we have searched for proteins that interact with IRS-4 by another method, that of coimmunoprecipitation with IRS-4 followed by separation of the associated proteins by SDS-PAGE and microsequencing of them by mass spectrometry. An advantage of this method over the screening of expression libraries and the yeast two-hybrid screen is that it provides a direct display of all of the main IRS-associated proteins and their relative amounts in the cell type of interest.

We chose IRS-4 in HEK293 cells for examination because less was known about the proteins that interact with IRS-4 and because IRS-4 is very abundant in this cell type (14).

Using this approach, we have identified a group of proteins that coimmunoprecipitated with IRS-4 and are thus likely to interact with it. We selected one of these proteins, known as IRAS, for further study. The cDNA encoding IRAS had previously been cloned in a screen of an expression library with antisera raised against the receptor for imidazoline compounds and named IRAS for imidazoline receptor antisera selected (15). By itself IRAS may not be an imidazoline receptor, because expression of the protein in COS and SF9 insect cells did not result in imidazoline binding (15). However, it is possible IRAS is part of an imidazoline receptor, because expression of IRAS in Chinese hamster ovary cells resulted in an increase in imidazoline-binding sites (15). In this study we provide evidence that full-length IRAS associates directly with IRS-4 and that this association does not affect insulin-stimulated tyrosine phosphorylation of IRS-4 or the binding of PI 3-kinase and PI 3,4,5-trisphosphate to IRS-4.
Grb2 to IRS-4. However, overexpression of IRAS enhanced the activation of ERK by insulin.

**EXPERIMENTAL PROCEDURES**

**Antibodies—**Antibodies were purchased from the following sources (listed with the catalog numbers in parentheses): PI 3-kinase (60-195), Tyr(P) (G1010-agarose, 16-101), IRS-1 (60-248), and IRS-2 (60-506) from Upstate Biotechnology, Inc.; Tyr(P) (RC20, horseradish peroxidase) from Transduction Laboratories; phosphorylated ERK1/2 (9101), phosphorylated Akt Threonine (9275), and phosphorylated Akt Serine (9271) from New England BioLabs; ERK1/2 (93), Akt 1/2 (8312), Grb2 (255), IRS-2 (1555), and HA epitope tag (7392) from Santa Cruz Biotechnology; and the T7 epitope tag (69522-3) from Novagen. Affinity-purified rabbit antibodies against the carboxy-terminal 16 aa of peptide IRS-4 and against aa 994–1197 of IRS-4 were the ones described in Ref. 14.

Four types of rabbit antibodies were generated against human IRAS. These were against an amino terminus peptide consisting of aa 7–21, a carboxy-terminal peptide consisting of aa 1395–1412, the glutathione S-transferase fusion protein with the PX domain of IRAS (aa 10–125), and the glutathione S-transferase fusion protein with the acidic domain of IRAS (aa 559–705). Each antibody was affinity-purified on the immobilized peptide or glutathione S-transferase fusion protein, as described in Ref. 17. All four antibodies immunoprecipitated IRAS, but none was effective for immunoprecipitation (data not shown). Unless noted otherwise, all of the experiments using an antibody against IRAS were performed with the one against the carboxy-terminal peptide because this antibody was most sensitive for immunoblotting.

**Plasmids—**Human IRS-4 cDNA in pcDNA3.1 was as described in Ref. 15. The following were generous gifts: human IRS-4 cDNA with an amino-terminal HA epitope tag in pCCEPL (18) from Dr. Derek LeRoith; SRHis vector, which places His6, T7, and Xpress tags at the amino terminus of cDNA inserts (19) from Dr. Shigeo Ohno; mouse IRS-1 with a Myc tag at its carboxy-terminal (20) from Dr. Bryan Wolf; mouse IRS-2 in CMVHis (21) from Dr. Xiao Sun; and rat IRS-3 with an HA epitope in pCEFL (18) from Dr. Derek LeRoith; Human IRAS cDNA in pcDNA3.1 was as described in Ref. 14.

**Identification of Proteins Coimmunoprecipitating with IRS-4—**Initially the proteins that coimmunoprecipitated with IRS-4 were examined on a small scale. Cleared lysate (0.5 ml of a lysate in 3% C12E9 containing half of a 10-cm plate) was incubated with 5 μg of antibody against the carboxy-terminal terminus of IRS-4 or with irrelevant IgG at 4 °C for 2 h, and the immunoadsorbents were collected on Pansorbin (5 μl; Calbiochem) or protein A-Sepharose (20 μl; Amershams Biosciences) for 2 h. The beads were washed three times with lysate buffer containing 0.3% C12E9. The immunoprecipitates were solubilized either by boiling the beads at 100 °C for 5 min in SDS sample buffer containing 20 mM dithiothreitol (reduced samples) or by mixing at room temperature with SDS sample buffer containing 8 M urea instead of dithiothreitol (nonreduced samples). The SDS samples were separated by SDS-PAGE on gradient gels of 5–12% and 5–15% for the reduced and nonreduced samples, respectively, together with known amounts of standard proteins (Bio-Rad). The separated proteins were electrophoretically transferred to nitrocellulose in 200 mA for 16 h at 25 °C. Tris, 190 mM glycine, 20% methanol, 0.005% SDS. The nitrocellulose was stained with colloidal gold total protein stain (Bio-Rad).

To isolate the coimmunoprecipitating proteins for microsequencing, the cleared lysate in 3% C12E9 derived from ten 10-cm plates of HEK293 cells was incubated with 50 μg of antibody against IRS-4 or of irrelevant IgG. The immunoprecipitates were collected on 50 μl of protein A-Sepharose, the beads were washed three times with lysate buffer, and proteins were released with 150 μl of SDS sample buffer under reducing or nonreducing conditions. The SDS samples were separated by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad). The protein bands that were present in the IRS-4 immunoprecipitate but not in the immunoprecipitate with the irrelevant IgG were excised from the gel for sequence analysis.

The protein bands were subjected to in-gel reduction, carboxylamidomethylation, and tryptic digestion (Promega). Multiple peptide sequences were determined in a single run by microcapillary reverse-phase chromatography directly coupled to a Finnigan LCQ quadrupole ion trap mass spectrometer equipped with a custom nanoelectrospray source. The column was packed in-house with 5 μm of C18 support into a New Objective one-piece 75-μm internal diameter column terminating in a 8.5-μm tip. The flow rate was 190 nL/min. The ion trap was programmed to acquire successive sets of three scan modes consisting of full scan mass spectra (MS) over alternating ranges of 395–800 m/z or 800–1300 m/z, followed by two data-dependent scans on the most abundant ion in those full scan scans. These data-dependent scans allowed the automatic acquisition of a high fragmentation (zoom scan) to determine charge state and the MS and MS/MS spectra for peptide sequence information. MS/MS spectra were acquired with a relative collision energy of 30% and an isolation width of 2.5 Da, and recurring ions were dynamically excluded. Interpretation of the resulting MS/MS spectra of the peptides was facilitated by programs developed at the Harvard Microchemistry Facility and by data base correlation with the algorithm Sequest (24, 25).
IRAS, a Protein Associated with IRS-4

RESULTS

Proteins Coimmunoprecipitating with IRS-4—To find proteins that interact with IRS-4, we immunoprecipitated IRS-4 from lysates of HEK293 cells with an antibody against the carboxyl terminus of the protein and analyzed for associated proteins by SDS-PAGE and protein staining. Because the relatively large amount of the precipitating antibody stained strongly and obscured other proteins in the same size range, the immunoprecipitates were examined both under reducing conditions, where the heavy and light chains of the antibody ran at ~200 kDa, and under nonreducing conditions, where the intact antibody ran at about 130 kDa. As shown in Fig. 1, a number of proteins, ranging in size from 30 kDa to greater than 200 kDa, were present in the IRS-4 immunoprecipitate but not the control immunoprecipitate. One protein at 66 kDa that was identified as heat shock protein 70 (see below) was present in both but appeared more abundant in the IRS-4 immunoprecipitate. In a similar experiment, the proteins that coimmunoprecipitated with IRS-4 from lysates of HEK293 cells treated with insulin for 10 min were compared with those associated in the unstimulated state under reducing conditions. No difference in the associated proteins was detected (data not shown).

To identify the proteins coimmunoprecipitating with IRS-4, the immunoprecipitation was carried out on a larger scale. The associated proteins at 200, 130, 116, 110, 97, 85, 66, 32, and 31 kDa were analyzed by ion trap tandem mass spectrometry as described under “Experimental Procedures.” Table I summarizes the results of this analysis. These protein bands were identified as: IRAS, a deubiquitinating enzyme, a WD repeat protein, a mixture of the catalytic subunit of PI 3-kinase and a fragment of IRS-4, another fragment of IRS-4, a mixture of the regulatory subunit of PI 3-kinase and heat shock protein 90, heat shock protein 70, 14-3-3 protein, and a mixture of 14-3-3 β and ζ proteins, respectively.

Association of IRAS with IRS-4—We decided to investigate the interaction of the protein IRAS with IRS-4, because the limited information about IRAS suggested that it might be an interesting signaling protein. IRS-4 was immunoprecipitated from lysates of untreated and insulin-treated HEK293 cells, and the immunoprecipitates, lysates, and depleted lysates were immunoblotted for IRS-4, IRS-3, and Tyr(P). Approximately 90% of the IRS-4 was depleted from the lysate by immunoprecipitation, and approximately the same percentage of the IRAS coimmunoprecipitated with the IRS-4 (Fig. 2A, compare lanes 11 and 12 versus lanes 5–10). The depleted IRS-4 and IRAS were recovered in the immunoprecipitates (lanes 1 and 2), and insulin treatment had no effect on the amount of IRAS that coimmunoprecipitated with IRS-4 (lanes 1 and 2). Control immunoprecipitations with irrelevant rabbit immunoglobulin yielded no IRS-4 or IRAS (lanes 3 and 4). To be certain that insulin treatment was effective, the samples were blotted for Tyr(P). As expected from our previous study (14), insulin treatment markedly increased the Tyr(P) content of IRS-4 (compare lane 1 with lane 2 and lanes 5–7 with lanes 8–10). No tyrosine phosphorylation of IRAS was detected.

The results presented above do not exclude the possibility that rather than being associated with IRS-4, IRAS was directly immunoprecipitated by the antibody against IRS-4. To exclude this possibility, we first attempted to determine whether IRS-4 coimmunoprecipitated with antibody against IRAS. Unfortunately, neither the antibody against a peptide from the carboxyl end of IRAS that was routinely used for

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**Table I**

| Size | Name | Accession number | Length | Peptides* |
|------|------|------------------|--------|-----------|
| kDa | | | | |
| 200 | IRAS | AAAC3104 | 1504 | 29 |
| 130 | Deubiquitinating enzyme | AAF32262 | 1087 | 16 |
| 116 | WD repeat protein | AAF82044 | 1121 | 13 |
| 110 | PI 3-kinase catalytic subunit | AAB9753 | 1068 | 3 |
| 97 | IRS-4 fragment | NP_003595 | 1257 | 17 |
| 85 | PI 3-kinase regulatory subunit | CCA65888 | 728 | 24 |
| 66 | Heat shock protein 90 | HHHU54 | 724 | 7 |
| 66 | Heat shock protein 70 | AAA62266 | 641 | 16 |
| 32 | 14-3-3 | P42655 | 255 | 5 |
| 31 | 14-3-3 | P31946 | 246 | 1 |
| 14-3-3 | | P29312 | 245 | 4 |

* Number sequenced. 
† Mixture of the two proteins. 
‡ Five peptides common to the β and ζ forms were also sequenced.
immunoblotting nor three other affinity-purified antibodies against other regions of IRAS (see “Experimental Procedures”) were effective in immunoprecipitation. However, further evidence for the association of IRAS with IRS-4 was obtained by showing that immunoprecipitation of IRS-4 with two other antibodies, one against a glutathione S-transferase fusion protein with aa 994–1197 of IRS-4 and the other against Tyr(P) (4G10-agarose), resulted in the coimmunoprecipitation of IRAS (data not shown). Moreover, we have coexpressed HA-tagged IRS-4 and IRAS in Cos7L cells through transient transfection, immunoprecipitated the IRS-4 with anti-HA antibody from lysate of these cells and found by immunoblotting that the immunoprecipitate contained IRAS as well as IRS-4 (Fig. 2), lysates of untreated or insulin-treated (10 min) HEK293 cells were immunoprecipitated with antibody against IRS-4 or irrelevant rabbit immunoglobulin (rIgG). The immunoprecipitates (IP), lysates (Lys), and lysates after immunoprecipitation (depleted lysates, dLys) were immunoblotted (IB) for IRAS, IRS-4, and Tyr(P), as described under “Experimental Procedures.” The 1× load was derived from 1 mg of cell lysate. A repetition of this experiment gave similar results. B, Cos7L cells (10-cm plates) were transiently transfected with 4 μg each of plasmids encoding IRAS and control vector pcDNA 3.1 (lanes 1 and 3) or 4 μg each of plasmids encoding IRAS and HA-tagged IRS-4 (lanes 2 and 4). The cells were lysed in nonionic detergent, and a portion of each lysate was immunoprecipitated with anti-HA. Samples of the immunoprecipitates (IP) and lysates (Lys) were immunoblotted for IRAS and IRS-4. The 1× load is equivalent to 100 μg of lysate protein.

**Fig. 2. Coimmunoprecipitation of IRAS with IRS-4.** A, lysates of untreated or insulin-treated (10 min) HEK293 cells were immunoprecipitated with antibody against IRS-4 or irrelevant rabbit immunoglobulin (rIgG). The immunoprecipitates (IP), lysates (Lys), and lysates after immunoprecipitation (depleted lysates, dLys) were immunoblotted (IB) for IRAS, IRS-4, and Tyr(P), as described under “Experimental Procedures.” The 1× load was derived from 1 mg of cell lysate. A repetition of this experiment gave similar results. B, Cos7L cells (10-cm plates) were transiently transfected with 4 μg each of plasmids encoding IRAS and control vector pcDNA 3.1 (lanes 1 and 3) or 4 μg each of plasmids encoding IRAS and HA-tagged IRS-4 (lanes 2 and 4). The cells were lysed in nonionic detergent, and a portion of each lysate was immunoprecipitated with anti-HA. Samples of the immunoprecipitates (IP) and lysates (Lys) were immunoblotted for IRAS and IRS-4. The 1× load is equivalent to 100 μg of lysate protein.

**Fig. 3. Overexpression of IRAS in HEK293 cells.** HEK293 cells were stably transfected with IRAS cDNA or vector, as described under “Experimental Procedures.” A, IRAS-overexpressing cell lines D5 and A2 and the vector control line were lysed in SDS sample buffer, and the lysates were immunoblotted for IRAS. The 1× load corresponds to 50 μg of protein. B, nonionic detergent lysates of the D5 and vector cell lines were immunoprecipitated with antibody against IRS-4; the immunoprecipitates were separated by SDS-PAGE, and the gel was stained with Coomassie Blue. The immunoprecipitates were derived from 3 mg of cell lysate. A repetition of these experiments gave similar results.

Expression of IRAS resulted in the most of the IRS-4 being associated with IRAS. In addition, it should be noted that overexpression of IRAS did not lead to an increase in the amounts of the other proteins in the IRS-4 immunoprecipitate. The relative amounts of these other proteins were about 1/5 or less of the amounts of IRAS and IRS-4, as assessed by protein staining (Figs. 1 and 3B). The fact that IRAS was the only protein in the IRS-4 immunoprecipitate from D5 cells present in an amount equivalent to IRS-4 indicates that IRAS associated directly with IRS-4 rather than through a third protein.

**Subcellular Distributions of IRAS and IRS-4**—Untreated and insulin-treated HEK293 cells overexpressing IRAS (D5 line) or containing vector were fractionated into cytosol and total organelles. The latter fraction was solubilized with nonionic detergent to yield solubilized membranes and a pellet that consists largely of nuclei and cytoskeleton. In agreement with our previous results (14), IRS-4 was located in all three fractions in roughly equal amounts (Fig. 4). Neither insulin treatment nor overexpression of IRAS altered the distribution of IRS-4. Moreover, overexpression of IRAS did not change the amount of IRS-4 in HEK293 cells. With both the vector and the D5 lines, the subcellular distribution of IRAS was coincident with that of IRS-4. In the experiments shown in Fig. 4, insulin treatment was for 2 min. The subcellular distributions of IRS-4 and IRAS were also compared for normal HEK293 cells in the untreated state and after 10 min of exposure to insulin. The distributions were similar to those in Fig. 4, and insulin treatment resulted in no change (data not shown).

**Effect of IRAS on Tyrosine Phosphorylation of IRS-4**—The effect of IRAS overexpression on the insulin-stimulated tyrosine phosphorylation of IRS-4 in the HEK293 cells was examined. The cells of the D5 and vector lines were untreated or treated with insulin for various times, lysed in SDS sample buffer, and immunoblotted for Tyr(P) (Fig. 5). The intensity of tyrosine phosphorylation in the basal state was the same in the two lines. Insulin treatment resulted in a 4-fold increase in phosphorylation within 2 min that persisted for at least 1 h. Overexpression of IRAS had no effect on the time course or extent of insulin-stimulated IRS-4 phosphorylation. Reprobing the membrane for IRS-4 again showed that the amount of

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**Fig. 2. Coimmunoprecipitation of IRAS with IRS-4.** A, lysates of untreated or insulin-treated (10 min) HEK293 cells were immunoprecipitated with antibody against IRS-4 or irrelevant rabbit immunoglobulin (rIgG). The immunoprecipitates (IP), lysates (Lys), and lysates after immunoprecipitation (depleted lysates, dLys) were immunoblotted (IB) for IRAS, IRS-4, and Tyr(P), as described under “Experimental Procedures.” The 1× load was derived from 1 mg of cell lysate. A repetition of this experiment gave similar results. B, Cos7L cells (10-cm plates) were transiently transfected with 4 μg each of plasmids encoding IRAS and control vector pcDNA 3.1 (lanes 1 and 3) or 4 μg each of plasmids encoding IRAS and HA-tagged IRS-4 (lanes 2 and 4). The cells were lysed in nonionic detergent, and a portion of each lysate was immunoprecipitated with anti-HA. Samples of the immunoprecipitates (IP) and lysates (Lys) were immunoblotted for IRAS and IRS-4. The 1× load is equivalent to 100 μg of lysate protein.

**Fig. 3. Overexpression of IRAS in HEK293 cells.** HEK293 cells were stably transfected with IRAS cDNA or vector, as described under “Experimental Procedures.” A, IRAS-overexpressing cell lines D5 and A2 and the vector control line were lysed in SDS sample buffer, and the lysates were immunoblotted for IRAS. The 1× load corresponds to 50 μg of protein. B, nonionic detergent lysates of the D5 and vector cell lines were immunoprecipitated with antibody against IRS-4; the immunoprecipitates were separated by SDS-PAGE, and the gel was stained with Coomassie Blue. The immunoprecipitates were derived from 3 mg of cell lysate. A repetition of these experiments gave similar results.
IRS-4 was almost the same in the D5 and vector lines. Similar results were obtained in an experiment comparing the tyrosine phosphorylation of IRS-4 in the A2 and vector lines.

**Effect of IRAS on IRS-4 Signaling Complexes**—We have previously shown that IRS-4 associates with PI 3-kinase and Grb2 in HEK293 cells (14). Although overexpression of IRAS did not alter tyrosine phosphorylation of IRS-4, it seemed possible that it might affect the formation of the complexes between IRS-4 and these signaling proteins. To investigate this possibility, IRS-4 was immunoprecipitated from lysates of unstimulated and insulin-treated HEK293 D5 and vector cells, and the immunoprecipitates were immunoblotted for the 85-kDa subunit of PI 3-kinase and Grb2, as well as for IRAS and IRS-4 (Fig. 6A). As we had found previously (14), PI 3-kinase was associated with IRS-4 in both the unstimulated and insulin-treated state, presumably because in HEK293 cells IRS-4 contains some Tyr(P) in the untreated state. On the other hand, insulin stimulated the association of Grb2 with IRS-4. Overexpression of IRAS had no significant effect on the association of either PI 3-kinase or Grb2 with IRS-4, even though, as expected, it resulted in approximately seven times as much IRAS present in the IRS-4 immunoprecipitate.

PI 3-kinase and Grb2 are considered to bind directly to IRS-4 through interaction of their SH2 domains with specific Tyr(P) motifs on IRS-4 (14). Consequently, the coimmunoprecipitation of IRAS, PI 3-kinase, and Grb2 with IRS-4 could be due to the formation of binary complexes of each protein with IRS-4, to the formation of ternary complexes of IRS-4 with IRAS and either PI 3-kinase or Grb2, and/or to the formation of a quaternary complex of all four proteins. To determine whether such a quaternary complex formed and if so how overexpression of IRAS affected it, we immunoprecipitated PI 3-kinase and Grb2 from lysates of unstimulated and insulin-treated HEK293 D5 and vector cells and immunoblotted the immunoprecipitates for IRAS, IRS-4, PI 3-kinase, and Grb2. As shown in Fig. 6B, the PI 3-kinase immunoprecipitate contained IRAS, IRS-4, and Grb2. This finding indicates that a quaternary complex of the four proteins occurs. In agreement with the results from the immunoprecipitnat of IRS-4, the amounts of IRS-4 and IRS-4 in the PI 3-kinase immunoprecipitate were increased only very slightly by insulin treatment, whereas the amount of Grb2 in the immunoprecipitate was increased markedly by insulin treatment. Overexpression of IRAS resulted in the expected increase of ~7-fold of IRAS in the PI 3-kinase immunoprecipitate but had no effect on the amounts of IRS-4 and Grb2 in the complex.

The composition of the Grb2 immunoprecipitates also indicated the formation of a quaternary complex (Fig. 6C). IRAS, IRS-4, and PI 3-kinase coimmunoprecipitated with Grb2, and insulin treatment markedly enhanced the amounts of all three proteins in the Grb2 immunoprecipitate to the same extent. Overexpression of IRAS increased the amount of IRS in the Grb2 immunoprecipitates but did not alter the amounts of IRS-4 and PI 3-kinase. Immunoblotting of the lysates used for immunoprecipitation showed that the HEK293 D5 and vector lysates contained the same amounts of PI 3-kinase and Grb2 (data not shown).

As described above, the relative amounts of the proteins in the IRS-4 immunoprecipitate from D5 cells indicated that IRAS does not associate directly with PI 3-kinase or Grb2. In the case of Grb2, this possibility could also be excluded because the amount of Grb2 coimmunoprecipitating with IRS-4 increased with insulin treatment, whereas the amount of IRAS did not (Fig. 6A). A similar argument cannot be made in the case of PI 3-kinase, because insulin treatment only slightly enhanced the association of PI 3-kinase with IRS-4. However, we have found by immunoblotting that immunoprecipitation of 90% of the IRS-4 from a lysate of HEK293 cells coimmunoprecipitated ~90% of the IRAS, whereas immunoprecipitation of 90% of the PI 3-kinase resulted in coimmunoprecipitation of less than 25% of the IRAS (Fig. 2 and data not shown). This result also indicates that IRAS does not bind directly to PI 3-kinase.

**Effect of IRAS on Activation of Kinases**—Even though overexpression of IRAS had no effect on insulin-stimulated tyrosine phosphorylation of IRS-4 or on complex formation with IRS-4, it seemed possible that it might effect the insulin activation of the ERK or Akt kinases. Activation of the ERK1/2 was examined by immunoblotting SDS lysates of untreated and insulin-treated cells with antibody specific for the phosphorylated, activated form. Insulin treatment caused the rapid appearance of the phosphorylated form of ERK1/2 (Fig. 7). The extent of ERK1/2 activation, as assessed by the intensity of the signal, was approximately four times greater in the D5 line of HEK293 cells than in the vector line (Fig. 7, lane 2 versus lane 7) and approximately two times greater in the A2 line than in the vector line (Fig. 7, lane 9 versus lane 7). The larger effect in the D5 line than the A2 line correlates with the greater extent of overexpression of IRAS in the D5 line (Fig. 3). All three lines expressed approximately the same amount of ERK1/2 (Fig. 7). These comparisons were made with cells treated for 2 min with insulin, because immunoblotting SDS samples of D5 and vector cells for phosphorylated ERK1/2 treated with insulin for various times showed that phosphorylation was maximal at this time and returned to near basal level in 60 min (data not shown).

To determine whether the effect of IRAS overexpression on insulin activation of ERK1/2 was dependent upon the association of IRAS and IRS-4, we examined ERK1/2 activation in Cos7L cells, which normally contain no IRAS and little or no IRAS-4, upon expression of IRAS, IRS-4, or both through transient transfection. Expression of the combination of IRAS and IRS-4 resulted in ~4-fold enhancement of insulin activation of ERK1/2 (Fig. 8, compare lanes 2 and lane 8), whereas expression of either IRAS or IRS-4 alone had little or no effect on
FIG. 6. Complex formation with IRS-4. Lysates of untreated and insulin-treated (2 min) D5 and vector HEK293 cells were immunoprecipitated with antibodies against IRS-4 (A), PI 3-kinase (B), and Grb2 (C), as described under “Experimental Procedures.” The immunoprecipitates (IP) were immunoblotted (IB) for IRS-4, IRS-2, PI 3-kinase, and Grb2. The 1× load was derived from 0.4 mg of lysate protein. A repetition of this experiment gave similar results. The signal for PI 3-kinase is a doublet, which may be due to the presence of both the α and β isotypes of the 85-kDa subunit. The slight increase in the amount of PI 3-kinase associated with IRS-4 in response to insulin (~25%) seen in A (first and second lanes) was not observed in the replicate experiment.

FIG. 7. Effect of stable IRAS overexpression on insulin activation of ERK in HEK293 cells. D5 and vector HEK293 cells were left untreated or treated with insulin for 2 min and dissolved in SDS sample buffer. The samples were immunoblotted for phosphorylated ERK1/2 (pERK) and for ERK1/2. The 1× load corresponds to 50 µg of protein. A repetition of this experiment gave similar results.

FIG. 8. Effect of transient IRAS and IRS-4 expression on insulin activation of ERK1/2 in Cos7L cells. Cos7L cells in 3.5-cm wells were transfected with 0.5 µg of plasmid encoding IRAS, IRS-4, or a combination of both. The total amount of plasmid was maintained at 1 µg throughout with pcDNA3.1 vector. The cells were treated with insulin or not for 2 min and then solubilized in SDS sample buffer, and the samples were immunoblotted for phosphorylated ERK1/2 (pERK), ERK1/2, IRS-4, and IRS-4. The 1× load corresponds to 3% of a 3.5-cm plate of cells for ERK1/2 and 1% of a plate for the other proteins. A repetition of this experiment gave similar results.

Insulin activation of ERK1/2 (Fig. 8, compare lane 2 and lane 4 or 6). The requirement of both IRAS and IRS-4 for enhancement of insulin activation of ERK1/2 strongly suggests that this enhancement is dependent upon their association. Further evidence for a role of IRAS in specifically augmenting insulin activation of ERK1/2 was obtained by comparing the effect of its overexpression in HEK293F cells by transient transfection upon insulin and EGF activation of ERK1/2. As expected from the results of stable transfection of HEK293 cells, overexpression of IRAS enhanced insulin activation of ERK1/2 (Fig. 9A, compare lane 2 and lane 4). However, it had no effect on EGF activation of ERK1/2 (Fig. 9B, compare lane 2 and lane 4).

Activation of Akt1/2 in the control and IRAS-overexpressing D5 HEK293 lines was examined by immunoblotting with antibodies specific for the phosphorylated Thr and Ser on the activated kinases, which are designated Thr308 and Ser473 for their locations on Akt 1. In this case immunoblotting SDS lysates yielded blots that were too messy for interpretation, and consequently we immunoprecipitated the Akt from lysates and then immunoblotted the immunoprecipitates. Untreated cells contained the phosphorylated forms of Akt; insulin treatment caused an ~1.5-fold increase in the phosphorylation of Akt Thr308 and a lesser increase in the phosphorylation of Akt Ser473 (Fig. 10). The IRAS-overexpressing D5 line showed the same degree of Akt phosphorylation on each site as the vector-transfected line in both the untreated and insulin-treated states. Reprobing of the blots for Akt1/2 showed that the two cell lines expressed the same amounts of Akt.

Domains of IRAS and IRS-4 That Interact—To determine the region of IRAS that interacts with IRS-4, we coexpressed T7 epitope-tagged sections of IRAS with IRS-4 in Cos7L cells by transient transfection and examined the anti-T7 immunoprecipitates from cell lysates for IRS-4. IRS-4 contains a PX domain at its amino terminus and an acidic region in its middle (15). Consequently IRAS was divided into its PX domain, the combined PH and PTB domains located at its amino terminus and an acidic region in its middle (23) and the carboxy-terminal segment (Fig. 11A). However, it had no effect on EGF activation of ERK1/2 (Fig. 9B, compare lane 2 and lane 4).

Activation of Akt1/2 in the control and IRAS-overexpressing D5 HEK293 lines was examined by immunoblotting with antibodies specific for the phosphorylated Thr and Ser on the activated kinases, which are designated Thr308 and Ser473 for their locations on Akt 1. In this case immunoblotting SDS lysates yielded blots that were too messy for interpretation, and consequently we immunoprecipitated the Akt from lysates.
mainly with C-IRS4 (Fig. 11B, right top panel). There was also a small amount of IRAS in the immunoprecipitate of the PH-PTB portion that was not detected in the immunoprecipitate with a control immunoglobulin (Fig. 11B, right top panel, compare IgG and PH-PTB). Association of IRAS with Other IRSs—To assess whether IRAS could associate with other IRSs, we coexpressed IRAS and each of the IRSs in Cos7L cells. Each IRS protein was then immunoprecipitated from its cell lysate, and the immunoprecipitate was immunoblotted for IRAS. In each case IRAS coimmunoprecipitated with the IRS (Fig. 12, right panel). A repetition of this experiment gave similar results.

DISCUSSION

In this study we have searched for novel proteins that may participate in insulin signaling by identifying proteins that communoprecipitated with IRS-4. Because of the great sensitivity of peptide sequencing by ion trap mass spectrometry, this approach is entirely feasible. The validity of this approach was established by the identification of the catalytic and regulatory subunits of PI 3-kinase in the IRS-4 immunoprecipitate. Previously we had shown by immunoblotting that IRS-4 associates with PI 3-kinase in HEK293 cells (Ref. 14 and Fig. 6). Moreover, the identification of the 14-3-3 proteins in the IRS-4 immunoprecipitate is consistent with earlier studies showing that 14-3-3 proteins associate with IRS-1 and IRS-2 (4, 5, 10).

In addition to PI 3-kinase and the 14-3-3 proteins, the IRS-4 immunoprecipitate specifically contained a number of other proteins: IRAS, a deubiquitinating enzyme, a WD domain protein, and heat shock protein 90. In this study we focused on characterizing the interaction of IRAS with IRS-4. Additional studies will be required to determine whether the other proteins in the IRS-4 immunoprecipitate interact directly with IRS-4 or one of its associated proteins and, if so, what effects they have on IRS-4 function.

Several types of evidence strongly indicate that IRAS is associated with IRS-4. First, IRAS communoprecipitated with IRS-4 using four different antibodies to communoprecipitate IRS-4. Second, IRAS was present together with IRS-4 in the immunoprecipitates of PI 3-kinase and Grb2, two proteins known to associate directly with specific Tyr(P) motifs in the IRSs via their SH2 domains. Moreover, the association of IRAS and IRS-4 is almost certainly a direct one rather than one through a third protein, because in the IRS-4 immunoprecipitate from the D5 cell line IRAS was the only protein specifically present in an amount equivalent to IRS-4.
In normal HEK293 cells, almost all of the IRAS was complexed with IRS-4, but because the amount of IRAS was only about 1/2 that of the IRS-4, only a small fraction of the IRS-4 was complexed with IRAS. Consequently, to investigate the role of IRAS in IRS-4 signaling, we overexpressed IRAS so that its level was approximately the same as that of IRS-4, and most of the IRS-4 was complexed with IRAS. Overexpression of IRAS had no effect on the insulin-stimulated tyrosine phosphorylation of IRS-4. Moreover, it did not affect of the amounts of PI 3-kinase or Grb2 complexed with IRS-4 in the untreated or insulin-treated state. Overexpression of IRAS also had no effect on insulin activation of Akt, a kinase that is downstream of PI 3-kinase (2), as assessed by immunoblotting with phosphospecific antibodies. Untreated cells contained activated phosphorylated Akt, and there was only a small increase in its amount in response to insulin. The presence of activated Akt in untreated cells was probably due to constitutive activation of PI 3-kinase, because in untreated HEK293 cells the IRS-4 contained some Tyr(P) and was complexed with PI 3-kinase.

The absence of an effect of overexpression of IRAS on Akt activation shows that it does not inhibit Akt activation. However, it is uncertain whether IRAS overexpression could enhance insulin-dependent Akt activation under the appropriate conditions, because it is not known whether Akt is fully activated in the insulin-treated normal HEK293 cells.

In contrast to the results with Akt, there was no detectable activated phosphorylated ERK in untreated HEK293 cells, and insulin treatment caused its activation. Moreover, overexpression of IRAS enhanced the insulin-stimulated activation of ERK by about 4-fold. Activation of ERK in response to insulin is initiated with the tyrosine phosphorylation of the IRSs and Shc by the activated insulin receptor. These proteins then bind Grb2, which is associated with Sos, the guanine nucleotide exchange protein for Ras. This association activates the exchange activity of Sos and so leads to an elevation of the GTP form of Ras, which in turn activates the mitogen-activated protein kinase cascade and thus results in activation of ERK (1). It remains to be determined how the overexpression of IRAS stimulated this signaling pathway. Overexpression of IRAS had no significant effect on the amounts of Grb2 or ERK in the cells, nor on the amount of Grb2 that associated with IRS-4 in response to insulin. The finding that the enhancement of insulin activation of ERK by IRS in Cos7 cells required coexpression of IRS-4 indicates that the effect depends upon the association of IRAS with IRS-4. Moreover, the fact that EGF activation of ERK in HEK293F cells was not enhanced by IRAS overexpression is also consistent with this conclusion. EGF activation of ERK also proceeds via Shc, Ras, and the mitogen-activated protein kinase cascade (26). Thus, if IRAS were functioning by interaction with one of these components, it would be expected to enhance EGF activation of ERK as well. A possible explanation for the effect of IRAS on insulin activation of ERK is that the IRS-4-Grb2-Sos complex is more active in stimulating guanine nucleotide exchange on Ras when the complex also contains IRAS.

Through expression of the various parts of IRAS and IRS-4, it was found that the main interaction between these proteins occurs via the carboxyl-terminal region of each. In light of a previous study by Burks et al. (13), this finding was somewhat surprising. These authors employed the yeast two-hybrid system to identify proteins that interact with the PH domain of IRS-1 and IRS-2. They found several proteins, each of which contained an acidic domain, although IRAS was not one of them. They showed that these proteins associated with IRS-1 and IRS-2 through binding of the acidic domain to the PH domain. Thus, on the basis of this study, we expected to find the acidic domain of IRAS interacting with the PH domain of IRS-4. It remains possible that such an interaction also contributes to the association. IRAS also associates with IRS-1, IRS-2, and IRS-3, although our data suggest that it associates considerably more weakly with them than with IRS-4. Thus, with these other IRSs the main interaction could be between the acidic domain and the PH domain.

While this work was in progress, a study appeared in which a portion of IRAS was found to interact with integrin α5 subunit by a screen in the yeast two-hybrid system (27). The study characterized a truncated version of IRAS without the PX domain at its amino terminus, which was named Nischarin. Overexpression of Nischarin caused reorganization of actin filaments and reduced cell migration dependent upon α5β1 integrin. The relation of these effects to the association of IRAS with IRS-4 remains to be established.

In the future it will be important to determine the cellular role of IRAS. The fact that IRAS is highly expressed in the brain (28) and IRS-4 is strongly expressed in the hypothalamus (29) suggests that the association of the two may play a role in the hypothalamus. In addition it will be important to establish whether IRAS is a portion of the brain imidazole receptor and, if so, whether the binding of an imidazole affects the interaction of IRS with IRAS-4. In this regard, transfection of IRAS cDNA into Chinese hamster ovary cells has been reported to produce binding sites with nanomolar affinity for mazonidine, the ligand most selective for the I1 imidazole receptor (15, 30). However, expression of IRAS in COS and SF9 cells did not lead to an increase in binding sites (15), and the D5 and A2 cell lines overexpressing IRAS described here showed no change in the number or affinity of imidazole binding sites.2

Acknowledgments—We thank Valeria R. Fantin for preliminary experiments on IRS-4-interacting proteins, Kerry A. Pierce and Eric Spooner for technical assistance in the peptide sequencing, and Michael Chen for preparation of the plasmid containing IRAS cDNA.

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