Interaction of Insulin Receptor Substrate-1 with the ø3A Subunit of the Adaptor Protein Complex-3 in Cultured Adipocytes*

(Received for publication, June 10, 1998, and in revised form, August 4, 1998)

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Signaling through the insulin receptor tyrosine kinase involves its autophosphorylation in response to insulin and the subsequent tyrosine phosphorylation of substrate proteins such as insulin receptor substrate-1 (IRS-1). In basal 3T3-L1 adipocytes, IRS-1 is predominantly membrane-bound, and this localization may be important in targeting downstream signaling elements that mediate insulin action. Since IRS-1 localization to membranes may occur through its association with specific membrane proteins, a 3T3-F442A adipocyte cDNA expression library was screened with non-tyrosine-phosphorylated, baculovirus-expressed IRS-1 in order to identify potential IRS-1 receptors. A cDNA clone that encodes ø3A, a small subunit of the AP-3 adaptor protein complex, was demonstrated to bind IRS-1 utilizing this cloning strategy. The specific interaction between IRS-1 and ø3A was further verified by in vitro binding studies employing baculovirus-expressed IRS-1 and a glutathione S-transferase (GST)-ø3A fusion protein. IRS-1 and ø3A were found to co-fractionate in a detergent-resistant population of low density membranes isolated from basal 3T3-L1 adipocytes. Importantly, the addition of exogenous purified GST-ø3A to low density membranes caused the release of virtually all of the IRS-1 bound to these membranes, while GST alone had no effect. These results are consistent with the hypothesis that ø3A serves as an IRS-1 receptor that may dictate the subcellular localization and the signaling functions of IRS-1.

Insulin exerts its specific biological effects in fat and skeletal muscle by binding to and activating its tyrosine kinase receptor present on the cell surface. Subsequent signaling from this receptor involves its autophosphorylation and tyrosine phosphorylation of substrate proteins that then act as docking sites for various SH2 domain-containing proteins. A major substrate of the insulin receptor tyrosine kinase is the insulin receptor substrate-1 (IRS-1). This protein was first identified as a 185-kDa phosphoprotein from insulin-stimulated Fao hepatoma cells (1), and it was subsequently cloned (2) and purified from rat liver (3) and mouse 3T3-L1 adipocytes (4, 5). In unstimulated rat and 3T3-L1 adipocytes, a significant fraction of IRS-1 is found associated with an intracellular membrane fraction (6–8). Upon insulin stimulation, tyrosine-phosphorylated IRS-1 recruits PI 3-kinase to these intracellular membranes (9), one mechanism hypothesized to account for insulin’s specific effects on increased glucose uptake (7, 10). Subsequently, and in a time- and dose-dependent manner, insulin causes the translocation of IRS-1-PI 3-kinase complexes from these intracellular membranes into the cytoplasm (6–8), an event that is accompanied by apparent serine/threonine phosphorylation of IRS-1 as well as other membrane components (6, 8, 11). Major questions that remain are how IRS-1 is localized to intracellular membranes, what the function(s) of such localization is, and how the membrane association of IRS-1 is regulated by insulin.

In order to answer these questions, more information is needed to establish the specific intracellular localization of IRS-1 within insulin-responsive cells. One mechanism by which proteins translocate between different subcellular compartments is via transport vesicles (for reviews, see Refs. 12–18). These vesicles possess specific structural coat proteins on their surfaces that selectively and efficiently drive and direct protein trafficking. For example, clathrin-coated vesicles (reviewed in Ref. 19) contain triskelia of clathrin proteins as well as heterotetrameric adaptor protein (AP) complexes (20–24). Clathrin-coated vesicles bud from two distinct membrane compartments, the plasma membrane and the trans-Golgi network (TGN), and the composition of the adaptor protein complex is dependent upon the source of the budding vesicle. AP-1 is present in TGN-derived vesicles, while AP-2 complexes associate with the plasma membrane (22, 23). AP-1 and AP-2 complexes consist of a b-adaptin subunit (b1 or b2), an a-AP (AP-2) or g-adaptin (AP-1) subunit, a m subunit (m1 or m2), and a ø subunit (ø1 or ø2) (22, 23). Functionally, a and g subunits specify associated vesicular proteins and the contents of the vesicle, while b subunits mediate clathrin attachment to the membrane (reviewed in Refs. 14 and 19). Functional roles for the smaller subunits (m, ø) are less well defined, although results from previous studies demonstrate an interaction between m subunits and tyrosine-based sorting signals (25–27). Additionally, deletion of the ø1 yeast homolog in Saccharomyces cerevisiae mutant with a defective clathrin heavy chain allele enhances the effects of certain clathrin-mediated processes (28).

Recently, a clathrin-independent complex was described that co-localizes with markers for both the TGN and endosomes (29–32). This complex, AP-3, contains a b-adaptin subunit with NTA, nitritrotiacetic acid; kb, kilobase pair(s); MES, [2-(N-morpholino)-ethanesulfonic acid].

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† Supported by Juvenile Diabetes Foundation International Postdoctoral Fellowship 396189.
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§ The abbreviations used are: IRS-1, insulin receptor substrate-1; AP, adaptor protein; TGN, trans-Golgi network; LDM, low density membrane; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; SSC, standard saline citrate; GST, glutathione S-transferase; BSA, bovine serum albumin; PI, phosphatidylinositol; kDa, kilodalton.
isosforms designated βA and B (32, 33), which is homologous to β-NAP, a neuron-specific, non-clathrin-associated phosphoprotein related to β-COP (34). AP-3 also possesses a δ-adaptin subunit, designated p160, which is homologous to the α- and γ-adaptins, a μ subunit, isosforms that are designated p47A and B, and εA and B subunit isoforms (29, 32). Functionally, each of the four subunits in the AP-3 complex of S. cerevisiae appears essential for cargo-selective transport to the yeast vacuole, since deletion of any of these subunits results in the mislocalization of alkaline phosphatase and the vacuolar t-SNARE, Vam3p, to a nonvacuolar compartment (35, 36). Additionally, it was demonstrated that the eye pigment defect in a double-stranded oligonucleotide containing the Drosophila melanogaster ganet mutant might be caused by compromised function of the δ subunit (37).

In the present studies, we sought to identify proteins that bind non-tyrosine-phosphorylated IRS-1 and hence might be candidates for directing its membrane localization. We report here that a cDNA clone encoding the adaptin subunit εA was isolated from a cultured adipocyte cDNA expression library based on its ability to bind radiolabeled, baculovirus-expressed IRS-1. The interaction between εA and IRS-1 was verified by in vitro binding studies using tagged, recombinant fusion proteins and by membrane receptor binding studies in which exogenous εA effectively competed for endogenous IRS-1 bound to adipocyte intracellular membranes. Finally, immunoblot analysis of 3T3-L1 adipocyte subcellular fractions revealed that εA and IRS-1 co-fractionate in detergent-insoluble low density membranes (LDMs). The identification of the adaptin subunit, εA, as a potential receptor for IRS-1 in adipocyte intracellular membranes may facilitate our understanding of the intracellular targeting of IRS-1 and its role in insulin signaling.

**EXPERIMENTAL PROCEDURES**

**Materials—** Baculovirus expression vector pAcHLTA and linearized BaculoGold DNA were from Pharmingen. Nickel-NTA resin was from Qiagen. [γ-32P]ATP (6000 Ci/mmol) and enhanced chomeliuminescence detection reagents were from NEN Life Science Products. The catalytic subunit of protein kinase A and glutathione-agarose were from Sigma. Prepacked PD-10 columns and pGEX2TK were purchased from Amersham Pharmacia Biotech. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Jerzy Lab Supply. Horseradish peroxidase-conjugated goat anti-rabbit antibody was from Boehringer Mannheim. Rabbit anti-IRS-1 was purchased from Novagen. A catalytic subunit of protein kinase A was obtained from Life Technologies, and the zymosan kinase D enzyme. The reaction was quenched by the addition of 350 μl of buffer containing 10 mM NaP, pH 8.0, 10 mM NaPP, and 10 mM EDTA. Labeled IRS-1 was separated from unincorporated [γ-32P]ATP on a PD-10 column equilibrated in buffer containing 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl. Peak fractions were determined by scintillation counting and SDS-polyacrylamide gel electrophoresis (PAGE) and were pooled.

**Northern Blot Analysis—** Total cellular RNA was isolated from murine adipocytes, rat fat pads, and 3T3-L1 fibroblasts using TRIzol reagent. Equal amounts of RNA were run on a agarose-formaldehyde gel and were subjected to autoradiography. Positive plaques were identified, isolated, and purified by further rounds of screening. Excision of Bluestreak phagemids was according to the manufacturer's protocol, and sequencing from both strands was performed using an automated sequencer (Applied Biosystems model 373). Nucleotide and deduced amino acid sequences were then subjected to data base searches to determine homology to known cDNAs and proteins.

**Expression Cloning of εA from a 3T3-F442A Murine Adipocyte cDNA Expression Library—** Approximately 106 plaques from a 3T3-F442A murine adipocyte cDNA expression library made in the λZapII system were screened with baculovirus-expressed IRS-1 protein labeled with [35S]methionine. The strategy was planned out according to the manufacturer's specifications and allowed to grow for 6 h at 37 °C. The agar was overlaid with nitrocellulose filters impregnated with 10 mM IPTG, and plaques were left to grow overnight at 37 °C. Plates were then chilled at 4 °C for 1 h before the filters were removed from the agar. Filters were washed three times in buffer 1 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 1 mM DTT) at room temperature for 15 min/wash. The filters were then blocked for 4–5 h at 4 °C in buffer 2 (10 mM Triss-HCl, pH 7.5, 250 mM NaCl, 5% nonfat dry milk, 1 mM DTT) and subsequently incubated overnight at 4 °C in fresh buffer 2 containing 106 cpm/ml 32P-labeled IRS-1. Filters were washed several times in buffer 1 at room temperature, air-dried, and subjected to autoradiography. Positive plaques were identified, isolated, and purified by further rounds of screening. Excision of Bluestreak phagemids was according to the manufacturer's protocol, and sequencing from both strands was performed using an automated sequencer (Applied Biosystems model 373). Nucleotide and deduced amino acid sequences were then subjected to data base searches to determine homology to known cDNAs and proteins.

**Construction and Affinity Purification of εA Fusion Protein—** In order to subclone εA into pGEX2TK, full-length εA cDNA was amplified by polymerase chain reaction using EcoRI-digested full-length εA cDNA as template and an oligonucleotide primer to introduce a 5′-NdeI site. The resulting 1.7-kb product was cloned into pGEX2TK, and a lysate was used to transform E. coli for screening. The resulting plasmid, pGEX2TK-εA, was transformed into E. coli strainicultures were supplemented with 0.2× SSC, 0.1% SDS for 30 min at 65 °C, and then subjected to autoradiography. A Northern blot of various murine adult tissues was probed with 3× 105 cpm/ml εA cDNA 32P-labeled by random priming (41). The filter was rinsed briefly at room temperature; washed with 0.1× SSC, 0.1% SDS for 30 min at 65 °C; and then subjected to autoradiography. A Northern blot of total cellular RNA isolated from murine fat pads, rat fat pads, rat skeletal muscle, 3T3-L1 fibroblasts, and 3T3-L1 adipocytes was hybridized at 42 °C with 3× 105 cpm/ml 32P-labeled by random priming. The filter was rinsed briefly at room temperature, washed with 0.2× SSC, 0.1% SDS for 30 min at 45 °C, and then subjected to autoradiography.

A high titer baculovirus stock was amplified according to Pharmingen’s specifications. The 3T3-L1 fibroblasts and 3T3-L1 adipocytes were plated and harvested as described in the text. The resulting supernatants were centrifuged and the pellet was washed once with STE buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM EDTA), frozen in ethanol/dry ice, and stored at −70 °C. The pellet was thawed rapidly and resuspended in STE containing 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml clarified lysates for 1 h at 4 °C. The resin was washed five times with 10 volumes of wash buffer, and the protein was eluted with buffer containing 50 mM NaP, pH 6.0, 300 mM NaCl, 10% glycerol, and 500 mM imidazole. Peak protein fractions were pooled and dialyzed at 4 °C against buffer containing 25 mM Hepes and 150 mM NaCl. The protein association assay was determined by the method of Bradford (38), and aliquots were frozen at −80 °C.

**Labeling of His-IRS-1 Protein—** 100 μg of purified IRS-1 was incubated in kinase buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 12 mM MgCl2, 1 mM dithiothreitol (DTT)), 1 unit/μl protein kinase A catalytic subunit, and 1 μCi of [γ-32P]ATP at 4 °C for 30 min in a final volume of 100 μl according to a previously published procedure (39). The reaction was quenched by the addition of 550 μl of buffer containing 10 mM NaP, pH 8.0, 10 mM NaPP, and 10 mM EDTA. Labeled IRS-1 was separated from unincorporated [γ-32P]ATP on a PD-10 column equilibrated in buffer containing 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl. Peak fractions were determined by scintillation counting and SDS-polyacrylamide gel electrophoresis (PAGE) and were pooled. The probe was kept at 0–4 °C and was used the same day for incubation with nitrocellulose filters in expression cloning procedures.

Expression Cloning of εA from a 3T3-F442A Murine Adipocyte cDNA Expression Library—Approximately 106 plaques from a 3T3-F442A murine adipocyte cDNA expression library made in the λZapII system were screened with baculovirus-expressed IRS-1 protein labeled with [35S]methionine. The strategy was planned out according to the manufacturer's specifications and allowed to grow for 6 h at 37 °C. The agar was overlaid with nitrocellulose filters impregnated with 10 mM IPTG, and plaques were left to grow overnight at 37 °C. Plates were then chilled at 4 °C for 1 h before the filters were removed from the agar. Filters were washed three times in buffer 1 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 1 mM DTT) at room temperature for 15 min/wash. The filters were then blocked for 4–5 h at 4 °C in buffer 2 (10 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5% nonfat dry milk, 1 mM DTT) and subsequently incubated overnight at 4 °C in fresh buffer 2 containing 106 cpm/ml 32P-labeled IRS-1. Filters were washed several times in buffer 1 at room temperature, air-dried, and subjected to autoradiography. Positive plaques were identified, isolated, and purified by further rounds of screening. Excision of Bluestreak phagemids was according to the manufacturer's protocol, and sequencing from both strands was performed using an automated sequencer (Applied Biosystems model 373). Nucleotide and deduced amino acid sequences were then subjected to data base searches to determine homology to known cDNAs and proteins.
leucocytes. Lysozyme was then added to a final concentration of 0.5 mg/ml, and the suspension was left on ice with occasional agitation for 20 min. The cell suspension was sonicated until it no longer viscous. Triton X-100 was added to a final concentration of 1%, and the suspension was incubated with gentle agitation on ice for 30 min. The suspension was centrifuged for 10 min at 12,000 × g at 4 °C. The pellet was resuspended in 100 mM Tris-HCl, pH 7.5, containing 2 mM NaCl and 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, and recentrifuged as above. This step was repeated; the pellet was resuspended in 6 M urea with 1 mM DTT and 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin; and the suspension was recentrifuged. The supernatant was dialyzed to remove urea and then incubated with glutathione-agarose beads for 1–2 h at 4 °C. The beads were washed extensively with STE buffer containing protease inhibitors, and bound fusion protein was eluted with an equal volume of buffer consisting of 50 mM Tris-HCl, pH 8.0, with 10 mM reduced glutathione. Aliquots were frozen at −20 °C.

In Vitro Binding of IRS-1 and o3A—Equal amounts of purified baculovirus-expressed, histidine-tagged IRS-1, and either purified GST-o3A or the negative controls, GST-Rab5C or GST alone, were incubated in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 2 h at 4 °C. Nickel-NTA beads, washed in TBS with 0.1% bovine serum albumin (BSA), were added to precipitate the IRS-1, and the beads were then washed extensively in buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM β-mercaptoethanol, 0.5% Tween 20, 0.1% BSA, 100 mM imidazole. The washed beads were boiled in SDS sample buffer, and bound proteins were resolved by SDS-PAGE and then transferred to nitrocellulose filters. Filters were blocked in TBS containing 0.1% Triton X-100, 3% dry milk, and 0.5% BSA and then incubated with anti-GST antibodies. Filters were incubated with horseradish peroxidase-conjugated antibodies to rabbit IgG, followed by detection by chemiluminescence.

Additionally, 32P-labeled IRS-1, prepared as described above, was incubated for 2 h at 4 °C with either GST-o3A or GST alone in equal amounts. In order to precipitate the GST fusion proteins, glutathione-agarose beads were added, and the beads were washed sequentially with wash buffer alone, wash buffer containing 0.5 mM NaCl, and finally with wash buffer alone. The beads were then boiled in SDS sample buffer, and bound proteins were resolved by SDS-PAGE. The gels were fixed, dried, and subjected to autoradiography for detection of bound, labeled IRS-1.

Cell Fractionation of 3T3-L1 Adipocytes—3T3-L1 fibroblasts were differentiated into adipocytes as described previously (42). Cells were serum-starved overnight in Dulbecco’s modified Eagle’s medium plus 0.5% BSA, and cellular fractions were prepared exactly as described (6). Protein concentration of the resuspended membrane fractions and the concentrated cytosol was determined by the method of Bradford (38).

For preparation of the Triton X-100-insoluble fraction, equal amounts of LDMs were resuspended in 0.25% (N-morpholino)ethanesulfonic acid (MES) buffer containing 50 mM MES, pH 6.5, 150 mM NaCl, 50 mM NaF, 100 μM vanadate, 30 mM NaPP, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. An equal volume of MES buffer containing 2% Triton X-100 was added to the LDMs so that the final concentration of Triton X-100 was 1%. The membranes were incubated on ice for 30 min and then centrifuged at 350,000 × g in a TL-100 ultracentrifuge (Beckman). The pellet was resuspended in MES buffer. Equal protein (30 μg) from each fraction was subjected to SDS-PAGE, transferred to nitrocellulose filters, and the filters were blocked as described above. Filters were then incubated with either anti-IRS-1 or anti-o3 antibodies. Filters were incubated with horseradish peroxidase-conjugated antibodies to rabbit IgG, followed by detection by chemiluminescence.

Release of Membrane-bound IRS-1 by GST-o3A—10 μg of LDM were resuspended in buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 100 μM vanadate, 30 mM NaPP, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Resuspended LDMs were incubated with buffer alone or increasing amounts (3–75 μg) of GST-o3A or 75 μg of GST alone for 2 h at 4 °C. The samples were centrifuged at 350,000 × g for 30 min in a TL-100 ultracentrifuge, and supernatants were transferred to fresh tubes. The pellets and equal aliquots of the supernatants were boiled in SDS sample buffer and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose filters, and the filters were incubated with anti-IRS-1 antibodies. Filters were incubated with horseradish peroxidase-conjugated antibodies to rabbit IgG, followed by detection by chemiluminescence.

RESULTS

It was previously demonstrated that in basal 3T3-L1 adipocytes, IRS-1 is predominantly associated with intracellular membranes (6–8) and that following insulin stimulation of these cells, PI 3-kinase is recruited to this membrane-bound IRS-1 (9). These findings are significant in that they suggest a discrete role for membrane-bound IRS-1 to deliver PI 3-kinase to a specific intracellular locale (7, 10, 43), a step that is perhaps critical to the observed functional role that PI 3-kinase plays in insulin-stimulated glucose transport (44–49). Evidence indicates that the interaction of IRS-1 with intracellular membranes does not occur through the pleckstrin homology domain of IRS-1 (50, 51), suggesting that interactions between IRS-1 domains and membranes may be protein/protein- as well as lipid/protein-mediated. In order to identify putative membrane-localized receptors for IRS-1 in cultured adipocytes, a 3T3-F442A murine adipocyte expression library was screened for proteins that could bind 32P-labeled non-tyrosine-phosphorylated IRS-1. DNA prepared from a purified positive plaque obtained using this screening strategy was sequenced, and the nucleotide and deduced amino acid sequences were subjected to data base searches to determine homology to known cDNAs and proteins. The 1.12-kb clone contains 76 nucleotides of 5′ noncoding sequence, an open reading frame of 582 nucleotides, and 466 nucleotides of 3′ noncoding sequence (Fig. 1). The open reading frame exhibits 95% homology at the nucleotide level and 100% identity at the amino acid level with human o3A (Fig. 1), a protein with a predicted molecular weight of 21,732 and an isoelectric point of 5.2 (30, 31). o3A was recently shown to be a small subunit of AP-3, an adaptin complex present in endosomes and the TGN (31, 32). Amino acid sequences that are underlined and designated as domains 1 and 2 are motifs found in the small subunits of AP-1, AP-2, and AP-3.

Northern blot analyses were performed to determine whether the expression pattern of o3A in murine tissues differs from that in human tissues. Results presented in Fig. 2A show a single hybridizing mRNA species with an apparent size of approximately 1.5 kb in all murine tissues, a size consistent with that reported for human o3A (30, 31). The tissue distribution of o3A in murine tissues differs substantially from the human profile in that the highest mRNA levels are present in testis, liver, lung, kidney, and brain with nondetectable levels present in heart, skeletal muscle, and spleen. In contrast, the abundance of o3A in different human tissues showed the highest mRNA levels to be present in heart and testis, intermediate levels to be present in brain, liver, skeletal muscle, and spleen, and lower levels to be present in kidney and lung (31). We next examined the abundance of o3A mRNA in different tissues and cells used as fat cell models. Fig. 2B illustrates that a 1.5-kb o3A mRNA species is present in high levels in cultured 3T3-L1 fibroblasts and that this mRNA species reproducibly increases 2-fold following differentiation of the fibroblasts into adipocytes. In contrast, o3A mRNA is not detected in total cellular RNA prepared directly from rat fat pads or mouse fat pads or in total cellular RNA from adipocytes prepared from rat fat pad. In order to verify that equal amounts of RNA were loaded in each lane of the Northern blot presented in Fig. 2, A and B, the blots were stripped and reprobed with β-actin cDNA (data not shown).

The results presented in Fig. 2, A and B, demonstrate that under high stringency conditions, o3A mRNA is not detectable in primary adipocytes or skeletal muscle prepared from adult mouse or rat. However, when a filter similar to that shown in Fig. 2B was hybridized with 32P-labeled o3A cDNA followed by low stringency washes, it was found that additional abundant mRNA species that are recognized by our probe exist in these
cell types (Fig. 2C). Most notably, a transcript of slightly slower mobility than the 1.5-kb s3A mRNA is the major hybridizing species in rat skeletal muscle under low stringency conditions. In addition, as shown in Fig. 2C, approximately equal amounts of both transcripts are present in primary adipocytes from rat and mouse using the lower stringency washing conditions.

In order to confirm that s3A binds IRS-1, the interaction between IRS-1 and s3A was examined in vitro binding experiments as depicted in Fig. 3. Equal amounts of purified baculovirus-expressed histidine-tagged IRS-1 and either purified GST-s3A or the negative controls, GST-Rab5C or GST alone (Fig. 3A), were incubated together, and then nickel-NTA beads were added to precipitate the IRS-1. Anti-GST immunoblot analysis of the beads (Fig. 3B) and the supernatants (data not shown) demonstrates that approximately 5-fold more GST-s3A than negative control proteins (GST and GST-Rab5C) binds immobilized histidine-tagged-IRS-1. Results from control experiments (data not shown) indicate that approximately 10% of the total GST-s3A binding to IRS-1 immobilized on the beads is nonspecific and occurs with the beads alone, while 70% of the total GST binding to the immobilized IRS-1 occurs nonspecifically. The results of the converse experiment are presented in Fig. 3, C and D. 32P-Labeled IRS-1 was incubated with either GST-s3A or the negative control GST immobilized onto glutathione-agarose beads as described under "Experimental Procedures," and bound and eluted IRS-1 was detected by autoradiography. 3-Fold more IRS-1 bound to GST-s3A than to GST alone (Fig. 3D), consistent with the results shown in Fig. 3B. Taken together, the data shown in Fig. 3 demonstrate that a specific interaction occurs between IRS-1 and s3A.

To examine the localization of s3A and IRS-1 in cultured
adipocytes, membrane and cytosolic fractions were prepared from 3T3-L1 adipocytes, and these subcellular fractions were immunoblotted with affinity-purified anti-peptide antibodies recognizing IRS-1 and both the A and B isoforms of 3A. As shown in Fig. 4 and as previously published (6–8), under basal conditions, most of the IRS-1 protein in 3T3-L1 adipocytes is present in the LDM fraction. Insulin stimulation causes a dose- and time-dependent decrease of IRS-1 in the LDMs with a nearly complete elimination of IRS-1 detected in the supernatants. Additionally, corresponding decreases in immunodetectable IRS-1 were observed in the cytosol (Fig. 4).

To investigate whether 3A functions as an IRS-1 receptor in vivo, a receptor competition binding assay was performed utilizing LDMs prepared from basal 3T3-L1 adipocytes. We hypothesized that if IRS-1 is bound to 3A in these membranes, then it should be possible to compete with endogenous 3A for binding to endogenous IRS-1 by adding exogenous GST-3A. Equal amounts of LDM protein were incubated with increasing amounts of GST-3A, the membranes were pelleted, and both the membrane pellets with increasing GST-3A resulted in increasing amounts of immunodetectable IRS-1 in the supernatants, while similar amounts of GST (Fig. 5) or another negative control, GST-Rab5c (data not shown), did not increase the IRS-1 detected in the supernatants. Additionally, corresponding decreases in immunodetectable IRS-1 were observed in the membrane pellets with increasing GST-3A. The addition of exogenous GST-3A to the LDMs did not result in selective

IRS-1 detection decreases in the LDMs with a nearly complete elimination of IRS-1 detected in the supernatants. Additionally, corresponding decreases in immunodetectable IRS-1 were observed in the cytosol (Fig. 4). These data presented are similar to observations published by Clark et al. (52, 53) that IRS-1 is present in detergent-resistant LDMs. The presence of both IRS-1 and 3A in the LDM fraction of cultured adipocytes is consistent with the demonstrated interaction of IRS-1 and 3A in the expression cloning format and the in vitro binding experiments, suggesting the hypothesis that 3A functions as the IRS-1 receptor in intracellular membranes of cultured adipocytes.
IRS-1 Interaction with σ3A

A Blot: Anti-IRS-1

Cytosol PM LDM Triton Pellet

IRS-1

B Blot: Anti-σ3

Ins: - - + - - + - +

Fig. 4. IRS-1 and σ3 co-localize to the same cellular fractions. 30 μg of each 3T3-L1 adipocyte fraction (with or without (+) / 100 nm insulin (Ins)) were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with anti-IRS-1 or affinity-purified anti-σ3, an antibody that was generated to a peptide sequence present in both the A and B isoforms of σ3 (supplied by Drs. E. Dell’Angelica and J. Bonifacino, National Institutes of Health). The fractions are as follows: cytosol, plasma membrane (PM), LDM, and Triton X-100-insoluble pellet. IRS-1 and σ3 are indicated by the arrows.

![Blot: Anti-IRS-1](image)

IRS-1 membrane pellets

![Blot: Anti-σ3](image)

supernatants post addition GST or GST-σ3A

![Blot: Anti-IRS-1](image)

GST (μg): - - - - - - - 75

GST-σ3A (μg): 0 5 25 50 75

Fig. 5. GST-σ3A causes release of IRS-1 from its endogenous σ3A receptor in low density membranes. 10 μg of low density membranes were incubated with buffer only or with increasing amounts of GST-σ3A (5–75 μg) or 75 μg of GST alone for 2 h at 4 °C. The membranes were then pelleted by centrifugation. Pellets and supernatants were resolved on a 6% SDS-PAGE gel and transferred to nitrocellulose. The filter was blocked and incubated with anti-IRS-1 followed by horseradish peroxidase-conjugated secondary antibody. Detection was by chemiluminescence. Bands corresponding to IRS-1 are indicated. The results are representative of three independent experiments.

The results presented in Fig. 5 demonstrate that exogenous σ3A can specifically displace IRS-1 from its endogenous intracellular membrane receptor in 3T3-L1 adipocytes, providing strong evidence that σ3A possesses structural features either the same as or very similar to those of the IRS-1 receptor present in these membranes.

Other examples exist whereby substrates of receptor tyrosine kinases exhibit specific interactions with proteins present in adaptin complexes. The ubiquitous Eps15 protein, initially described as a substrate of the epidermal growth factor receptor, is constitutively associated with AP-2 (57). Additionally, Okabayashi et al. (58) found that Shc, another substrate of the activated insulin receptor, interacts with AP-2 complex adaptins in vitro (58). Although neither study identified which specific adaptin subunit was responsible for the direct interaction with its respective protein, it was conclusively demonstrated in the former study that GST-Eps15 precipitated AP-2 complexes from lymphocytes, epithelial cells, and fibroblasts, while in the latter study, GST-Shc associated tightly with intact AP-2 holocomplexes from bovine brain lysates.

The Northern blot results presented in Fig. 2, A and B, in conjunction with the Northern analyses of human σ3 subunits presented in the study by Dell’Angelica et al. (31) demonstrate that various forms of σ3 mRNA are expressed at moderate to high levels in a variety of cultured cells and tissues, including 3T3-L1 cultured adipocytes. In contrast, high stringency Northern blot analysis of mRNAs from primary rodent adipocytes and skeletal muscle, two well described systems in which to study insulin’s major effects, showed undetectable levels of σ3A mRNA (Fig. 2B). If binding to σ3A ensures proper membrane localization of IRS-1 so that it can mediate its biological effects, a stoichiometric association between σ3A and IRS-1 should exist. Since both primary rodent adipocytes and skeletal muscle contain abundant levels of IRS-1, we would expect at least detectable levels of σ3A in these cell types. Probing and washing a similar filter to that shown in Fig. 2B using lower stringency conditions revealed the presence of the 1.5-kb σ3A transcript as well as an abundant, slower migrating transcript in primary rodent adipocytes and skeletal muscle (Fig. 2C). We are currently pursuing the identification of additional, possibly novel, isoforms of σ3 present in insulin-responsive tissues.

The exact structure and function for AP-3, as well as the identification of the cellular compartments to which it localizes, are not yet defined. Similar to the clathrin-associated adaptin complexes, AP-1 and AP-2, the AP-3 complex is composed of four distinct subunits: β3A, which is more related to the neuronal β-NAP than to β1 or β2; δ; μ3; and σ3 (29, 31–33). While electron microscopy studies have not been performed on AP-3, bulk protein release from these membranes compared with GST or GST-Rab6C addition, since a portion of the supernatants from each condition were run on a gradient gel and silver-stained (data not shown), and the protein profile for all three conditions appeared the same. These results indicate that σ3A binding to IRS-1 releases it from its endogenous receptor in adipocyte intracellular membranes.

DISCUSSION

Several observations underscore the importance of subcellular localization of key elements in insulin signaling pathways. It was initially observed by Kelly (9) that insulin stimulation of adipocytes results in the redistribution of cytosolic PI 3-kinase to an intracellular membrane fraction. This evidence was later followed by the observations that tyrosine-phosphorylated residues within IRS-1 bind SH2 domains present in PI 3-kinase (54–56) and that IRS-1 in basal 3T3-L1 adipocytes is localized to intracellular membranes (6–8). Thus, it is the tyrosine phosphorylation of this membrane-bound IRS-1 that recruits PI 3-kinase to intracellular membranes upon insulin stimulation. Novel findings presented in this study indicate IRS-1 may be localized to the intracellular membranes of basal 3T3-L1 adipocytes through an interaction with σ3A, the small subunit of the AP-3 adaptin complex. The interaction between IRS-1 and σ3A was initially demonstrated by screening a cultured adipocyte cDNA expression library, and it was subsequently confirmed by in vitro binding studies using purified fusion proteins (Fig. 3). Both IRS-1 and σ3A are present in detergent-resistant structures present in LDMs (Fig. 4), thus localization of the two proteins is consistent with σ3A acting as a receptor for IRS-1.

Finally, the results presented in Fig. 5 demonstrate that exogenous σ3A can specifically displace IRS-1 from its endogenous intracellular membrane receptor in 3T3-L1 adipocytes, providing strong evidence that σ3A possesses structural features either the same as or very similar to those of the IRS-1 receptor present in these membranes.

We are currently pursuing the identification of additional, possibly novel, isoforms of σ3 present in insulin-responsive tissues.

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sequence homologies of AP-3 complex subunits with the subunits of AP-1 and AP-2 suggest that all three complexes share overall similarity in their structural features (32). Differences do exist, however, and probably it is these differences that most contribute to the distinct subcellular localizations of AP-1, AP-2, and AP-3 and to their purported functions.

Immunofluorescence microscopy demonstrates that the α3-containing complex is predominantly concentrated in a juxtanuclear area, suggesting that it is associated with the TGN, yet α3 immunostaining also extends far into the periphery of the cell (31).2 An identical immunostaining pattern has also been observed for δ (32). Extensive co-localization of peripheral AP-3 staining with endosomal markers such as the transferrin receptor suggests that the AP-3 complex functions in peripheral regions of the cell but at sites that are clearly distinct from the plasma membrane where AP-2 is localized. Since IRS-1 is specifically associated with LDMS in nonstimulated 3T3-L1 adipocytes, while very little IRS-1 (~3%) is detected in the plasma membrane fraction (6, 8), the observed interaction between IRS-1 and α3A is consistent with AP-3, and not AP-2, involvement in the localization of IRS-1. Immunoblot analyses indicate that β3A and α3 are not detectable in purified clathrin-coated vesicles (29, 31–33), further distinguishing the α3-containing AP-3 complex from AP-1 and AP-2, both established yet subunits of AP-3 complex subunits and IRS-1 is specific for a α3- and not a α1- and/or α2-containing complex.

It has been shown that in 3T3-L1 adipocytes, membrane-bound IRS-1 is tyrosine-phosphorylated by insulin receptors present at the cell surface, yet under conditions where the endocytosis of activated insulin receptors is inhibited, IRS-1 still becomes tyrosine-phosphorylated (6). This finding, as well as results from others (8, 53), suggests that in basal adipocytes, IRS-1 is tethered to components that are proximal to, but that lie beneath, the plasma membrane. In support of this idea, the pleckstrin homology domain and the phosphotyrosine binding domain of IRS-1 have been shown to function in the efficient coupling between IRS-1 and the activated insulin receptor in the plasma membrane (60–63), yet there is no direct evidence to suggest that these domains are responsible for IRS-1 localizing to intracellular membranes. Although our data are consistent with the findings of Clark et al. (52, 53) that IRS-1 present in the LDMS fraction is in a detergent-resistant compartment, the authors of those studies concluded that IRS-1 was possibly interacting with the cell cytoskeleton. The evidence underlying their conclusion was based on similarities between structures present in electron micrographs of their IRS-1-containing fraction and 5–15 nm intermediate filament cytoskeletal elements. However, a detailed investigation of whether IRS-1 specifically associates with cytoskeletal elements was not provided in this study. Besides the cytoskeleton, other subcellular compartments, including caveolae and clathrin-containing vesicles, have been demonstrated to be detergent-resistant. Thus, the precise intracellular localization of IRS-1 remains to be determined. The observed interaction between IRS-1 and α3A and the immunolocalization of AP-3 at the cell periphery are consistent with IRS-1 localizing to AP-3-containing structures in the cell.

The localization of IRS-1 to AP-3 may provide the specificity for subsequent signaling events, such as the insulin-stimulated recruitment and activation of PI 3-kinase and its downstream targets. It has been demonstrated that epidermal growth factor and platelet-derived growth factor also stimulate PI 3-kinase, yet in the case of these other growth factors, PI 3-kinase is recruited directly to the respective transmembrane receptor. Activated epidermal growth factor receptors have been shown to bind AP-2 complexes (64), an interaction mediated by the ability of the μ2 subunit to recognize the tyrosine endocytic signal within the cytoplasmic tail of the receptor (12, 65). Both α3A and α3B show a high degree of homology to the μ subunits of AP-3 (p47A and B), in the region of the μ subunit that recognizes and binds tyrosine-based sorting signals. Using the yeast two-hybrid system, however, this region in both α subunits was shown not to interact with this motif, indicating that α subunits have their own distinct function (31).

The adaptin complexes are thought to be part of the cellular machinery involved in protein sorting (12, 17). It was recently reported that the 3’ phosphorylated lipid products of PI-3 kinase enhance the in vitro recognition of tyrosine-based sorting signals for μ2, suggesting that these lipid products may function in vivo to regulate AP-2-mediated protein trafficking events (65). Substantial evidence exists to indicate that PI 3-kinase is a critical component of protein trafficking in eukaryotes, including reports that missorting of platelet-derived growth factor and CSF receptors occurs when PI 3-kinase binding sites are deleted from the receptor structure (66, 67). Also, specific inhibitors of PI 3-kinase cause alterations in the targeting of proteins to their proper cellular destinations (68). Hence, it is notable that inhibition of insulin-stimulated PI 3-kinase by both wortmannin and LY29004 abolishes the insulin-stimulated translocation of GLUT4 from its intracellular storage compartment within the endosomal system to the cell surface (44, 45). It will be interesting to determine whether the lipid products generated by PI-3 kinase bound to IRS-1 also function in vivo to enhance AP-3-mediated trafficking events, perhaps contributing to the regulation of insulin-stimulated glucose transport. In addition, insulin treatment of 3T3-L1 adipocytes causes the translocation of membrane-bound IRS-1 into the cytosol (6–8), an event that recently was shown to be accompanied by phosphorylation of component(s) in the intracellular membranes (8). We are currently investigating whether insulin-stimulated phosphorylation regulates the association of IRS-1 with α3A.

The data in this present study indicate that IRS-1 binds α3A, and they are consistent with the notion that α3A may function as the IRS-1 receptor in intracellular membranes of cultured adipocytes. Defining a specific role for α3 subunits and for the AP-3 holocomplex in intact insulin-responsive cells in future experiments may therefore provide new insights into mechanism(s) of insulin-stimulated bioeffects including the glucose transport.

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