Insulin stimulation of 3T3-L1 adipocytes results in rapid activation of the insulin receptor tyrosine kinase followed by autophosphorylation of the receptor and phosphorylation of insulin receptor substrate 1 (IRS-1), its major substrate. The insulin receptor resides mostly at the cell surface of 3T3-L1 adipocytes under basal conditions, while about two-thirds of IRS-1 fractionates with intracellular membranes and one-third fractionates with cytosol. To test whether insulin receptor internalization is required for optimal tyrosine phosphorylation of IRS-1, 3T3-L1 adipocytes and CHO-T cells were incubated at 4°C which inhibits receptor endocytosis but not its tyrosine kinase activity. Under these conditions, tyrosine phosphorylation of IRS-1 in the low density microsome fraction in response to insulin was as intense as that observed at 37°C, indicating that endocytosis of insulin receptors is not necessary for tyrosine phosphorylation of IRS-1 to occur. Surprisingly, at 37°C, insulin action on 3T3-L1 adipocytes progressively decreased the amount of IRS-1 protein associated with the low density microsome fraction and increased that in the cytosol. This redistribution of IRS-1 from the low density microsome fraction to the cytosol in response to insulin was accompanied by decreased electrophoretic mobility of IRS-1 on SDS-polyacrylamide gel electrophoresis. Incubation of adipocytes at 4°C blocked the appearance of tyrosine-phosphorylated IRS-1 in the cytosol. Taken together, these data indicate that insulin receptors phosphorylate IRS-1 at the cell surface, perhaps in coated pits which are included in the low density microsome fraction. The results also suggest a desensitization mechanism in which the tyrosine-phosphorylated membrane-bound IRS-1, associated with signaling molecules such as phosphatidylinositol 3-kinase, is released into the cytoplasm in concert with its serine/threonine phosphorylation.

Intrinsic tyrosine kinase activity of the cell surface insulin receptor is required to mediate its biological actions suggesting the importance of its protein substrates in signal transduction. One such cellular substrate is the insulin receptor substrate 1 (IRS-1) for recent reviews, see Refs. 1–3). The amino acid sequences deduced from cloned rat, mouse, and human IRS-1 cDNAs (4–6) indicate the presence of multiple potential sites of phosphorylation by both tyrosine and serine/threonine protein kinases. In the intact cell, insulin stimulation causes rapid tyrosine phosphorylation of IRS-1 at multiple sites (7). These phosphotyrosine-containing sequences within IRS-1 serve as binding sites for a number of proteins containing Src homology 2 (SH2) domains (1–3). Proteins recruited to IRS-1 in this manner include the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (8, 9), the tyrosine phosphatase Syp (10), and the adaptor proteins Grb2 (11, 12) and Nck (13). The enzymatic activities of both PI 3-kinase (8) and Syp (14) are markedly increased upon their binding to IRS-1. Thus, IRS-1 is thought to serve as a docking protein which facilitates the actions of various signaling proteins in initiating their biological responses.

Recent evidence directly implicates IRS-1 and PI 3-kinase in one of the major metabolic actions of insulin, glucose transport. The binding of p85 to IRS-1 is required for activation of the p110 catalytic subunit of PI 3-kinase which is responsible for catalyzing the phosphorylation of phosphoinositides at the D-3 position of the inositol ring (8, 15). PI 3-kinase also possesses a serine kinase activity (16, 17) which phosphorylates both p85 and IRS-1 (18). Insulin causes stimulation of PI 3-kinase activity in isolated rat adipocytes (19), 3T3-L1 adipocytes (20), and cells overexpressing the insulin receptor (21–23). Specific inhibitors of both the lipid and serine kinase activities of PI 3-kinase abolish insulin-stimulated glucose transport and the translocation of GLUT4 in insulin-responsive cells (18, 24, 25). Furthermore, the introduction of IRS-1 antisense constructs into GLUT4-transfected fat cells decreases the sensitivity to insulin of GLUT4 glucose transporter movement to the cell surface, and this effect can be overcome by co-expressing IRS-1 in these cells (26).

Studies of the insulin receptor indicate that critical structural element(s) exist in its cytoplasmic domain to propagate signaling to components in the pathway leading to glucose transport. Specifically, point mutations of Tyr-960 in the NPXY motif located within the juxtamembrane sequence of the receptor which do not alter receptor kinase activity or the rate of 125I-insulin endocytosis abolish the phosphorylation of IRS-1, the activation of PI 3-kinase, and insulin-stimulated glucose transport in cells expressing these mutant receptors (27–32). Interestingly, a direct interaction between the amino terminus of IRS-1 and the region of the insulin receptor containing phosphorylated Tyr-960 has been shown utilizing the yeast two-hybrid system (33). However, it is unclear how IRS-1 interacts with the insulin receptor, an integral plasma membrane protein, in intact cells.

The distribution of IRS-1 in rat fat cells was recently shown to be mostly cytosolic, but approximately 20% of the IRS-1 was associated with intracellular membranes (34). The IRS-1 in both these subcellular fractions was tyrosine-phosphorylated following insulin stimulation. The authors of this work pro-
posed that internalized, activated insulin receptors are responsible for the phosphorylation of the IRS-1 located in the intracellular membranes. A previous study indicated that basal PI 3-kinase activity in rat fat cells appears to be cytosolic while essentially all of the insulin-stimulated PI 3-kinase activity, presumably in IRS-1-PI 3-kinase complexes, exists in microsomal membranes (19), a heterogeneous mixture of Golgi membranes and endosomes. It was further determined that the activated complexes of IRS-1 and PI 3-kinase were present in vesicles distinct from those containing the insulin-regulated glucose transporters GLUT4 and those containing internalized insulin receptors (35). Hence, these studies suggest that activated IRS-1-PI 3-kinase complexes are specifically localized to intracellular membranes, but it remains unclear as to where these complexes are initially formed and how these complexes are targeted to this site.

The present studies were designed to determine whether insulin receptors must first internalize into endosomes in order for IRS-1 to become tyrosine-phosphorylated. We took advantage of the fact that, at low temperature, endocytosis of the insulin receptor is inhibited (36, 37) while its tyrosine kinase activity remains functional. Our results demonstrate that IRS-1 in 3T3-L1 adipocytes undergoes insulin-dependent tyrosine phosphorylation at both 4°C and 37°C, indicating that receptor internalization is not required for its phosphorylation. We also show that, under basal conditions, the majority of IRS-1 in 3T3-L1 adipocytes is present in the low density microsomal fraction, and that following insulin stimulation, tyrosine-phosphorylated IRS-1 is released from these membranes into the cytosol. Furthermore, this release is accompanied by a change in the electrophoretic mobility of IRS-1, suggesting that serine/threonine phosphorylation of IRS-1 is occurring. These data are consistent with the hypothesis that in 3T3-L1 adipocytes, IRS-1 undergoes directed intracellular routing which is regulated by insulin.

EXPERIMENTAL PROCEDURES

Materials—4G10 anti-phosphotyrosine monoclonal antibody and rabbit polyclonal anti-IRS-1 used for immunoblotting were purchased from UBI. CT-1 anti-insulin receptor monoclonal antibody was a gift from Dr. Kenneth Siddle and was utilized as purified IgG, prepared from mouse ascites. Rabbit polyclonal anti-IRS-1 used for immunoprecipitation was prepared by injecting a peptide of the carboxy-terminal 15 amino acids derived from the sequence of rat liver IRS-1 conjugated to keyhole limpet hemocyanin into New Zealand White rabbits. An IgG fraction from the resultant serum was prepared by Protein A-Sepharose chromatography. Horseradish peroxidase-conjugated secondary antibodies were purchased from Boehringer Mannheim. Control mouse and rabbit immunoglobulins, Protein A-Sepharose, and goat anti-mouse Sepharose were from Sigma. Rhodamine and fluorescein isothiocyanate-conjugated anti-mouse were from Tago. [γ-32P]ATP and Renilla

et al. [29]P]ATP and Renais-

sance enhanced chemiluminescence reagent were purchased from DuPont NEN. Centriprep-10 concentrators were obtained from Amicon. Bicinchoninic acid (BCA) protein determination kit was from Pierce. Phosphatidylinositol and phosphatidylyserine were purchased from Avanti Lipids. PE STL G thin layer chromatography plates were purchased from Whatman.

Cell Culture—3T3-L1 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin sulfate. Two days postconfluence, differentiation was induced by incubating the cells for 3 days in DMEM supplemented with 10% fetal bovine serum, 5 μg/ml insulin, 1 μM deoxymethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. Cells were subsequently incubated for 3 days in DMEM supplemented with 10% fetal bovine serum and 5 μg/ml insulin. Thereafter, the cells were maintained in DMEM plus 10% fetal bovine serum with medium changes every 3 days. Differentiated adipocytes were routinely used for experiments between day 8 and day 14, at which time >90% of the cells displayed the adipocyte phenotype. CHO-T cells expressing the human insulin receptor were grown in F12 medium supplemented with 10% fetal calf serum.

Preparation of Cell Fractions—Differentiated 3T3-L1 adipocytes were grown in 15-cm² dishes and were serum-starved for 12–18 h in DMEM supplemented with 0.5% bovine serum albumin (BSA). For experiments at 4°C, the medium was aspirated and replaced with 4°C unsupplemented DMEM for 15 min. Cells were then stimulated with the indicated concentrations of insulin for the indicated times at either 4°C or 37°C. The insulin-containing medium was aspirated, and the cells were washed three times with media at either 4°C or 37°C with Buffer A (20 mM Tris- HCl, pH 7.5, 1 mM EDTA, 25 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 100 μM Na3VO4, 1 μM NaPP, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). Cell fractions were then prepared using modifications of previously described methods (38). All procedures were performed at 0-4°C. Briefly, cells were homogenized for 30 s with a motor-driven Teflon-glass homogenizer in 24 ml of ice-cold Buffer A, and the homogenate was centrifuged at 16,000 × g for 20 min. The resulting pellet containing the plasma membranes was resuspended in 6 ml of Buffer A, homogenated with 10 strokes in a glass dounce homogenizer (pestle A), and this was layered onto a sucrose cushion consisting of 20 ml Tris-HCl, pH 7.5, 1 mM EDTA, 1.12 M sucrose. The supernatant was centrifuged at 100,000 × g for 1 h. The band containing plasma membranes was collected, diluted with 20 ml of Buffer A, and recentrifuged for 15 min at 250,000 × g to pellet the plasma membranes. The final plasma membrane pellet was resuspended in Buffer A to a concentration of approximately 1-2 mg/ml. The 16,000 × g supernatant was centrifuged for 20 min at 48,000 × g. The 48,000 × g supernatant was centrifuged for 1.5 h at 250,000 × g to obtain a pellet containing low density microsomes. The final low density microsome pellet was resuspended in Buffer A to a concentration of approximately 1.5-3 mg/ml. The supernatant from this 250,000 × g centrifugation step was concentrated using a Centriprep-10 apparatus which had been previously blocked for 1 h with 5% Tween 80 and exhaustively washed with water to remove traces of the detergent.

CHO-T cells were grown in 15-cm² dishes and were serum-starved for 12-18 h in F12 media supplemented with 0.5% BSA. Cell fractions from CHO-T cells were then prepared as described above except that the first homogenization step consisted of 10 strokes with a motor-driven Teflon/ glass homogenizer in 24 ml of ice-cold Buffer A.

Immunoprecipitation of Insulin Receptor—Low density microsomal and plasma membrane fractions were prepared from 3T3-L1 adipocytes as described. 125 μg of protein from each fraction were resuspended in Buffer B (50 mM HEPES, pH 7.5, 1% Brij-96, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.5 mM Na3VO4, 30 mM NaPP, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) and were preincubated by incubating for 90 min with 20 μl of packed goat anti-mouse Sepharose at 4°C. Samples were centrifuged at 16,000 × g for 30 s, and the supernatants were transferred to fresh tubes. CT-1 anti-insulin receptor IgG (5 μg) was added to the precleared cell fractions and incubated overnight at 4°C. 20 μl of packed goat anti-mouse Sepharose were added to each tube and incubated for an additional 90 min at 4°C. The rose-bound immune complexes were washed five times in Buffer B, solubilized in sample buffer containing 2-mercaptoethanol with heating at 100°C for 5 min, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using polyacrylamide gels as described by Laemmli (39).

SDS-PAGE Electrophoresis and Immunoblotting—Cell fractions were thawed on ice, solubilized in sample buffer containing 2-mercaptoethanol with heating at 100°C for 5 min, and resolved by SDS-PAGE (39). Proteins were transferred electrophoretically to nitrocellulose essentially as described by Towbin et al. (40). The nitrocellulose was blocked with either 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% BSA, 0.1% Triton X-100 (anti-phosphotyrosine antibody) or 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3% nonfat dry milk, 0.5% BSA, 0.1% Triton X-100 (anti-IRS-1). The nitrocellulose was then incubated with gentle agitation in primary antibody according to supplier's specifications for 2 h at room temperature, washed several times in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Following several washes in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, bound antibodies were visualized using enhanced chemiluminescence.

Immunofluorescence—CHO-T cells were grown on glass coverslips and were serum-starved for 12-18 h in F12 media supplemented with 0.5% BSA. For experiments at 4°C, the medium was aspirated and replaced with 4°C unsupplemented DMEM for 15 min. Cells were then stimulated with 1 μM insulin for the indicated times at either 4°C or 37°C. The insulin-containing medium was aspirated, the cells were
Insulin Regulation of Membrane-associated IRS-1

RESULTS

Insulin-stimulated Tyrosine Phosphorylation of IRS-1 Occurs at 4°C—To determine whether tyrosine phosphorylation of IRS-1 occurs at 4°C, subcellular fractions were prepared from 3T3-L1 adipocytes stimulated with insulin at 37°C or at 4°C, a temperature at which insulin receptors are not expected to internalize. The subcellular fractions were subjected to immunoblot analysis utilizing anti-phosphotyrosine antibody (Fig. 1). The major phosphorylated band, which corresponded to the molecular weight of IRS-1, was densitometrically scanned, and the data was normalized per cell, and the results were displayed graphically (Fig. 2). As shown in Figs. 1 and 2, tyrosine-phosphorylated IRS-1 present in the low density microsome fraction of 3T3-L1 adipocytes treated with insulin at 4 min increases to identical levels at both temperatures, suggesting that tyrosine phosphorylation of IRS-1 occurs in the absence of insulin receptor internalization at 4°C. Between 15 and 60 min of insulin stimulation at 37°C, a 2-fold decrease in tyrosine-phosphorylated IRS-1 is observed in the low density microsome fraction of cells with a concomitant 2-fold increase in cytosolic tyrosine-phosphorylated IRS-1. In contrast, additional insulin stimulation at 4°C causes a time-dependent increase in the tyrosine-phosphorylated IRS-1 associated with the low density microsome fraction. Strikingly, there is no tyrosine-phosphorylated cytosolic IRS-1 after stimulation with insulin at 4°C.

As demonstrated in Fig. 1, levels of tyrosine-phosphorylated IRS-1 present in the plasma membrane fractions at both temperatures following insulin stimulation are substantially (approximately 20-fold) lower than levels present in the low density microsome fractions under identical conditions. Thus, the results of the plasma membrane fractions are not displayed graphically in Fig. 2.

Tyrosine-phosphorylated Insulin Receptors Are Present in the Low Density Microsome Fraction at Both 4°C and 37°C—Also shown in Fig. 1 are tyrosine-phosphorylated insulin receptors present in the subcellular fractions. The amount of tyrosine-phosphorylated insulin receptor β subunit detected in the plasma membrane fraction at 4°C is significantly lower relative to the amount of tyrosine-phosphorylated insulin receptor β subunit in the plasma membrane fraction at 37°C. There are nonspecific anti-phosphotyrosine immunoreactive bands which co-migrate with the 95-kDa insulin receptor β subunit in the

washed with ice-cold phosphate-buffered saline (PBS) consisting of 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 3.35 mM KCl, 170 mM NaCl, 0.68 mM CaCl₂, and 0.49 mM MgCl₂ and were then fixed at 4°C for 10 min in 4% formaldehyde. The cells were washed several times with ice-cold PBS and were then permeabilized for 6 min with −20°C methanol. The cells were washed several times with PBS and were then blocked for 1 h with 2% BSA in PBS. Cells were incubated for 3 h with 20 μg/ml anti-phosphotyrosine antibody, 1.5 μg/ml anti-IR IgG, or 20 μg/ml mouse IgG as negative control. Cells were washed with PBS containing 0.05% Triton X-100 and were then incubated for 30 min with fluorescein isothiocyanate or rhodamine-conjugated anti-mouse. Cells were washed, postfixed in 4% formaldehyde for 10 min, and mounted. Immunofluorescence was visualized by digital imaging microscopy using a thermoelectrically cooled charged-coupled device camera. Each image was corrected for lamp intensity variations and photobleaching.

Assay of PI 3-Kinase Activity—Low density microsome and cytosol fractions were prepared from 3T3-L1 adipocytes treated with 1 μM insulin for 15 min at 4°C or 37°C as described above. These fractions were then subjected to PI 3-kinase activity assay according to previously published procedures (41). Briefly, 125 μg of protein from each fraction were resuspended in Buffer C containing 50 mM HEPES, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.5 mM Na₃VO₄, 30 mM NaPPi, 2 μg/ml aprotinin, and 5 μg/ml leupeptin. Anti-IRS-1 IgG was incubated with 20 μl of packed Protein A-Sepharose in 1 ml of Buffer C for 2 h at 4°C. Antibody-bound Protein A-Sepharose was then washed several times with Buffer C. Resuspended adipocyte fractions were added to the anti-IRS-1-bound Protein A-Sepharose and incubated overnight at 4°C. Immune complexes were washed five times in Buffer C, washed twice in 10 mM Tris-HCl, pH 7.5, containing 500 mM LiCl, once in 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 100 mM NaCl, and once in 20 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 0.5 mM EDTA, 10 mM MgCl₂, and 120 μM adenosine. Immune complexes were resuspended in 40 μl of the latter buffer, 100 μM phosphatidylinositol, 100 μM phosphatidylserine, and 10 μM [γ-32P]ATP, and the reaction was incubated for 10 min at 30°C. The reaction was quenched with HCl, extracted with methanol/chloroform (1:1), and 10 μl were spotted onto PE SII G plates. Separation of phosphatidylinositol monophosphates by borate thin layer chromatography (42) was performed.

Fig. 1. Insulin causes tyrosine phosphorylation of IRS-1 present in the low density microsome fraction of 3T3-L1 adipocytes at both 4°C and 37°C. Plasma membrane (PM), low density microsome (LDM), and cytosolic fractions were prepared from 3T3-L1 adipocytes treated with (+) or without (−) 1 μM insulin for 15 or 60 min at 4°C or 37°C by the methods described under “Experimental Procedures.” Protein (25 μg) from each fraction was resolved by SDS-PAGE on 7.5% gels and electrophoretically transferred to nitrocellulose for 8 h at 150 mA. The filters were blocked and subsequently incubated with 0.5 μg/ml 4G10 anti-phosphotyrosine antibody as described under “Experimental Procedures.” Bands corresponding to IRS-1 (160 kDa) and the β subunit of the insulin receptor (IR, 95 kDa) are designated with arrowsheads. The immunoblots presented are representative of five experiments.

Fig. 2. Tyrosine phosphorylation of IRS-1 in low density microsome and cytosol of 3T3-L1 adipocytes treated with insulin at 4°C and 37°C. The data shown in Fig. 1 for IRS-1 were quantitated using a scanning densitometer. The data were normalized by adjusting the numerical value obtained from densitometric scanning to the total protein from each subcellular fraction prepared from an equal number of cells and are depicted as arbitrary units.
low density microsome fraction, thus making it difficult to
determine the relative levels of tyrosine-phosphorylated insu-
lin receptor present in this fraction at the two temperatures. In
order to clarify the levels of tyrosine-phosphorylated insulin
receptor present in the low density microsome fraction and to
confirm the levels of tyrosine-phosphorylated insulin receptor
present in the plasma membrane fraction, insulin receptors
were quantitatively immunoprecipitated from subcellular frac-
tions prepared from 3T3-L1 adipocytes utilizing conditions that
were identical with those in the previous experiment. Immuno-
precipitates were immunoblotted with anti-phosphotyrosine
antibody as shown in Fig. 3. Bands corresponding to the 95-
kd subunit of the insulin receptor (IR, 95 kDa) is designated with an
arrowhead.

Tyrosine phosphorylation of the insulin receptor in the
plasma membrane fraction increases in a time-dependent man-
ner at both 4 °C and 37 °C. There are 4-fold more tyrosine-
phosphorylated insulin receptors in the plasma membrane
fraction at 37 °C relative to 4 °C following a 15-min insulin
stimulation suggesting that tyrosine phosphorylation of recep-
tors is slower at 4 °C. Tyrosine phosphorylation of insulin recep-
tors in the plasma membrane fraction increases slightly with increased exposure to insulin at 37 °C, while the increase in
tyrosine phosphorylation of insulin receptors in the plasma
membrane fraction at 4 °C is greater. This is most likely due to
the fact that, at 37 °C, tyrosine-phosphorylated insulin recep-
tors are being internalized in a time-dependent fashion, and
hence their numbers would be expected to decrease in the
plasma membrane fraction and increase in the low density
microsome fraction. This is reflected in the data shown in the
bottom panel of Fig. 4. Levels of tyrosine-phosphorylated insu-
lin receptor in the low density microsome fraction from cells
stimulated at 37 °C are slightly increased over time. Our data
indicate the presence of tyrosine-phosphorylated insulin re-
ceptors in the low density microsome fraction at both 37 °C
and at 4 °C.

To confirm that insulin receptors are not significantly inter-
nalized following insulin stimulation at 4 °C, we performed
immunofluorescence microscopy using CHO-T cells. We first
tested whether insulin stimulation of CHO-T cells at 4 °C
would result in the tyrosine phosphorylation of IRS-1 present
in the low density microsome fraction as observed in 3T3-L1
adipocytes. Confluent CHO-T cells were stimulated with insu-
lin at 4 °C or 37 °C for 15 min and 60 min, and subcellular
fractions were prepared and immunoblotted with anti-phos-
photyrosine antibody. As the results presented in Fig. 5 dem-
strate, tyrosine-phosphorylated IRS-1 is present in the low
density microsome fractions prepared from cells stimulated
with insulin at both 4 °C and 37 °C. Additionally, similar to the
effect observed in 3T3-L1 adipocytes, a substantial amount
of tyrosine-phosphorylated IRS-1 is present in the cytosol at
37 °C following insulin stimulation. Thus, our findings
show that CHO-T cells are similar to 3T3-L1 adipocytes in
that IRS-1 associated with the low density microsome fraction
can be tyrosine-phosphorylated in an insulin-dependent
manner at 4 °C.

Insulin Receptor Internalization Is Inhibited at 4 °C—Immuno-
fluorescence microscopy of CHO-T cells stimulated with in-
sulin at 4 °C and 37 °C was performed (Fig. 6). A weak, fibrillar
pattern of intracellular staining, similar to the staining ob-
served for focal adhesions, is observed in cells in the absence
of insulin at both 4 °C and 37 °C when anti-phosphotyrosine
antibody is incubated with fixed, permeabilized CHO-T cells (Fig.
6, B and E). Cells stimulated with insulin at 4 °C and then
incubated with anti-phosphotyrosine antibody display intense
cell surface staining (Fig. 6C). In contrast, cells stimulated
with insulin at 37 °C do not show this intense staining at the cell
periphery. Rather, they exhibit bright intracellular staining
suggesting that at 37 °C internalization of tyrosine-phosphoryl-
ated insulin receptors has occurred (Fig. 6F). To confirm that
internalization of insulin receptors is inhibited at 4 °C in
CHO-T cells following insulin stimulation, fixed, permeabilized
cells were incubated with a monoclonal antibody generated
against the carboxyl terminus of the insulin receptor. As shown
in Fig. 6, bright staining is observed at the surface of cells at

FIG. 3. Insulin receptors in 3T3-L1 adipocytes are tyrosine-
phosphorylated at both 4 °C and 37 °C in response to insulin.
Plasma membrane (PM) and low density microsomes (LDM) were
prepared from 3T3-L1 adipocytes treated with (+) or without (−) 1 μM
insulin for 15 or 60 min at 4 °C or 37 °C by the methods described under
"Experimental Procedures." Protein (125 μg) from each fraction was
solubilized in HEPES buffer containing 1% Brij and incubated with 5 μg
of anti-insulin receptor IgG. Samples were incubated with goat anti-
mouse Sepharose and then washed several times. The immune
pellets were solubilized in sample buffer containing reductant, resolved
by SDS-PAGE on 7.5% gels, and were then electrophoretically trans-
ferred to nitrocellulose for 8 h at 150 mA. The filters were blocked and
subsequently incubated with 0.5 μg/ml 4G10 anti-phosphotyrosine as
described under "Experimental Procedures." The band corresponding to
the β subunit of the insulin receptor (IR, 95 kDa) is designated with an
arrowhead.

FIG. 4. Insulin receptor tyrosine phosphorylation in the
plasma membranes and low density microsomes of 3T3-L1 adi-
pocytes treated with insulin at 4 °C and 37 °C. The data shown in
Fig. 3 for insulin receptor were quantitated using a scanning densitom-
eter. The data were normalized by adjusting the numerical value ob-
tained from densitometric scanning to the total protein from each
subcellular fraction prepared from an equal number of cells and are
depicted as arbitrary units.
Insulin causes tyrosine phosphorylation of IRS-1 present in the low density microsome fraction of CHO-T cells at both 4 °C and 37 °C. Low density microsome (LDM) and cytosolic fractions were prepared from CHO-T cells treated with (+) or without (−) 1 μM insulin for 15 or 60 min at 4 °C or 37 °C by the methods described under "Experimental Procedures." Low density microsome protein (25 μg) and cytosolic protein (75 μg) were resolved by SDS-PAGE on 7.5% gels and were electrophoretically transferred to nitrocellulose for 8 h at 150 mA. The filters were blocked and subsequently incubated with 0.5 μg/ml 4G10 anti-phosphotyrosine antibody as described under "Experimental Procedures." Bands corresponding to IRS-1 (160 kDa) are designated with an arrowhead. The immunoblots presented are representative of two experiments.

Insulin Causes a Temperature-dependent Release of IRS-1 Protein from the Low Density Microsome Fraction into the Cytosol—To investigate in more detail the localization of IRS-1 in intracellular membranes, subcellular fractions identical with those utilized in Fig. 1 were immunoblotted with anti-IRS-1 antibody. The bands corresponding to IRS-1 were densitometrically scanned, the data were normalized per cell, and the results are presented in Figs. 7 and 8. Under basal conditions, approximately 62% of the IRS-1 protein in 3T3-L1 adipocytes is present in the low density microsome fraction, approximately 35% is present in the cytosol, and approximately 3% is in the plasma membrane fraction. Remarkably, at 37 °C, IRS-1 protein in the low density microsome fraction is decreased 80% with increasing exposure to insulin with a simultaneous 2.5-fold increase in IRS-1 protein in the cytosol. In addition, the small amount of IRS-1 protein in the plasma membrane fraction is decreased 30% at 37 °C. At 4 °C, this apparent insulin-independent release of membrane-associated IRS-1 into the cytosol is completely inhibited. A decreased electrophoretic mobility of IRS-1 protein present in the low density microsome and cytosolic fractions following 15-min and 60-min insulin stimulations at 37 °C is clearly evident in Fig. 7. In contrast, this electrophoretic shift is not observed when cells are incubated with insulin at 4 °C. As the levels of tyrosine phosphorylation of IRS-1 in the low density microsome fractions are identical following a 15-min insulin stimulation at 4 °C and 37 °C (see Figs. 1 and 2), this electrophoretic shift suggests that serine/threonine phosphorylation may be occurring on IRS-1 at 37 °C.

The concentration dependence of insulin in inducing the release of IRS-1 protein from the low density microsome fraction into the cytosol is shown in Fig. 9. 3T3-L1 adipocytes were stimulated with insulin at the indicated concentrations at 37 °C, subcellular fractions were prepared and immunoblotted with anti-IRS-1. The IRS-1 bands were scanned, and the data were normalized per cell and presented graphically. The data demonstrate that with increasing insulin concentration, there is an increasing loss of IRS-1 protein from the low density microsome fraction with a corresponding increase in cytosolic IRS-1. It appears that 10 nM insulin results in maximal loss of IRS-1 protein from the low density microsome fraction since further increases in cytosolic IRS-1 do not further increase the effect although increases in cytosolic IRS-1 have still not reached a plateau by 1 μM insulin. The percentages of IRS-1 protein present in the cytosolic and low density microsome fractions under basal conditions and following 1 μM insulin stimulation in this experiment were similar to those observed previously (see Fig. 8).

PI 3-Kinase Associates with Cytosolic IRS-1 in a Temperature-dependent Manner—To compare the PI 3-kinase activity associated with the IRS-1 present in the low density microsome fraction and cytosol of 3T3-L1 adipocytes stimulated with insulin at 4 °C and at 37 °C, subcellular fractions prepared from cells stimulated with insulin at the two temperatures were immunoprecipitated with anti-IRS-1 and then assayed for PI 3-kinase activity. As shown in Fig. 10, PI 3-kinase activity is associated with the low density microsome fraction in response to insulin at both 4 °C and at 37 °C. The levels of PI 3-kinase activity associated with the low density microsome fractions correlate well with the levels of tyrosine-phosphorylated IRS-1 present in this fraction (see Figs. 1 and 2). This observation is similar to results obtained in primary rat adipocytes where activation of PI 3-kinase correlated closely with the extent of tyrosine-phosphorylated IRS-1 present in low density vesicles (35). Fig. 10 also illustrates that approximately 5-fold more PI 3-kinase activity is present in IRS-1 immunoprecipitates of cytosols prepared from cells stimulated with insulin at 37 °C than from cells stimulated with insulin at 4 °C.

DISCUSSION

Our present approach to determine whether insulin receptor internalization is required for tyrosine phosphorylation of IRS-1 in intact 3T3-L1 adipocytes and CHO-T cells depends upon the premise that insulin receptors cannot internalize at low temperature while their tyrosine kinase activity is functional. Immunofluorescence microscopy presented here (Fig. 6) directly documents that activated insulin receptors remain largely at the cell surface at 4 °C, consistent with previously published reports that insulin receptor internalization is inhibited at low temperature (36, 37). Thus, our results demonstrating that IRS-1 undergoes similar levels of insulin-dependent tyrosine phosphorylation at 4 °C versus 37 °C (Figs. 1 and 2 for 3T3-L1 adipocytes and Fig. 5 for CHO-T cells) show that insulin receptor internalization is not necessary for IRS-1 tyrosine phosphorylation in these cells. A previous study suggested that internalization of activated insulin receptors is required for the tyrosine phosphorylation of IRS-1 in microsomal membranes (34). This conclusion was based on the observations that there are 5–6-fold more tyrosine-phosphorylated insulin receptors in low density microsomes relative to plasma membranes shortly after exposure to insulin, and that both the kinase activity associated with these receptors and the phosphorylation state of IRS-1 in the low density microsomes paralleled the phosphorylation content of the receptors. These correlations were quite striking. However, we have clearly demonstrated in two differ-
ent cell types that, under conditions where insulin receptor internalization is inhibited (4°C), insulin elicits levels of tyrosine-phosphorylated IRS-1 that are identical with those observed under physiological conditions (37°C) where receptor internalization does occur.

If insulin receptor internalization is not required for tyrosine phosphorylation of IRS-1 in the low density microsome fraction, which is thought to consist of intracellular membranes, what is the mechanism for such phosphorylation? Results presented in Figs. 3 and 4 demonstrate that there are tyrosine-phosphorylated insulin receptors present in the low density microsome fraction following insulin stimulation of adipocytes at 4°C even though internalization of receptors is inhibited. Results using autoradiographic electron microscopy have demonstrated that at low temperature, insulin-bound receptor complexes redistribute at the cell surface and cluster within coated pits (43) although this occurs more slowly than at 37°C. Clathrin-coated vesicles are present in the low density microsome fraction of adipocytes. Therefore, a likely explanation for the presence of tyrosine-phosphorylated receptors in the low density microsome fraction at low temperature is that clathrin-coated vesicles are included in this fraction. These considerations lead us to propose that a pool of the membrane-bound IRS-1 protein is associated with coated pits, and that tyrosine phosphorylation of IRS-1 by the insulin receptor can occur in these structures. Consistent with this concept, we found that significant amounts of IRS-1 protein in the low density microsome fraction of adipocytes are resistant to Triton X-100 extraction, as is clathrin (data not shown). As depicted in the model of Fig. 11, IRS-1 phosphorylation in coated pits may then be followed by its endocytosis and its delivery to endosomal sites where PI 3-kinase or other associated proteins may act. This hypothesis is not inconsistent with the possibility that IRS-1 may also be phosphorylated by internalized insulin receptors in intracellular membrane compartments at 37°C as suggested by Kublaoui et al. (34).

The mechanism by which IRS-1 might interact with membranes is unknown. Activated insulin receptors cannot account

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**Fig. 6.** Internalization of tyrosine-phosphorylated insulin receptors is inhibited at 4°C in CHO-T cells. CHO-T cells grown on coverslips were treated with (+) or without (−) 1 μM insulin for 60 min at 4°C or 37°C by the methods described under “Experimental Procedures.” Cells were fixed, permeabilized, and blocked as described under “Experimental Procedures.” Cells were incubated with 20 μg/ml 4G10 anti-phosphotyrosine, 1.5 μg/ml anti-insulin receptor IgG1, or 20 μg/ml mouse IgG as negative control, washed, and then incubated with fluorescein isothiocyanate- (ANTI-P-TYR) or rhodamine- (ANTI-IR) conjugated anti-mouse. Cells were washed, postfixed, and mounted. Top panel: A, nonimmune mouse IgG, 4°C; B, anti-phosphotyrosine, −ins, 4°C; C, anti-phosphotyrosine, +ins, 4°C; D, nonimmune mouse IgG, 37°C; E, anti-phosphotyrosine, −ins, 37°C; F, anti-phosphotyrosine, +ins, 37°C. Bottom panel: G, nonimmune mouse IgG, 4°C; H, anti-insulin receptor, −ins, 4°C; I, anti-insulin receptor, +ins, 4°C; J, nonimmune mouse IgG, 37°C; K, anti-insulin receptor, −ins, 37°C; L, anti-insulin receptor, +ins, 37°C.

**Fig. 7.** Insulin causes a temperature-dependent release of IRS-1 protein from the low density microsome fraction of 3T3-L1 adipocytes into the cytosol. Plasma membrane (PM), low density microsome (LDM), and cytosolic fractions were prepared from 3T3-L1 adipocytes treated with (+) or without (−) 1 μM insulin for 15 or 60 min at 4°C or 37°C by the methods described under “Experimental Procedures.” Protein (25 μg) from each fraction was resolved by SDS-PAGE on 7.5% gels and electrophoretically transferred to nitrocellulose for 8 h at 150 mA. The filters were blocked and subsequently incubated with 2 μg/ml anti-IRS-1 as described under “Experimental Procedures.” Bands corresponding to IRS-1 (160 kDa) are designated with arrowheads. The immunoblots presented are representative of three experiments.

R. Heller-Harrison and M. Czech, unpublished results.
for the putative IRS-1 binding component in membranes because IRS-1 is associated with low density microsome in the absence of insulin (Figs. 7 and 8). IRS-1 contains a structural domain, the pleckstrin homology (PH) domain, that is thought to represent an interaction motif (44). The PH domain in β-adrenergic receptor kinase has been shown to bind heterotrimeric G protein βγ subunits at the plasma membrane, thus recruiting β-adrenergic receptor kinase to the cell surface (45, 46). Similarly, the region containing the PH domain in oxysterol-binding protein is responsible for its binding to Golgi membranes (44). The PH domains of several proteins have been shown to interact selectively with phosphatidylinositol derivatives (47). An important hypothesis for future investigation is whether the PH domain in IRS-1 is required for its association with membranes in the low density microsome fraction.

The present results also demonstrate a striking regulatory action of insulin on IRS-1 binding to low density membranes. In control 3T3-L1 adipocytes, approximately two-thirds of the IRS-1 protein is associated with intracellular membranes, while approximately one-third is cytosolic (Figs. 7 and 8). Insulin stimulation at 37°C causes a substantial decrease in the levels of IRS-1 protein in the low density microsome fraction with a concomitant increase in IRS-1 protein in the cytosol. The insulin concentration required to elicit this release of IRS-1 protein into the cytosol is within the range of concentrations required to observe biological effects such as glucose transport regulation in these cells (Fig. 9). In addition, this effect is observed in 3T3-L1 adipocytes as early as 3 min following stimulation with insulin at 37°C.
stimulation with 100 nM insulin (data not shown) which corre-
grates temporally with insulin-induced translocation of GLUT4

glucose transporters to the plasma membrane (34). Taken
together, these results strongly suggest that this novel effect of
insulin that causes dissociation of IRS-1 from low density mem-

branes is of physiological importance. A likely role for this
phenomenon is desensitization of the insulin effect. Thus, as
depicted in the model of Fig. 11, subsequent to delivery of
tyrosine-phosphorylated PI 3-kinase-associated IRS-1 to spe-
cific sites within intracellular membranes, IRS-1 is released for
recycling back to its location of tyrosine phosphorylation. Such
a mechanism might operate in concert with dephosphorylation
of phosphotyrosine sites on IRS-1 by tyrosine phosphatases.
Interestingly, we find PI 3-kinase-associated IRS-1 in the
cytosol following insulin treatment (Fig. 10), indicating that
IRS-1 release from membranes occurs prior to its complete
dephosphorylation of tyrosine phosphates.

Some apparent diversity of results has been reported with
regard to the amount of tyrosine-phosphorylated IRS-1 that is
present in the cytosol of adipocytes. A previous study (35)
suggested that in primary rat adipocytes activation of PI 3-ki-
nase correlated closely with the extent of tyrosine-phosphoryl-
ated IRS-1 present in low density microsomes, but no PI 3-ki-
nase activity bound to IRS-1 in cytosol was observed. However,
another study showed clearly detectable tyrosine-phosphoryl-
ated IRS-1 in the cytosolic fraction of rat adipocytes after
incubation with insulin (34). Our findings in 3T3-L1 adipocytes
are consistent with this latter report. Insulin stimulation of
these cultured adipocytes at physiological temperature caused
a time-dependent diminution of tyrosine-phosphorylated IRS-1
in the low density microsome fraction, and the simultaneous
appearance of tyrosine-phosphorylated IRS-1 in the cytosol
(Figs. 1 and 2). Insulin-stimulated PI 3-kinase activity associ-
ated with the low density microsome fractions correlates well
with the levels of tyrosine-phosphorylated IRS-1 present in this
fraction (Fig. 10). We also observed PI 3-kinase activity asso-
ciated with tyrosine-phosphorylated IRS-1 in the cytosol of
3T3-L1 adipocytes following insulin stimulation. Although we
have no direct data indicating whether the cytosolic IRS-1-PI 3-
kinase complexes in the cytosol are required for biological
actions of insulin, our data strongly suggest that these cytosolic
complexes are derived from released membrane-bound

components. As depicted in the model of Fig. 11, our findings are consist-
ent with the hypothesis that phosphorylation of IRS-1 on ser-
inethreonine residues is involved in the mechanism of IRS-1
release from intracellular membranes. Thus, following insulin
stimulation at 37 °C in both 3T3-L1 adipocytes and in CHO-T
cells, the tyrosine-phosphorylated IRS-1 present in the low
density microsome fraction exhibits decreased electrophoretic
mobility (Figs. 1 and 5). This decreased electrophoretic mobility of
IRS-1 coincides with the decreased amount of IRS-1 associated
with the low density microsome fraction and the appear-
ance of tyrosine-phosphorylated IRS-1 in the cytosol. Moreover,
both the release of IRS-1 into the cytosol and the elec-

trophoretic mobility shift in IRS-1 due to insulin are abolished at
low temperature (Figs. 7 and 8). The electrophoretic mobility
shift in IRS-1 at 37 °C may not be explained by tyrosine phos-
phorylation of IRS-1 because this also occurs at 4 °C. IRS-1 is
known to be phosphorylated on serine within its PH domain in
response to insulin (48). Additionally, PI 3-kinase has been
shown to be a dual specificity enzyme in that it possesses both
lipid and serine kinase activities (16, 17), and IRS-1 was dem-
onstrated to be an insulin-dependent substrate for this serine
kinase activity of PI 3-kinase in intact cells (18). We hypothe-
size that insulin may regulate the intracellular localization of
IRS-1 by causing it to be phosphorylated by PI 3-kinase and
subsequently released from intracellular membranes into the
cytosol. Alternatively, perhaps other serine/threonine protein
kinases are involved in IRS-1 phosphorylation in response to
insulin. Further work is required to determine whether serine/
threonine phosphorylation of IRS-1 is required for the release
mechanism and which protein kinase(s) may be involved.

It is noteworthy that in contrast to results obtained with
3T3-L1 adipocytes, increased tyrosine-phosphorylated IRS-1
was detected in the cytosol of CHO-T cells stimulated with
insulin at 4 °C (Fig. 5). However, this cytosolic tyrosine-phos-
phorylated IRS-1 in CHO-T cells does not exhibit decreased
electrophoretic mobility, as does that prepared from 3T3-L1 or
CHO-T cells stimulated with insulin at 37 °C (Figs. 1 and 5).
One possibility is that tyrosine-phosphorylated IRS-1 in
CHO-T cells binds more weakly to intracellular membranes
relative to 3T3-L1 adipocytes.

In summary, the results presented here provide two new
insights into the dynamics of IRS-1 function in insulin receptor
signaling. First, based on the marked tyrosine phosphorylation
of IRS-1 in response to insulin at low temperature, we can
conclude that insulin receptor endocytosis is not required for
initiating the IRS-1 signaling pathway. This is consistent with
studies showing that in certain cell types, mutant insulin
receptors which fail to undergo internalization are still effective
in mediating downstream biological effects (49). Secondly,
based on the striking redistribution of IRS-1 protein in subcel-

lular fractions in response to insulin, we suggest that IRS-1
cycles between membrane-bound and cytosolic locations in an
insulin-regulated manner. This recycling may reflect an impor-
tant physiological mechanism for the release of IRS-1 signaling
complexes from targeted membrane sites where regulatory
events may occur.

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