Intracellular Localization of Phosphatidylinositide 3-kinase and Insulin Receptor Substrate-1 in Adipocytes: Potential Involvement of a Membrane Skeleton

Sharon F. Clark,* Sally Martin, Amanda J. Carozzi, Michelle M. Hill, and David E. James

*The Center for Molecular and Cellular Biology, and the Department of Physiology and Pharmacology, University of Queensland, Brisbane, Queensland, 4072, Australia

Abstract. Phosphatidylinositide (PI) 3-kinase binds to tyrosyl-phosphorylated insulin receptor substrate-1 (IRS-1) in insulin-treated adipocytes, and this step plays a central role in the regulated movement of the glucose transporter, GLUT4, from intracellular vesicles to the cell surface. PDGF, which also activates PI 3-kinase in adipocytes, has no significant effect on GLUT4 trafficking in these cells. We propose that this specificity may be mediated by differential localization of PI 3-kinase in response to insulin versus PDGF activation. Using subcellular fractionation in 3T3-L1 adipocytes, we show that insulin- and PDGF-stimulated PI 3-kinase activities are located in an intracellular high speed pellet (HSP) and in the plasma membrane (PM), respectively. The HSP is also enriched in IRS-1, insulin-stimulated tyrosyl-phosphorylated IRS-1 and intracellular GLUT4-containing vesicles. Using sucrose density gradient sedimentation, we have been able to segregate the HSP into two separate subfractions: one enriched in IRS-1, tyrosyl-phosphorylated IRS-1, PI 3-kinase as well as cytoskeletal elements, and another enriched in membranes, including intracellular GLUT4 vesicles. Treatment of the HSP with nonionic detergent, liberates all membrane constituents, whereas IRS-1 and PI 3-kinase remain insoluble. Conversely, at high ionic strength, membranes remain intact, whereas IRS-1 and PI 3-kinase become freely soluble. We further show that this IRS-1–PI 3-kinase complex exists in CHO cells overexpressing IRS-1 and, in these cells, the cytosolic pool of IRS-1 and PI 3-kinase is released subsequent to permeabilization with Streptolysin-*O*, whereas the particulate fraction of these proteins is retained. These data suggest that IRS-1, PI 3-kinase, as well as other signaling intermediates, may form preassembled complexes that may be associated with the actin cytoskeleton. This complex must be in close apposition to the cell surface, enabling access to the insulin receptor and presumably other signaling molecules that somehow confer the absolute specificity of insulin signaling in these cells.

CELL proliferation, differentiation, and metabolism are regulated by an extensive array of hormones, cytokines, and growth factors. Although the biological endpoints of these regulatory mechanisms are quite unique, there is tremendous overlap during the intermediate steps comprising the signal transduction machinery. For example, insulin, EGF, PDGF, haematopoietic factors including B cell receptor antigen and several interleukins, and clotting factors, such as thrombin, all stimulate the Ras/mitogen–activated protein kinase (MAPK)[1] and the phosphatidylinositide (PI) 3-kinase–Akt pathways (Kucera and Rittenhouse, 1990; Cambier and Campbell, 1992; Gold et al., 1992; Lev et al., 1992; Izuhara and Harada, 1993; Kazlauskas, 1994). Yet each of these factors initiates a specific biological response in accord with the physiological requirements of the cell. Thus, a major challenge is to establish how different hormones and growth factors achieve their biological specificity, in view of this apparent redundancy in signaling. One way of achieving specificity in multicellular organisms is via the cell-specific expression of different receptors. Another, is by a combinatorial mechanism of signaling. This latter mechanism, which is

Address all correspondence to David E. James, Department of Physiology and Pharmacology, University of Queensland, Brisbane, Queensland 4072, Australia. Tel.: (617) 3365 4986. Fax: (617) 3365 4430. E-mail: D.James@cmcb.uq.edu.au

1. *Abbreviations used in this paper*: anti-pY, anti-phosphotyrosine; β-OG, β-octylglucoside; CYT, cytosol; HDM, high density microsomes; HSP, high speed pellet; IC, intracellular; IR, insulin receptor; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; M/N, mitochondria/nuclei; PI, phosphatidylinositide; PIP, phosphatidylinositol 3′-phosphate; PM, plasma membrane; SLO, Streptolysin-*O*; T_x-100, Triton X-100.

still probably the most commonly held view, has been supported by the realization that each of the signaling molecules that make up these signaling pathways belong to large gene families (Marshall, 1994; Toker and Cantley, 1997). In unicellular organisms, such as yeast, there are at least three isoforms of both MAPK and MAPK-kinase, and each isoform is located on distinct downstream pathways that coordinate a particular biological action in response to unique stimuli (Posas and Saito, 1997).

Another level of specificity may be derived from the intracellular location of different signaling molecules. For example, several growth factors stimulate the translocation of MAPK from the cytosol to the nucleus, where it is presumably capable of directly accessing downstream targets involved in transcriptional regulation (Gonzalez et al., 1993). Other signaling molecules, such as Raf and PI 3-kinase, also move from the cytosol to membrane compartments in response to activation (Susa et al., 1992; Traverse et al., 1993). Presumably the intracellular targeting of these proteins facilitates their interaction with the appropriate downstream substrates and/or signaling molecules. Indeed, the expression of Raf and PI 3-kinase isoforms, containing membrane-targeting motifs, results in the constitutive activation of these proteins and downstream signaling cascades (Stakoe et al., 1994; Klippel et al., 1996). In the case of PI 3-kinase, indiscriminate membrane targeting results in the activation of multiple signaling pathways, thus underscoring the absolute need to preserve the correct compartmentalization of this molecule (Klippel et al., 1996). This raises the possibility that the recruitment of signaling molecules to their precise site of action within the cell may be a central feature of biological specificity. PI 3-kinase represents one example where pleiotropism may be achieved by regulating its site of action, because it has been reported that this enzyme can be recruited to a variety of intracellular locations, such as the plasma membrane (PM; Susa et al., 1992), the microtubule organization centre (Kapeller et al., 1993), intracellular vesicles (Heller-Harrison et al., 1996), or cytoskeleton (Zhang et al., 1992), in response to different growth factors.

The present studies focus on the regulation of glucose metabolism by insulin. Insulin triggers a number of intracellular metabolic pathways in muscle and adipose tissue, resulting in increased glycogen and/or lipid storage. The rate of glucose entry into these cells is an important rate determining step for each of these processes, and so this has become the focal point of numerous studies. Insulin stimulates glucose transport in adipocytes by as much as 30-fold, in a process that involves the regulated exocytic movement of a facilitative glucose transporter (GLUT4) from intracellular membranes to the cell surface (James et al., 1989; Slot, 1991). This is an ideal biological step with which to dissect the specificity of downstream signaling pathways because it is uniquely regulated by insulin and other hormones, such as EGF, PDGF, or thrombin are incapable of initiating this response (Robinson et al., 1993; Vandenberghe et al., 1994; Wiese et al., 1995).

One signaling protein that has been implicated in insulin-regulated glucose transport is PI 3-kinase (Kotani et al., 1995). PI 3-kinase is a member of a lipid kinase family and consists of two subunits: a p85 regulatory and a p110 catalytic subunit (Hiles et al., 1992). The p85 subunit contains two SH2 domains and an SH3 domain that facilitate the interaction of this enzyme with other proteins. Insulin induces a rapid and marked accumulation of the reaction products of PI 3-kinase activity, phosphatidylinositol 3,4 bisphosphate, and phosphatidylinositol 3,4,5 trisphosphate, in insulin-sensitive cells (Kelly et al., 1992). The time course of insulin-activated PI 3-kinase parallels that of insulin-stimulated glucose transport (Kelly et al., 1992). In addition, potent PI 3-kinase inhibitors, such as the fungal metabolite, wortmannin, and the synthetic compound, LY294002, inhibit both insulin-stimulated glucose transport and the translocation of GLUT4 to the cell surface (Okada et al., 1994). Consistent with these studies, dominant negative mutants of the p85 regulatory subunit block insulin-triggered GLUT4 translocation in adipocytes (Kotani et al., 1995), whereas constitutively activated PI 3-kinase isoforms promote GLUT4 translocation in these cells (Katagiri et al., 1996). Collectively, these studies provide compelling evidence in favor of a role for PI 3-kinase in the regulation of glucose transport by insulin.

One of the challenges in defining a specific role for PI 3-kinase in the regulation of glucose transport is that other growth factors, such as PDGF, also stimulate PI 3-kinase activity in adipocytes, but have no significant effect on glucose transport in these cells (Wiese et al., 1995). The mode of PI 3-kinase activation is quite different for these two hormones, however, in that PI 3-kinase binds directly to the PDGF receptor in response to PDGF (Kazlauskas et al., 1992), whereas, in response to insulin, PI 3-kinase associates with one of the major substrates of the insulin receptor, insulin receptor substrate-1 (IRS-1; Sun et al., 1991). IRS-1 is a 160-kD protein that is phosphorylated on multiple tyrosine residues in response to insulin. Two of these phosphorylation sites exist within a consensus motif (YMXM) for the binding of the p85 subunit of PI 3-kinase (Sun et al., 1992). IRS-1 also contains a phosphotyrosyl-binding domain that appears to facilitate interaction with the insulin receptor (Gustafson et al., 1995). In addition, IRS-1 contains a pleckstrin homology domain that is also required for insulin-dependent phosphorylation (Yenush et al., 1996). IRS-1 is rapidly phosphorylated in intact cells after insulin stimulation (maximal within 20–40 s), and it is believed that a significant proportion of IRS-1 (~50%) undergoes phosphorylation in response to a maximal insulin challenge (Madoff et al., 1988). It has been suggested that phosphorylation of IRS-1 occurs at the cell surface because efficient phosphorylation of this protein can be achieved under conditions where internalization of the insulin receptor is inhibited (Heller-Harrison et al., 1995). Despite this claim, the intracellular location of IRS-1 is not known. IRS-1 does not contain a transmembrane domain, nor is there any evidence for posttranslational modification of the protein that would enable it to associate with membranes. In CHO cells that have been transfected with the IRS-1 cDNA, the protein appears largely cytosolic as determined by immunofluorescence microscopy (Sun et al., 1992). Subcellular fractionation studies in adipocytes, however, suggest that IRS-1 is predominantly membrane associated (Kelly and Ruderman, 1993). Establishing the intracellular distribution of IRS-1 and PI 3-kinase in adipocytes may provide clues concerning the basis for the biological specificity of insulin action in these cells.

In the present study, we have used differential centrifugation and sucrose density gradient sedimentation to study the distribution of signaling proteins in adipocytes. We show that insulin and PDGF recruit PI 3-kinase to distinct intracellular locations: with PDGF, the major increase was observed in the PM, whereas with insulin, PI 3-kinase was recruited to a high speed pellet fraction (HSP) that also contained IRS-1 and the intracellular pool of GLUT4. Using flotation and velocity sedimentation analysis, we were able to further fractionate the HSP fraction into two discrete components, one comprising intracellular membranes enriched in GLUT4 and the γ-adaptin subunit of the Golgi clathrin-coat, and another containing large protein complexes and cytoskeletal elements. IRS-1 and PI 3-kinase were enriched in the latter fraction, which was detergent insoluble, but readily dissociated at high ionic strength. Based on these observations, we propose that signaling molecules such as IRS-1 and PI 3-kinase may be specifically targeted to a proteinaceous matrix in adipocytes that may be held in place via interactions with the cytoskeleton. Based on the specificity with which insulin acts upon these effectors at this location, we suggest that this matrix must be readily accessible to the PM, and uniquely affords activation of appropriate downstream targets, such as GLUT4 translocation.

Materials and Methods

Reagents

3T3-L1 murine fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). All tissue culture reagents were obtained from GIBCO BRL (Gaithersburg, MD), except for FCS, which was purchased from Trace Biosciences (Clayton, Australia). Insulin was purchased from Calbiochem Corp. (San Diego, CA) and PDGF.B was purchased from Boehringer Mannheim (Mannheim, Germany) and Upstate Biotechnology Inc. (Lake Placid, NY). Acrylamide/bis-acrylamide (29:1) was obtained from Bio-Rad (Hercules, CA), and BSA was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Unless specified, all other biochemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). Streptolysin-*O* (SLO) was obtained from S. Bhakdi (University of Mainz, Mainz, Germany). BCA reagent, used in protein assays, was obtained from Pierce (Rockville, IL).

Antibodies

The antibodies used in this study were obtained from the following sources: anti-phosphotyrosine antibody (4G10) from B. Druker (Oregon Health Sciences University, Portland, OR); anti–IRS-1 polyclonal antibody from G. Lienhard (Dartmouth University, Dartmouth, UK); anti-Grb2 polyclonal antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-mSos polyclonal antibodies from D. Bowtel (Peter MacCallum Cancer Institute, Melbourne, Australia); anti–γ-adaptin polyclonal antibodies from M. Robinson (Cambridge University, Cambridge, UK); anti-Shc and anti-$p85^{PAN}$ polyclonal antibodies were from Upstate Biotechnology Inc. The GLUT4 antibody (R820) has been described in detail previously (James et al., 1989).

Cell Culture

3T3-L1 fibroblasts were cultured in DME supplemented with 10% newborn calf serum, L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin sulfate (50 mg/ml). Cells were grown to confluence and differentiated to form adipocytes, as previously described (Piper et al., 1991). Adipocytes were routinely used at 10–20 d postdifferentiation. Before experiments, 3T3-L1 adipocytes were incubated in Krebs-Ringer phosphate solution (2.5 mM Hepes, pH 7.4, 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 10 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 0.6 mM Na_2HPO_4), supplemented with 2% BSA for 2 h at 37°C to establish basal conditions.

CHO cells, overexpressing the insulin-receptor (IR) and IRS-1 (CHO/IR/IRS-1; Sun et al., 1992), were a generous gift from M. White (Harvard University, Cambridge, MA). CHO/IR/IRS-1 cells were routinely cultured in DME containing 10% FCS, L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin sulfate (50 mg/ml) supplemented with geneticin (0.8 mg/ml). Before experiments, confluent CHO/IR/IRS-1 cells were brought to basal conditions by incubation in DME, free of additives, for 2 h at 37°C.

Cell Permeabilization

In some experiments, cells cultured in 100 mm culture dishes were permeabilized by treatment of cells with the bacterial endotoxin, SLO. Preliminary experiments established that the concentration of SLO required to facilitate propidium iodide staining of the nucleus in 100% of CHO cells and adipocytes was 0.5 μg/ml and 2.0 μg/ml, respectively. Before permeabilization, cells were brought to basal conditions, as outlined above, and washed once in prechilled intracellular (IC) buffer (20 mM Hepes, pH 7.2, 140 mM potassium glutamate, 5 mM EGTA, 5 mM MgCl, 5 mM NaCl). To facilitate the binding of SLO to the cell surface, cells were incubated with the appropriate concentration of SLO in 2 ml of IC buffer for 5 min at 4°C, and then washed once with cold IC buffer. Permeabilization of cells was initiated by incubating cells in 2 ml of IC buffer containing 1 mg/ml BSA, 1 mM dithiothreitol, and an ATP regeneration system (40 IU creatine phosphokinase, 5 mM creatine phosphate, 1 mM ATP) at 37°C for 15 min. Cells were subsequently washed, and subcellular fractions obtained as outlined below.

Subcellular Fractionation of 3T3-L1 Adipocytes

Whole cell lysates were prepared from 3T3-L1 adipocytes cultured in 35 mm tissue culture dishes. After growth factor treatment, cells were rinsed twice in ice cold PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4). Washed cells were frozen in liquid N_2, and stored at −70°C. Immediately before assay, frozen cell monolayers were scraped into 500 μl of ice cold lysis buffer A (20 mM Tris, pH 7.5, 1 mM EDTA, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM PMSF) containing phosphatase inhibitors (2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM *tetra*-sodium pyrophosphate, and 1 mM ammonium molybdate), and mixed for 15 min at 4°C. Cell lysates were centrifuged at 12,500 *g* in a microfuge (Beckman Instruments, Inc., Palo Alto, CA) for 15 min at 4°C, and the resultant supernatant cleared of fat particles by filtration through a 0.45-μm filtration unit (Millipore Corp., Bedford, MA).

Subcellular fractions were prepared from 3T3-L1 adipocytes, cultured in 100 mm tissue culture plates, as previously described (Piper et al., 1991), with minor modifications. After growth factor treatment, cells were rinsed rapidly in ice cold TES (20 mM Tris, pH 7.4, 5 mM EDTA, 250 mM sucrose), and then harvested in ice cold TES containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 250 μM PMSF), and phosphatase inhibitors, as noted above. The suspension was homogenized by 10 passages through a cylinder cell homogenizer (H and Y Enterprise, Redwood City, CA) and subcellular fractions prepared by sequential differential centrifugation steps. Briefly, the homogenate was subjected to centrifugation at 13,000 *g* using an SS-34 rotor (Sorvall Instruments Division, Newton, CT) at 4°C for 20 min. The resultant pellet was washed and resuspended in 1 ml of TES, layered onto a 1.12 M sucrose cushion and centrifuged at 77,000 *g* for 1 h using an SW-41 rotor (Beckman Instruments Inc.) to obtain PM. The 13,000 *g* supernatant was centrifuged at 30,000 *g* using an SS-34 Rotor (Sorvall Instruments Division) for 30 min to obtain a pellet designated high density microsomes (HDM). The supernatant was cleared of remaining fat particles by filtering through a 0.45-μm filter (Millipore Corp.) and centrifuged at 175,000 *g* using a Ti-80 rotor (Beckman Instruments Inc.) to obtain a supernatant, designated cytosol (CYT) and high speed pellet (HSP). In previous studies, this pellet has been referred to as the low density microsomal fraction (Piper et al., 1991). However, the studies described herein suggest that a large proportion of the protein in this fraction is not directly associated with microsomal membranes, in which case, HSP is a more appropriate designation.

In some experiments the effects of growth factors on the subcellular distribution of GLUT4 was assessed using the PM lawn technique (Robinson et al., 1992). Briefly, adipocytes cultured on coverslips were treated with or without growth factor, and then sonicated to remove the majority of cell components except for fragments of plasma membrane that remained attached to the coverslip. Coverslips were then incubated in GLUT4 specific antisera, followed by a second incubation with FITC-con-

jugated secondary antibody. Immunolabeling was visualized by fluorescence microscopy using an Axioscope fluorescent microscope (Zeiss, Oberkochen, Germany).

Subcellular Fractionation of CHO Cells

An abbreviated differential centrifugation procedure was used to obtain subcellular fractions from CHO/IR/IRS-1 cells. Untreated or insulin-treated cells were washed twice in prechilled fractionation buffer (HES: 20 mM Hepes, pH 7.4, 1 mM EDTA, 250 mM sucrose), and homogenized in HES, supplemented with protease and phosphatase inhibitors, by 15 passages with a teflon homogenizer. The homogenate was then subjected to centrifugation at 15,000 *g* at 4°C for 15 min using an SS-34 rotor (Sorvall Instruments Division). The 15,000 *g* supernatant was centrifuged at 175,000 *g* at 4°C for 1 h 15 min using a Ti-80 rotor (Beckman Instruments Inc.) to yield a pellet designated HSP, and a supernatant designated CYT. Fractions were subjected to immunoblotting or velocity sedimentation analysis as described below.

Immunoprecipitation and PI 3-Kinase Assay

Whole cell lysates (500 μg), subcellular membrane fractions (25–100 μg of protein), and CYT (200 μg of protein) prepared from treated cells, were diluted in lysis buffer to a final volume of 500 μl and subjected to immunoprecipitation by incubation with an anti-phosphotyrosine antibody (4G10) prelinked to protein A/G beads (Pierce). Immunoprecipitations were performed for 2–4 h, or overnight at 4°C. Immunoprecipitated complexes were collected by centrifugation at 13,000 *g* for 10 s and washed sequentially in 1% NP-40 in PBS, pH 7.4, and 50 mM LiCl in 100 mM Tris, pH 7.5, and then washed twice in 100 mM NaCl in 10 mM Tris, pH 7.5. In some experiments, an aliquot of immunocomplexes was removed during the last wash and retained for Western blotting analysis. The remaining immunocomplexes were assayed for PI 3-kinase activity as previously outlined (Hara et al., 1994), using phosphatidylinositol as substrate. The reaction products were spotted onto silica-60–coated plates (Merck, Darmstadt, Germany) and subjected to TLC. The radio-labeled phospholipid product was visualized by autoradiography and then excised from silica plates and quantitated by scintillation counting (Packard 1900CA; Packard Instrument Co. Inc., Downers Grove, IL). Radio-labeled phosphatidylinositol 3-phosphate (PIP) was identified on the basis of its disappearance in wortmannin-treated cells (data not shown).

Immunoblotting

Proteins were subjected to SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore Corp.). Membranes were incubated with either 5% skim milk powder or 3% BSA in TBST (20 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween-20), and subsequently incubated with primary antibody, washed and incubated with the appropriate horseradish-peroxidase-linked secondary antibodies (Amersham Intl., Little Chalfont, UK) for 1 h at room temperature. To visualize immunoreactive proteins, membranes were incubated with enhanced chemiluminescence substrate, (Amersham Intl.) or Supersignal (Pierce), and subjected to autoradiography by exposing each membrane to film (X-OMAT; Fuji, Tokyo, Japan).

Treatment of 3T3-L1 Adipocyte HSP Fractions with Detergent or Ionic Buffer

The HSP fraction was prepared from nontreated and insulin-treated 3T3-L1 adipocytes, in fractionation buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 250 mM sucrose; HES), containing protease and phosphatase inhibitors as detailed above, and divided into five aliquots of equal volume. Aliquots were incubated for 1 h on ice, with one of each of the five following solutions to give the final detergent or NaCl concentrations as indicated: HES alone, pH 7.4; 1% Triton X-100 (T_x-100) in HES, pH 7.4; 60 mM β-octylglucoside in HES, pH 7.4; 1 M NaCl in HES, pH 7.4; and 0.5 M NaCl in HES, pH 7.4. The insoluble component of the HSP, resulting from each treatment, was pelleted by centrifugation at 175,000 *g*, using a TLA 100.3 rotor (Beckman Instruments Inc.) at 4°C for 1 h 15 min. The resultant pellets were solubilized with Laemmli sample buffer and stored at −20°C overnight before Western blotting analysis.

Detergent-treated HSP was also prepared for electron microscopy analysis, using a slightly modified procedure. The 30,000 *g* supernatant was obtained from untreated or insulin-stimulated adipocytes using the subcellular fractionation method, as described above. Aliquots of supernatant were mixed with an equal volume of fractionation buffer or fractionation buffer containing 2% T_x-100 and incubated for 1 h on ice. The insoluble components were collected by centrifugation, as noted above. Immunoblotting showed that the relevant proteins were present in the detergent insoluble components of the HSP, regardless of the method used (data not shown).

Sucrose Gradient Fractionation

In both flotation and velocity sedimentation analysis, gradients or sucrose layers were prepared using chilled stock sucrose solutions in buffer (20 mM Hepes, pH 7.4, 1 mM EDTA) containing phosphatase and protease inhibitors, as above. All sucrose concentrations are expressed as a percentage (wt/vol). For flotation analysis, HSP prepared from untreated or insulin-treated cells, was resuspended in 200 μl of HES. An aliquot of resuspended membranes (165 μl) was mixed with 835 μl of 70% sucrose solution to give a final concentration of 60% sucrose. This was then successively overlaid with 1 ml of 50% sucrose, 1 ml of 30% sucrose, 1 ml of 10% sucrose, and 400 μl of 5% sucrose to give a final volume of 4.4 ml. Gradients were centrifuged (90,000 *g* for 18 h) at 4°C using an SW60 rotor (Beckman Instruments Inc.). Gradient fractions (400 μl) were collected by piercing the bottom of each tube with a 27-in gauge needle. The pellet was resuspended in either 400 μl of HES or lysis buffer, as appropriate. Aliquots of each fraction were then subjected to immunoblotting (25 μl) or immunoprecipitation and PI 3-kinase assay (100 μl).

Velocity sedimentation analysis of the HSP fraction was performed in continuous (5–30%) sucrose gradients (4.2 ml). The HSP fraction was prepared from untreated or insulin-treated cells, and resuspended in 5% sucrose in 20 mM Hepes, 1 mM EDTA, and layered on top of the gradient. Gradients were centrifuged (90,000 *g* for 90 min) at 4°C using an SW60 rotor (Beckman Instruments Inc.). Fractions from each gradient were collected as noted above, and aliquots (25 μl) subjected to immunoblotting. For electron microscopy, fractions of interest were combined and diluted in buffer, supplemented with protease and phosphatase inhibitors, to give a final concentration of 250 mM (isotonic) sucrose. Each sample was then subjected to centrifugation at 175,000 *g* using a TLA 100.3 rotor (Beckman Instruments Inc.) at 4°C for 1 h 15 min. Pellets obtained from this centrifugation were prepared for electron microscopy.

Electron Microscopy

The HSP fraction, the detergent insoluble components of the HSP, or sedimentable material from velocity gradient fractions of the HSP, were fixed in 2% paraformaldehyde in PBS. Paraformaldehyde-fixed material was randomly adsorbed to formvar-coated copper grids lightly coated with carbon. Dry grids were incubated on 10 μl drops of HSP suspensions for 10 min. All grids were then washed for 7 × 2 min with ultra-pure water and stained with 0.4% uranylacetate in 0.015 M oxalic acid, pH 7–8/1.8%, methylcellulose for 5 min. Grids were dried and viewed using a Jeol 1010 transmission electron microscope.

Results

Effects of Insulin versus PDGF on Glucose Transport and PI 3-Kinase in 3T3-L1 Adipocytes

In agreement with previous studies (Wiese et al., 1995), insulin stimulated glucose transport by 8- to 10-fold in 3T3-L1 adipocytes, whereas in response to PDGF, the increase was <2-fold (data not shown). To determine if this was due to a differential effect of these growth factors on GLUT4 translocation, adipocytes were incubated with insulin or PDGF or without any additions, and cell surface levels of GLUT4 examined. Consistent with the transport data, insulin caused a substantial increase in cell surface GLUT4, whereas, with PDGF, there was no significant effect (Fig. 1). In contrast, PI 3-kinase activity measured in anti-phosphotyrosine (anti-pY) immunocomplexes was increased by 8- and 10-fold of insulin and PDGF, respectively (Fig. 2). The increases in PI 3-kinase activity correlated with an increase in the binding of the p85 subunit of

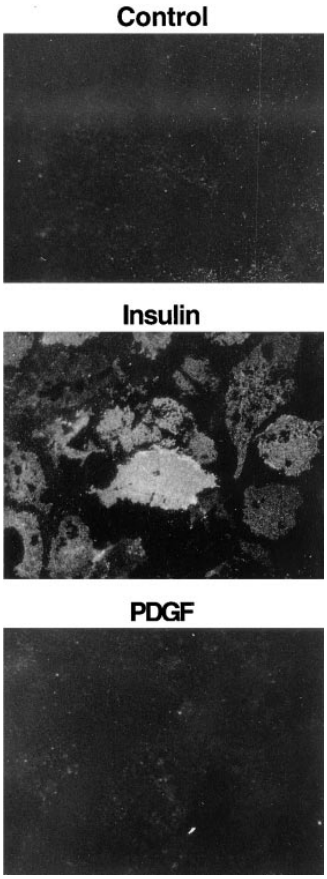

Figure 1. Effect of insulin and PDGF on GLUT4 translocation in adipocytes. 3T3-L1 adipocytes cultured on coverslips were brought to basal conditions and incubated with insulin (1 μM), or PDGF (100 ng/ml), or no additions (*Control*) for 15 min at 37°C. Cells were washed and sonicated to yield plasma membrane fragments (PM lawns). PM lawns were immunolabeled with anti-GLUT4 antibody and visualized using immunofluorescence microscopy. The results shown are representative of three independent experiments.

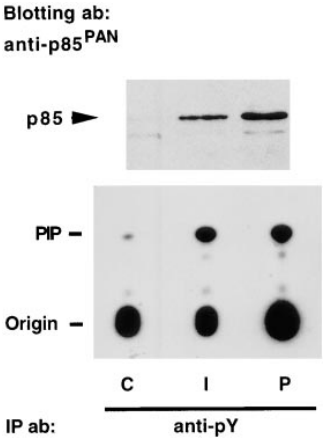

Figure 2. Effect of insulin and PDGF on PI 3-kinase activity. Whole cell lysates from 3T3-L1 adipocytes incubated with insulin (*I*; 1 μM) or PDGF (*P*; 100 ng/ml) or no additions (*C*), were subjected to immunoprecipitation (*IP*) using anti-phosphotyrosine antibodies (*anti-pY*). Immunoprecipitates were then subjected to either SDS-PAGE and immunoblotting with anti-p85^{PAN} polyclonal antibodies (*top panel*), or analysis of PI 3-kinase activity using phosphatidylinositol as substrate (*bottom panel*). The radio-labeled product, phosphatidylinositol 3′-phosphate (PIP) was separated from unincorporated radiolabel (*Origin*) using thin layer chromatography. Similar results were obtained in four independent experiments.

PI 3-kinase to tyrosyl-phosphorylated proteins, in response to insulin or PDGF (Fig. 2). These data are in agreement with previous observations (Wiese et al., 1995).

Differential Localization of PI 3-kinase in Response to Insulin versus PDGF

To study the effects of insulin versus PDGF on the intracellular location of PI 3-kinase, a series of subcellular fractionation studies, using 3T3-L1 adipocytes, was performed. Adipocytes provide a useful model system for these studies, because a relatively simple fractionation procedure has been established using differential centrifugation that enables the isolation of fractions enriched in markers specific for PM; mitochondria/nuclei (M/N); endosomes and endoplasmic reticulum (HDM); and intracellular membranes comprising recycling endosomes, the Golgi apparatus, and intracellular GLUT4 storage vesicles (HSP; Simpson et al., 1983; James and Pilch, 1988; Piper et al., 1991; Martin et al., 1996). The supernatant contains all elements that do not sediment at 175,000 *g* and is designated the CYT. In most studies, the M/N fraction was excluded from analysis because preliminary experiments showed that the relevant signaling molecules were not present in this fraction at significant levels.

PI 3-kinase activity was measured in anti-pY immunocomplexes obtained from subcellular fractions of cells that had been incubated with either insulin or PDGF (Fig. 3 *A*). In the basal state, phosphotyrosine immunoprecipitable PI 3-kinase activity was predominantly located in the cytosol. In response to insulin, the largest increase in PI 3-kinase activity was detected in the HSP (9–11-fold compared to basal, $n = 3$), and this fraction contained the largest proportion of total cellular PI 3-kinase activity (53%; Fig. 3 *C*). Insulin also caused a small but significant increase in PI 3-kinase activity in CYT, HDM, and PM fractions. In response to PDGF, the distribution of PI 3-kinase was significantly different to that observed with insulin. The predominant PDGF-stimulated PI 3-kinase activity was found in the PM (>40-fold compared to basal, $n = 3$) This fraction also contained the majority (56%), of the total cellular PI 3-kinase activity detected in response to PDGF. PDGF also induced a significant increase in the HDM but had no significant effect on PI 3-kinase activity in either cytosol or HSP. These changes in general, paralleled the distribution of anti-phosphotyrosine immunoprecipitable p85 (Fig. 3 *B*).

Given that the above measurements were performed on anti-pY immunoprecipitates, we could not be certain that these measurements truly reflected the distribution of PI 3-kinase within adipocytes. To address this, the distribution of the p85 subunit of PI 3-kinase was analyzed by immunoblotting with an antibody raised against p85 (anti-p85 ^{PAN}). The predominant band that was consistently labeled with this antibody in adipocytes had an average mobility in SDS-PAGE of 85 kD (Fig. 4 *A*). However, other bands of average mobility corresponding to ~100, 45, and 55 kD were routinely observed. The latter two bands likely correspond to previously reported isoforms or splice variants of this enzyme that have been shown to cross-react with the p85 antibody used in these studies (Inukai et al., 1997). In the absence of hormone, the majority of immunoreactive p85 was found in the cytosol and HSP. With insulin there was a two- to threefold increase in the immunoreactive p85 in the HSP fraction, commensurate with a decrease in the cytosol and relatively little change in the PM. In response to PDGF, there was a fivefold increase in immunoreactive p85 in the PM, and no change in either the HSP fraction or cytosol. The differential distribution of immunoreactive p85 in different fractions of cells treated with insulin versus PDGF is similar to that observed in anti-pY immunocomplexes (Fig. 3 *B*). We did not observe any significant change in the distribution of ei-

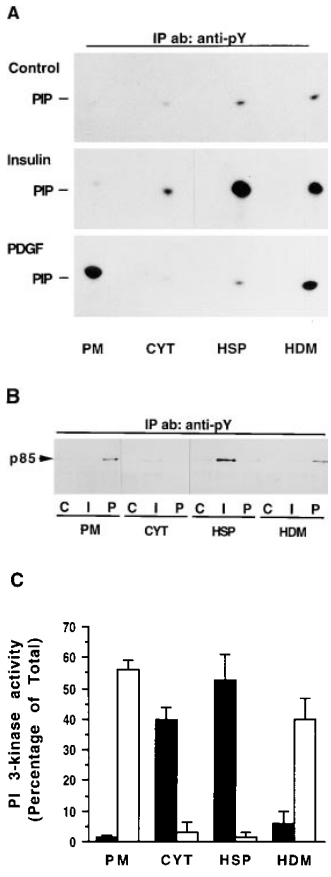

Figure 3. Effect of insulin and PDGF on the subcellular distribution of PI 3-kinase activity. Adipocytes were brought to basal conditions and incubated with insulin (*I*) or PDGF (*P*) or no additions (*C*). Cells were then homogenized and subjected to differential centrifugation to obtain fractions enriched in plasma membrane (*PM*), endoplasmic reticulum and endosomal markers (*HDM*), cytosol (*CYT*), and a high speed pellet fraction (*HSP*). Aliquots of each fraction (*PM* 25 μg; *HSP*, 100 μg; *HDM*, 75 μg; *CYT* 200 μg) representing on average 3/5, 2/5, 3/5, and 1/20 of these fractions in whole cells, respectively, were subjected to immunoprecipitation using anti-phosphotyrosine antibodies (*anti-pY*). Immunoprecipitates were then used for measurement of (*A*) PI 3-kinase activity, or (*B*) immunoreactive p85 using a polyclonal anti-p85^{PAN} antibody. Quantitation of the distribution of PI 3-kinase activity among subcellular fractions is shown in (*C*). Total PI 3-kinase activity in each fraction from insulin (■), versus PDGF (□), treated cells was calculated and expressed as percent of total after subtracting basal values. The mean values (± SEM) from three independent experiments are shown. *PIP*, phosphatidylinositol 3′-phosphate.

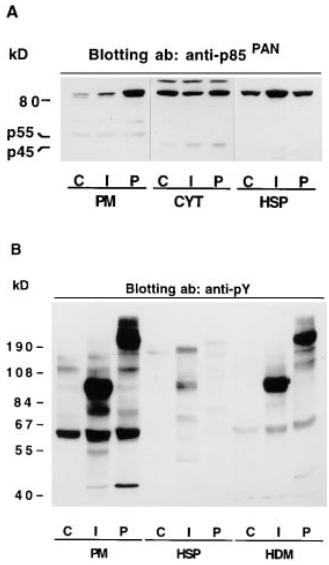

Figure 4. Effects of insulin versus PDGF on subcellular distribution of PI 3-kinase and tyrosyl-phosphorylated proteins in 3T3-L1 adipocytes. Adipocytes were brought to basal and incubated with insulin (*I*), or PDGF (*P*), or no additions (*C*). Cells were washed, homogenized and subcellular fractions prepared. Aliquots of each fraction (25 μg of protein) were analyzed by SDS-PAGE and immunoblotted with antibodies specific for (*A*) the p85 subunit of PI 3-kinase (*anti-p85^{PAN}*) or (*B*) phosphotyrosine (*anti-pY*). There was no significant change in the distribution of p85 in the HDM with either insulin or PDGF (not shown). Tyrosyl phosphorylation could not be detected in the cytosol using this procedure (not shown). Results are representative of two experiments.

ther the p45 or p55 immunoreactive species, suggesting that these proteins do not respond to these growth factors. The p55 band was enriched in the PM, whereas p45 was enriched in the cytosol (Fig. 4 *A*).

The profile and subcellular distribution of tyrosine phosphorylated proteins observed in response to insulin, was distinctly different from that observed in response to PDGF (Fig. 4 *B*). Tyrosyl-phosphorylated IRS-1 (180 kD) was enriched in the HSP, in agreement with previous studies (Kelly and Ruderman, 1993; Heller-Harrison et al., 1995). Several other insulin-regulated tyrosyl phosphoproteins in the molecular mass range 50–150 kD were also detected in the HSP. The 95-kD species presumably corresponds to the IR β-subunit. In agreement with previous studies, this protein is highly enriched in the PM (Kublaoui et al., 1995). The identity of the other insulin-regulated phosphotyrosine proteins in the HSP is not known. Insulin also stimulated tyrosine phosphorylation of proteins of molecular mass 60 and 53 kD in the PM, that may correspond to p60 (IRS-3; Lavan et al., 1997) and another recently identified insulin receptor-tyrosine kinase substrate, p53/p58 (Yeh et al., 1996), respectively. None of these insulin-stimulated phosphotyrosine proteins were observed in PDGF-treated cells (Fig. 4 *B*). The major phosphotyrosine protein (195 kD) observed in PDGF-treated cells was enriched in the PM and HDM and has an identical electrophoretic mobility to the immunoreactive murine PDGF receptor (data not shown).

Subcellular Distribution of IRS-1, Grb2, mSos, and Shc

As described above, PI 3-kinase and tyrosyl-phosphorylated IRS-1 are enriched in the HSP fraction isolated from adipocytes, in agreement with previous studies (Kelly and Ruderman, 1993; Heller-Harrison et al., 1995; Kublaoui et al., 1995). As shown in Fig. 5, immunoreactive IRS-1 is also enriched in this fraction. It is noteworthy that the IRS-1 antibody labels two proteins in the HSP of average mobility 165 and 120 kD. The upper of these two bands has a relative molecular mass which corresponds to IRS-1 and undergoes an apparent shift in molecular mass from 165 to 180 kD in response to insulin. It has previously been documented that IRS-1 undergoes a gel shift subsequent to phosphorylation (Sun et al., 1992). The lower band likely represents an unrelated protein that cross-reacts with the antibody, as opposed to IRS-1 degradation, because we failed to observe any evidence for tyrosine phosphorylation of a protein in this region of the gel (Fig. 4 *B*). In some experiments, insulin resulted in a redistribution of IRS-1 from the HSP fraction to cytosol, consistent with previous studies (Heller-Harrison et al., 1995). Presumably, the insulin-induced redistribution of IRS-1 to the cytosol accounts for the phosphotyrosine immunoprecipitable PI 3-kinase activity detected in the cytosolic fraction from insulin-treated cells, as shown above (Fig. 3, *A* and *C*). We did not observe any significant change in the subcellular distribution of IRS-1 in response to PDGF treatment (data not shown).

Insulin is known to activate numerous signaling molecules in adipocytes. These include, Grb2, another SH2 domain containing protein, that reportedly binds to IRS-1 in response to insulin (Sun et al., 1993) ; mSOS, a Grb2-binding protein (Baltensperger et al., 1993) that acts as a GTP

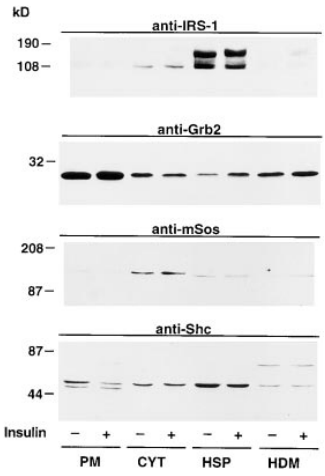

Figure 5. Subcellular distribution of IRS-1, Grb2, mSos, and Shc isoforms in adipocytes under basal and insulin-treated conditions. Adipocytes were brought to basal conditions and incubated in the absence (−) or presence(+) of insulin (1 μM) for 15 min at 37°C. Cells were washed, homogenized and subcellular fractions prepared. Aliquots of each fraction (25 μg of protein), the plasma membrane (*PM*), cytosol (*CYT*), high speed pellet (*HSP*), and high density microsomal (*HDM*) fractions, were analyzed by SDS-PAGE and immunoblotted with antibodies specific for IRS-1, Grb2, mSos, or Shc, respectively. The results shown are representative of two independent experiments.

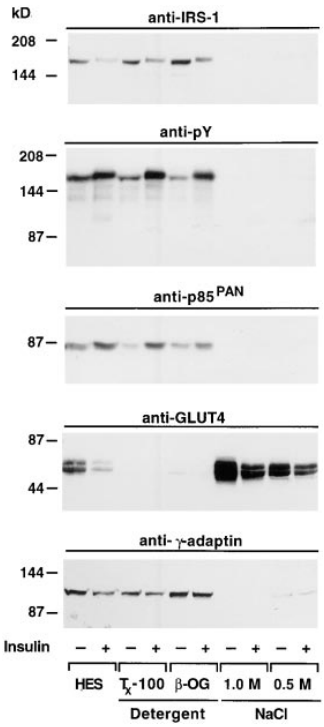

Figure 6. Effects of nonionic detergents and high ionic strength on the high speed pellet (HSP) fraction from adipocytes. Adipocytes were brought to basal conditions and incubated in the absence or presence of insulin (1 μM), and then subjected to differential centrifugation to generate the HSP fraction. The HSP was incubated in buffer (Hepes, pH 7.4, 1 mM EDTA, 250 mM sucrose) or the same buffer containing 1% Triton X-100 (*T_x-100*), 60 mM β-octylglucoside (*β-OG*), 1.0 M NaCl, or 0.5 M NaCl, at 4°C for 1 h, and then subjected to centrifugation at 175,000 *g*. The resultant pellets were analysed by SDS-PAGE and immunoblotted with antibodies specific for IRS-1, phosphotyrosine (*anti-pY*), PI 3-kinase (*anti-$p85^{PAN}$*), GLUT4, and γ-adaptin, respectively. The results are representative of three independent experiments.

exchange factor for p21 Ras; and Shc, another insulin-regulated docking protein (Skolnik et al., 1993). We next examined the distribution of these proteins in adipocytes to determine if the localization of IRS-1 and PI 3-kinase to the HSP fraction is specific or, alternatively, reflects a general depot for signaling proteins (Fig. 5). In support of the former, none of these proteins, with the exception of one of the Shc isoforms, was enriched in the HSP fraction. Grb2 was found in all subcellular fractions in 3T3-L1 adipocytes, but was particularly enriched in the PM. In response to insulin, there was a significant increase in the level of Grb2 in the PM and HSP fractions, perhaps due to its interaction with tyrosine-phosphorylated p60 (IRS-3) and IRS-1, which are enriched in each of these fractions, respectively (Fig. 4 *B*). mSOS was highly enriched in the cytosol and did not undergo a change in subcellular distribution in response to insulin stimulation. Hence, unless mSos dissociated from Grb2 during the isolation of these fractions, it would appear that Grb2 must bind to additional proteins in adipocytes other than mSOS, and thus potentially recruits unique signaling proteins to either the PM or HSP fractions.

The subcellular distribution of Shc was of particular interest because this molecule encodes a similar function to IRS-1, acting as a docking protein for downstream signaling proteins (Skolnik et al., 1993). Furthermore, three different Shc isoforms have been reported, the individual functions of which remain to be elucidated (Pelicci et al., 1992). All three isoforms were detected in 3T3-L1 adipocytes (Fig. 5) and, intriguingly, the subcellular distribution of each was completely different. The 66-kD isoform was enriched in the HDM with lower amounts also found in the PM. The 46-kD isoform was highly enriched in the PM (Fig. 5) and M/N (data not shown) while the 52-kD isoform was more widely distributed, with the majority located in the cytosol and the HSP. Insulin did not significantly affect the subcellular distribution of any Shc isoform. These data clearly show that signaling proteins have widely varying subcellular distributions in adipocytes and this may have important implications for the specificity of their biological actions. Moreover, the distribution of Grb2, mSos, and Shc did not parallel that of IRS-1. This implies that IRS-1 must be maintained at this location by a specific association with another protein or membrane.

Effects of Detergents and High Ionic Strength on IRS-1 and PI 3-kinase Solubility

To further define the nature of the interaction of IRS-1 and PI 3-kinase with the HSP, we examined the effects of high ionic strength and different detergents on the solubility of these proteins (Fig. 6). Immunoreactive IRS-1, tyrosyl-phosphorylated IRS-1, and PI 3-kinase remained largely insoluble in the presence of the two nonionic detergents, T_x-100 and β-octylglucoside (β-OG). Under exactly the same conditions, the polytopic membrane protein, GLUT4, was completely solubilized. In contrast, denaturing detergents such as SDS, completely solubilized both IRS-1 and PI 3-kinase (data not shown). It has been suggested that specialized vesicular compartments including clathrin-coated vesicles and caveolae remain insoluble in certain detergents (Pearse, 1982; Kurchalia et al., 1992). In this regard, it was of interest that the γ-adaptin subunit of clathrin-coated vesicles was also largely insoluble in T_x-100 and β-OG (Fig. 6). However, as shown in Fig. 7, a large proportion of the γ-adaptin in this fraction is not associated with membranes and so this presumably represents preassembled adaptor complexes that can be sedimented. It is highly unlikely that the detergent insoluble fraction, containing IRS-1 and PI 3-kinase, represents caveolae because these organelles are generally quite soluble in the nonionic detergent β-OG (Brown and Rose, 1992). Moreover, we have shown that caveolin, a major constituent of caveolae, is not enriched in our HSP fraction, but remains

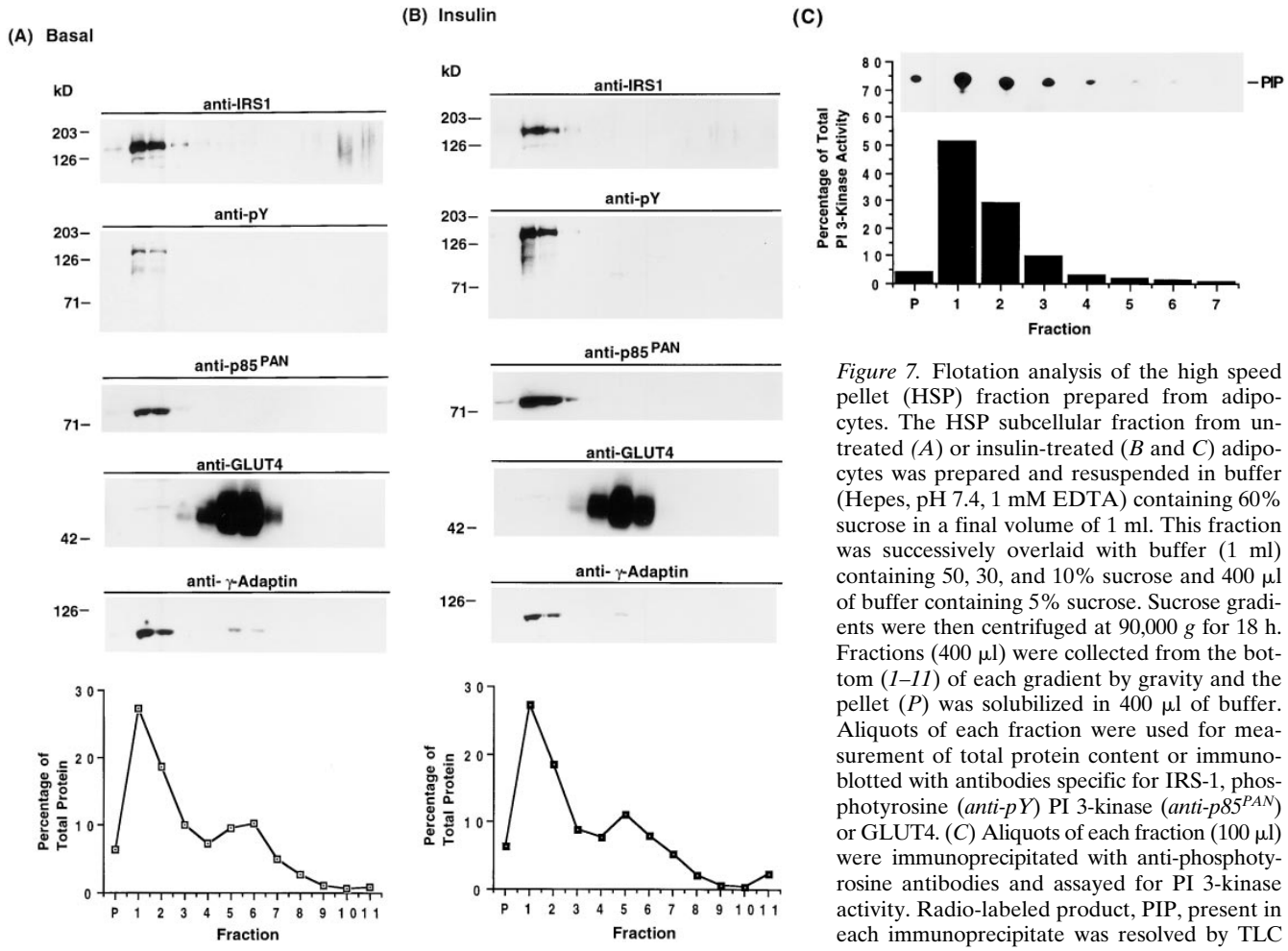

Figure 7. Flotation analysis of the high speed pellet (HSP) fraction prepared from adipocytes. The HSP subcellular fraction from untreated (*A*) or insulin-treated (*B* and *C*) adipocytes was prepared and resuspended in buffer (Hepes, pH 7.4, 1 mM EDTA) containing 60% sucrose in a final volume of 1 ml. This fraction was successively overlaid with buffer (1 ml) containing 50, 30, and 10% sucrose and 400 μl of buffer containing 5% sucrose. Sucrose gradients were then centrifuged at 90,000 *g* for 18 h. Fractions (400 μl) were collected from the bottom (*1–11*) of each gradient by gravity and the pellet (*P*) was solubilized in 400 μl of buffer. Aliquots of each fraction were used for measurement of total protein content or immunoblotted with antibodies specific for IRS-1, phosphotyrosine (*anti-pY*) PI 3-kinase (*anti-p85^{PAN}*) or GLUT4. (*C*) Aliquots of each fraction (100 μl) were immunoprecipitated with anti-phosphotyrosine antibodies and assayed for PI 3-kinase activity. Radio-labeled product, PIP, present in each immunoprecipitate was resolved by TLC (*inset*), excised, and quantitated by scintillation counting. The result shown depicts the percentage of PI 3-kinase activity immunoprecipitated from the pellet (*P*) and fractions (*1–7*), relative to total activity present in the entire gradient. Immunoprecipitates of fractions 8–11 contained <1% of total PI 3-kinase activity present in the entire gradient, and is not shown. Results are representative of two independent experiments.

attached to the plasma membrane. In addition, the minor pool of caveolin found in the HSP, is substantially solubilized by 1% T_x-100 (Hill, M., S. Clark, D. James, manuscript submitted for publication). In the presence of high ionic strength, both IRS-1 and PI 3-kinase were completely solubilized, suggesting that these proteins are retained in the HSP fraction via ionic interactions (Fig. 6).

PI 3-kinase–IRS-1 Complexes Are Not Associated with Membranes within the Adipocyte HSP

Specialized lipid micro-domains have been described, based on their resistance to solubilization with various nonionic detergents (Simons and Ikonen, 1997). These domains may be enriched in certain membrane lipids, such as glycolipids that confer detergent insolubility. One of the features that defines these types of structures, aside from detergent insolubility, is their buoyancy in hypertonic sucrose, due to the presence of lipid (Brown and Rose, 1992). In contrast, protein complexes and cytoskeletal elements do not exhibit this property (Fra et al., 1994). To determine if IRS-1 and PI 3-kinase are localized to a specialized lipid microdomain, the HSP fraction was loaded at the bottom of a discontinuous sucrose gradient and centrifuged for 18 h (Fig. 7). The HSP was resolved into two separate peaks of protein using this approach. The major peak (peak 1), which contained ~65% of HSP protein, remained at the bottom of the gradient in the 60% sucrose fraction. The second peak (peak 2) contained ~33% of HSP protein and was found in the middle of the gradient corresponding to the 30/50% sucrose interface. The distribution and recovery of protein in peaks 1 and 2 were not significantly different when HSP fractions were harvested from basal versus insulin-treated cells (Fig. 7, *A* and *B*). IRS-1 and PI 3 kinase were found exclusively in peak 1 both in basal and insulin-treated cells. Notably, we observed in these studies an insulin-dependent loss of IRS-1 from the HSP fraction that was presumably due to the redistribution of this protein to the cytosol (Figs. 5 and 6). Consistent with the studies described above (Fig. 4), there was a significant increase in IRS-1 tyrosine phosphorylation with insulin and an increase in PI 3-kinase associated with the HSP. However, these effects of insulin were only observed in peak 1, suggesting that neither IRS-1 nor PI 3-kinase

are associated with lipid-containing domains. As a control for the flotation analysis we also immunoblotted each fraction with an antibody specific for GLUT4. As expected, GLUT4-containing membranes quantitatively floated to a position in the gradient, corresponding to peak 2, proportional to the buoyant density of these membranes. In addition, it was noted that the amount of GLUT4 recovered in peak 2 from insulin-treated cells was significantly less than that from basal cells consistent with its movement from the HSP to the plasma membrane (see Fig. 1). This indicates that peak 2 contains the insulin-regulatable GLUT4 storage vesicles. Fractions were also immunoblotted with an antibody specific for γ-adaptin. Although the majority of this protein remained in peak 1, presumably representing preformed adaptor complexes, a significant amount was also found in peak 2. Hence, this indicates that the HSP contains coated vesicles and these remain intact during this fractionation procedure. A tyrosine-phosphorylated protein of M_r 95 kD, presumably corresponding to the β subunit of the IR, was also detected in peak 2 isolated from insulin-treated cells (Fig. 7 *B*).

These studies suggest that we have been able to segregate the HSP into two distinct fractions corresponding to peaks 1 and 2. Peak 1 contains the majority of protein as well as IRS-1 and PI 3-kinase, whereas peak 2 contains membranes and vesicles as determined by the presence of GLUT4, the IR and γ-adaptin. To rule out the possibility that a small amount of IRS-1 or PI 3-kinase, that is undetectable by immunoblotting, may remain associated with membranes in peak 2, we also measured PI 3-kinase activity in phosphotyrosine immunoprecipitates of each fraction from flotation gradients. This assay is much more sensitive than blotting and should provide a more quantitative measure of the distribution of this protein. Consistent with the distribution of immunoreactive p85 (Fig. 7 *B*), the majority (95%) of insulin-stimulated PI 3-kinase activity was recovered in peak 1 and the activity present in peak 2 was not significantly different from background (Fig. 7 *C*). Hence, these data indicate that PI 3-kinase does not associate with membranes within the HSP.

One possibility is that IRS-1 and PI 3-kinase complexes may dissociate from membranes during the flotation analysis and, thus, remain at the bottom of the gradient as free protein. If this were the case, it would be expected that these proteins would now behave as cytosolic proteins, and not sediment under the conditions that were originally used to generate this fraction. To address this possibility, the 60% sucrose fractions (F1 and F2) that comprised peak 1 from flotation gradients were diluted with buffer to achieve an isotonic sucrose concentration and resedimented. As shown in Fig. 8, a large proportion of both IRS-1 and PI 3-kinase were recovered in the pellets (P1 and P2) from both of these fractions. Thus, these data suggest that IRS-1 and PI 3-kinase are not associated with membranes in the HSP fraction and that their presence in this fraction must reflect an interaction with other proteins that render them detergent insoluble.

Velocity Sedimentation Analysis of the HSP

To further characterize the nature of the IRS-1–PI 3-kinase complex in adipocytes, we next subjected the HSP to velocity sedimentation analysis. This procedure separates particles on the basis of size rather than density. Under these conditions the majority of protein (68%) in the HSP remained at the top of the gradient (fractions 8–10) and, consistent with the flotation analysis, this peak contained the majority of IRS-1, tyrosyl-phosphorylated IRS-1 and PI 3-kinase (Fig. 9, *A* and *B*). Insulin stimulation did not substantially alter the velocity sedimentation profile of total protein nor IRS-1 (Fig. 9 *B*). However, we again observed an insulin-dependent loss of IRS-1 from the HSP which was presumably due to the re-distribution of this protein to the cytosol (Fig. 7, *A* and *B*). The insulin-stimulated increase in PI 3-kinase in the HSP cosedimented with the pool of tyrosyl-phosphorylated IRS-1. The majority (>80%) of immunoreactive GLUT4 in the HSP sedimented in zones (fractions 2–7) of the gradient that were devoid of IRS-1 and PI 3-kinase. There was a slight overlap between the GLUT4 peak and the IRS-1–PI 3-kinase peak. However, in view of the flotation studies described above, this almost certainly corresponds to the limited resolution of this technique and does not reflect colocalization of these proteins. Hence, using this technique, it would appear that IRS-1 and PI 3-kinase that remain in the upper portion of the velocity gradient, are not associated with membranes that sediment deeper into the gradient.

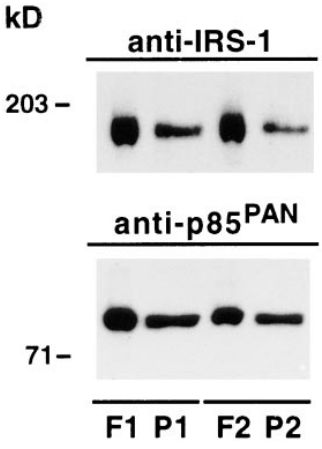

Figure 8. Resedimentation of IRS-1 and PI 3-kinase subsequent to sucrose gradient centrifugation. The HSP fraction from insulin-treated cells was subjected to flotation analysis as described in Fig. 7. Fractions 1 (*F1*) and 2 (*F2*) comprising Peak 1 from this gradient were made isotonic with buffer (20 mM Hepes, pH 7.4, 1 mM EDTA), and centrifuged at 175,000 *g* for 1 h 15 min. The corresponding pellets (*P1* and *P2*), together with an equivalent amount of the starting fractions (*F1* and *F2*), were subjected to SDS-PAGE and immunoblotted with specific antibodies for IRS-1 and PI 3-kinase (*anti-p85^{PAN}*). Identical results were also obtained using HSP isolated from basal cells and are not shown.

IRS-1 and PI 3-Kinase Have Similar Properties in Other Cell Types

IRS-1 is expressed at high levels in insulin-sensitive cell types, including adipocytes and myocytes (White, 1994). To determine if the association of IRS-1 and PI 3-kinase with insoluble proteinaceous particles is only a feature of these cell types, we performed similar experiments, to those described above, in CHO cells. CHO cells do not normally express significant levels of either IR or IRS-1 and this, in part, accounts for their lack of insulin sensitivity. Hence, we used CHO cells that have been stably transfected with the IR and IRS-1 cDNAs (Sun et al., 1992). Consistent with previous studies (Heller-Harrison et al.,

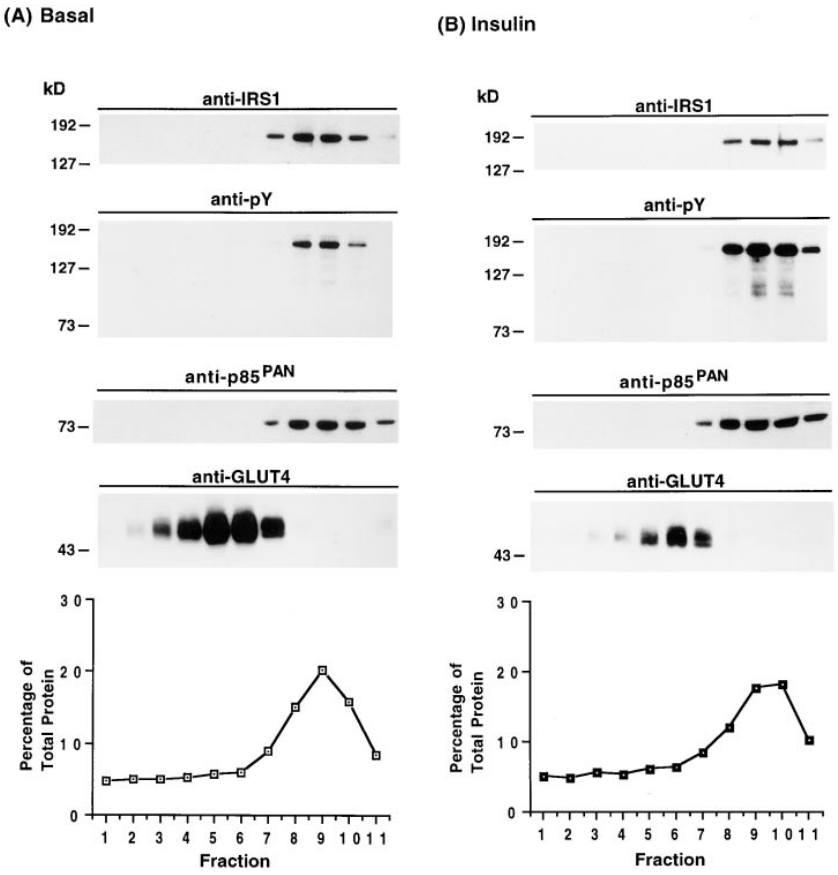

Figure 9. Sedimentation velocity analysis of the HSP from 3T3-L1 adipocytes. The HSP fraction obtained from basal (*A*), or insulin-treated adipocytes (*B*), was resuspended in buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 200 μl) containing 5% sucrose, and overlaid on to a 5–30% continuous sucrose gradient. Gradients were centrifuged at 90,000 *g* for 90 min, and fractions (400 μl) collected from the bottom (*1–11*) of each gradient by gravity. Aliquots of each fraction were assayed for protein content, or resolved by SDS-PAGE (30 μl) and subjected to immunoblotting with antibodies specific for IRS-1, phosphotyrosine (*anti-pY*), PI 3-kinase (*anti-p85^{PAN}*), and GLUT4. The results are representative of two independent experiments.

1995), we observed that a significant fraction of both IRS-1 and PI 3-kinase in these cells was found in an HSP fraction (see Fig. 11). The CHO/IR/IRS-1 cell HSP was next subjected to velocity gradient analysis and fractions were immunoblotted as described above (Fig. 10, *A* and *B*). The distribution of IRS-1, tyrosyl-phosphorylated IRS-1, and p85 in CHO cells was almost identical to that observed in adipocytes (Fig. 9). Moreover, insulin induced a significant increase in IRS-1 tyrosine phosphorylation and recruitment of PI 3-kinase in CHO/IR/IRS-1 cells that was commensurate with that observed in adipocytes (Fig. 10, *B*). Hence, these data suggest that the nonmembrane associated, insolubility of IRS-1 and PI 3-kinase that presumably results from the association of these proteins with a large protein complex, is a feature of many cells, and can be recapitulated using recombinant IRS-1 expressed in a cell type that normally expresses low levels of this protein.

Maintenance of the IRS-1–PI 3-Kinase Complex in Permeabilized Cells

The cytosolic or nonsedimentable pool of both IRS-1 and PI 3-kinase in CHO/IR/IRS-1 cells is significantly larger than is observed in adipocytes and can be readily detected by immunoblotting. Hence, this provided a useful system with which to experimentally define the existence of the two pools, cytosolic versus insoluble, of IRS-1 and PI 3-kinase in the intact cell. If the insoluble sedimentable pool of IRS-1 and PI 3-kinase represents a large preformed protein complex within the cell, then it should be retained after permeabilization. Whereas, provided the pores are sufficiently large, the cytosolic component of these proteins should be released from the cell under the same experimental conditions. The bacterial endotoxin, SLO, provides a useful reagent to achieve this purpose because it introduces large pores in the plasma membrane, leaving the remainder of the cell completely intact. As shown in Fig. 11, the majority of IRS-1 and immunoreactive p85 found in the cytosolic fraction was released from cells subsequent to permeabilization with SLO. In contrast, a substantial proportion of immunoreactive p85 and IRS-1, associated with the HSP fraction, was retained in permeabilized cells. We have also shown that a significant proportion of IRS-1 and PI 3-kinase is retained in SLO-permeabilized 3T3-L1 adipocytes (data not shown). However, because we do not observe a significant amount of IRS-1 in the cytosol of these cells, we have not been able to examine the release of this pool subsequent to permeabilization and, as is shown in the case of the CHO/IR/IRS-1 cells, this provides a very useful control. Notwithstanding, these studies collectively demonstrate that the insoluble fraction of IRS-1 and PI 3-kinase, which we have isolated biochemically in these studies, can also be experimentally defined as a large complex that is retained in cells, even under conditions where the cytosolic component of these same proteins is freely permeable.

Electron Microscopy Analysis of the HSP

We have previously shown that the HSP fraction isolated from adipocytes contains numerous vesicles ranging in diameter from 70–200 nm (James and Pilch, 1988; Martin et al.,

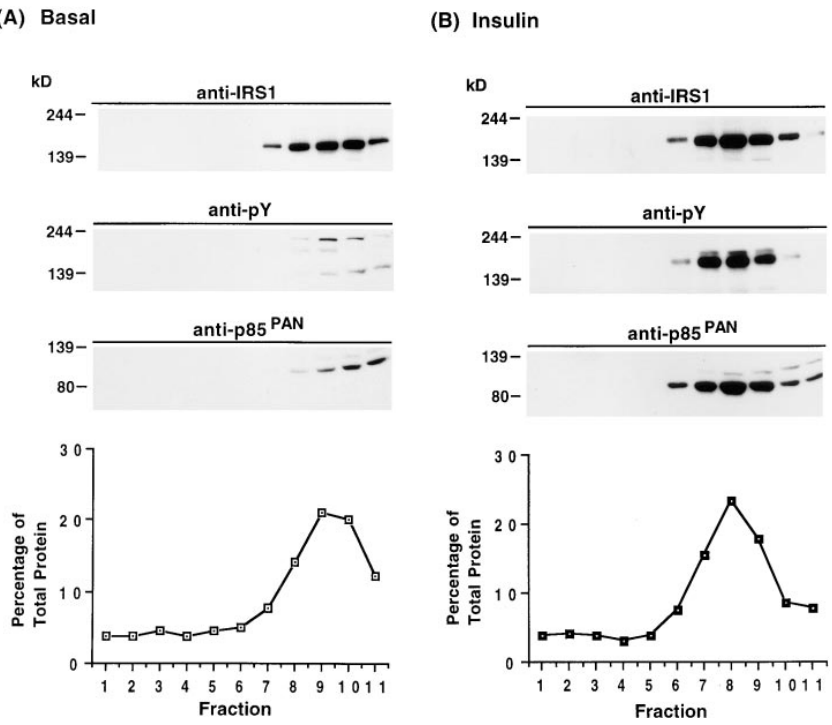

Figure 10. Sedimentation velocity analysis of the HSP from CHO/IR/IRS–1 cells. The HSP fraction obtained from basal (*A*), or insulin-treated (*B*), CHO cells overexpressing the insulin receptor (IR) and IRS-1, was resuspended in buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 200 μl) containing 5% sucrose, and overlaid on to a 5–30% continuous sucrose gradient. Gradients were centrifuged at 90,000 *g* for 90 min, and fractions (400 μl) collected from the bottom (*1–11*) of each gradient by gravity. Aliquots of each fraction were assayed for protein content, or resolved by SDS-PAGE (30 μl) and subjected to immunoblotting with antibodies specific for IRS-1, phosphotyrosine (*anti-pY*), and PI 3-kinase (*anti-p85^{PAN}*).

1996). Immunogold labeling studies have revealed that these vesicles are enriched in a variety of membrane and coat-associated proteins including GLUT4, VAMP2, and the mannose-6-phosphate receptor, (Martin et al., 1996). As shown in Fig. 12, electron microscopy of the IRS-1–PI 3-kinase peak and the GLUT4 peak, obtained by velocity centrifugation of the 3T3-L1 adipocyte HSP (Fig. 9), revealed that the latter, but not the former, was highly enriched in vesicles (Fig. 12, *A* and *B*). Intriguingly, long, filamentous structures with an estimated diameter of 5–15 nm, that resemble cytoskeletal elements (Kries and Vale, 1993), were preferentially retained in the gradient peak enriched in IRS-1 and PI 3-kinase. These structures were decorated with electron opaque particles with an estimated diameter of 10–50 nm, that, presumably, correspond to large protein complexes (Fig. 12 *A*). Similar filamentous structures were present in the T_x-100–insoluble component of the HSP, although in this instance, they appeared to be less labyrinthine and were decorated with large electron dense sacs (Fig. 12 *C*). These filamentous structures are present in the HSP, isolated from a variety of cell types, including both rat adipocytes and 3T3-L1 adipocytes, but appear more elongated in the HSP isolated from rat adipocytes (data not shown). Immunolocalization of either IRS-1 or PI 3-kinase in these fractions have been unsuccessful and so it remains to be determined if these proteins are directly associated with these cytoskeletal elements.

Discussion

PI 3-kinase plays a central role in numerous biological processes, including thrombin activation of platelets, apoptosis, growth factor–induced cell proliferation, differentiation, membrane ruffling, and insulin regulation of glucose metabolism (Toker and Cantley, 1997). Understanding how this enzyme acts as the focal point for such a diverse range of biological actions remains a major challenge. In the present study, we have addressed this problem by focusing on the insulin regulation of glucose transport in adipocytes. This provides a useful paradigm because considerable evidence supports a role for PI 3-kinase in insulin action and the regulation of glucose transport in adipocytes is quite specific to the hormone, insulin (Robinson et al., 1993; Vandenberghe et al., 1994; Wiese et al., 1995). An anomalous observation, that potentially confounded the specific role of PI 3-kinase in this process, was that PDGF also activates PI 3-kinase in adipocytes, quantitatively to the same extent as insulin, yet this growth factor is incapable of activating glucose transport or GLUT4 translocation to the cell surface (Wiese et al., 1995).

A major difference between insulin and PDGF signaling is that insulin uses a putatively soluble substrate, IRS-1, as a docking site for downstream signaling intermediates, such as PI 3-kinase, whereas PDGF uses its receptor for this purpose (Kazlauskas et al., 1992). Hence, we reasoned that this difference may confer specificity at the level of intracellular compartmentation, thus enabling PI 3-kinase to access a unique array of downstream effectors in either case. Using subcellular fractionation analysis, we verified this notion and showed that, in response to PDGF, PI 3-kinase was recruited to the plasma membrane, whereas, in the case of insulin, PI 3-kinase was recruited to an intracellular fraction, designated as HSP, that was quite distinct from the plasma membrane. The PDGF-activated receptor is, of course, enriched in the plasma membrane fraction, whereas IRS-1 is enriched in the HSP, consistent with the original hypothesis. These observations, which represented the impetus for these studies, has since been reported by two other groups and largely confirms the central tenet that the cellular location of PI 3-kinase may somehow confer biological specificity (Nave et al., 1996; Ricort et al., 1996).

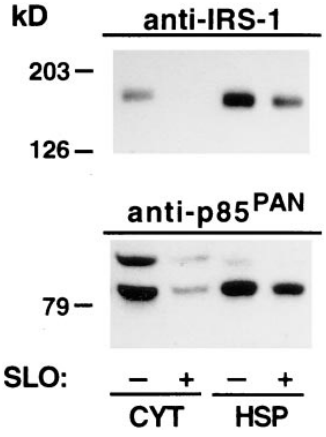

Figure 11. Retention of IRS-1 and PI 3-kinase within permeabilized CHO cells. CHO cells overexpressing IR and IRS-1 were brought to basal conditions and then incubated in the absence (−) or presence (+) of Streptolysin-*O* (*SLO*) to perforate the cells. Cells were washed, homogenized, and subjected to differential centrifugation to obtain the high speed pellet (*HSP*) and cytosolic (*CYT*) fractions. Aliquots of each fraction (15 μg of protein) were subjected to SDS-PAGE and immunoblotted with antibodies specific for IRS-1 and PI 3-kinase (*anti-p85^{PAN}*).

To further define the molecular basis for this differential compartmentalization of PI 3-kinase, we focused our efforts on characterizing the composition of the adipocyte HSP and, in particular, in defining the nature of the interaction of both IRS-1 and PI 3-kinase with this fraction. Importantly, IRS-1 was enriched in the HSP under basal conditions. Given that the bulk of tyrosyl-phosphorylated IRS-1 in cells treated with insulin remained associated with the HSP, it is more than likely that IRS-1 interacts with the insulin receptor in this fraction (Figs. 4 *B* and 5). Intriguingly, although insulin recruits PI 3-kinase from the cytosol to the HSP, presumably due to increased binding to tyrosyl-phosphorylated IRS-1, there is a significant component of PI 3-kinase found in the HSP in basal cells (Fig. 4 *A*). This may reflect PI 3-kinase bound to tyrosyl-phosphorylated IRS-1 because we could detect a phosphotyrosine protein of similar relative molecular mass in the HSP from basal cells (Figs. 4 *B* and 5). Apart from this, however, there is no a priori reason to suggest that PI 3-kinase is only located at this site by coupling with IRS-1. Indeed, the preassembly of these proteins in a complex may facilitate the necessary fidelity required to ensure that these proteins can rapidly associate in response to insulin. The HSP also contains other signaling molecules such as the 52-kD isoform of Shc, (Fig. 5) as well as other insulin-regulated phosphoproteins of unknown identity (Fig. 4 *B*; Hill, M., S. Clark, and D. James, manuscript submitted for publication). However, Grb2, the 46 and 66 kD isoforms of Shc (Fig. 5), MAPK (Hill, M., and D. James, unpublished observations), the tyrosyl-phosphorylated insulin receptor and two other insulin-regulated tyrosyl phosphoproteins of relative molecular mass p60 and p53 (Fig. 4 *B*) are not enriched in this fraction, suggesting that this is not simply an aggrophore of signaling intermediates.

In addition to IRS-1 and PI 3-kinase, the HSP is also enriched in the intracellular GLUT4 storage compartment (Fig. 7; James et al., 1988). Hence, this raises the provocative notion that PI 3-kinase may directly bind to these vesicles, thus, regulating their exocytic movement. Indeed, evidence has been documented in favor of this model (Heller-Harrison et al., 1996). However, intuitively, this seems unlikely because PI 3-kinase also mediates the activation of the pp70 ribosomal protein S6-kinase, independently of glucose transport (Fingar et al., 1993). In addition, PI 3-kinase mediates the activation of the serine kinase, protein kinase B, (Akt/PKB), that has also been implicated in the regulation of glucose transport in adipocytes, thus, denoting an important role for PI 3-kinase as an intermediate in this process (Kohn et al., 1996). We have obtained several independent experimental observations in the present studies to suggest that neither IRS-1 nor PI 3-kinase are directly associated with GLUT4-containing membranes, nor any membrane in the adipocyte HSP fraction. First, IRS-1 and PI 3-kinase are insoluble in nonionic detergents, whereas membrane proteins such as GLUT4 are completely soluble under these conditions (Fig. 6). Second, flotation analysis using sucrose density gradient sedimentation of the HSP resolved this fraction into two discrete peaks: one containing membranes, including GLUT4 vesicles, and the other containing non-membrane-associated proteins. IRS-1 and PI 3-kinase were exclusively found in the latter fraction and did not overlap with the peak of GLUT4 (Fig. 7). Third, using velocity sedimentation, the majority of PI 3-kinase and IRS-1 were segregated from vesicles containing GLUT4 and other proteins (Fig. 9). Finally, we were unable to detect the presence of vesicular structures in gradient fractions enriched in IRS-1 and PI 3-kinase (Fig. 12). Conversely, these fractions were enriched in cytoskeletal elements, which, based on their diameter (>10 nm), resembled cytoskeletal filaments. Thus, the present studies do not support the concept that insulin stimulates the recruitment of PI 3-kinase to the intracellular GLUT4 compartment as described previously (Heller-Harrison et al., 1996). In this report, it was suggested that this association may be very transient, occurring at early times after insulin addition. However, we have isolated the HSP from adipocytes incubated as described in the forementioned study, and subjected this fraction to gradient analysis as described above (Fig. 7), and yet find no evidence for a direct association between these signaling proteins and GLUT4 (data not shown). It is also unlikely that these proteins dissociated from membranes during the course of our isolation procedure because we were able to resediment both IRS-1 and PI 3-kinase subsequent to flotation analysis (Fig. 8).

We have not clearly established, from the present studies, the molecular basis by which IRS-1 and PI 3-kinase are targeted to what appears to be an insoluble protein matrix. However, several observations support their interaction with the cytoskeleton. First, the sucrose gradient fractions which contain these proteins are highly enriched in cytoskeletal elements as determined by electron microscopy (Fig. 12). Second, the cytoskeleton is reportedly insoluble in nonionic detergents, readily sediments using ultra-centrifugation, and can be disrupted by high ionic strength (Kries and Vale, 1993). IRS-1 and PI 3-kinase exhibited each of these properties (Figs. 4–6). Third, the cytoskeleton would remain intact subsequent to permeabilization of cells with SLO, and this is consistent with the maintenance of a pool of IRS-1 and PI 3-kinase within SLO-permeabilized CHO/IR/IRS-1 cells (Fig. 11). Finally, it has been shown that PI 3-kinase is highly enriched in cytoskeletal fractions isolated from other cell types including platelets (Zhang et al., 1992) and fibroblasts (Kapeller et al., 1993).

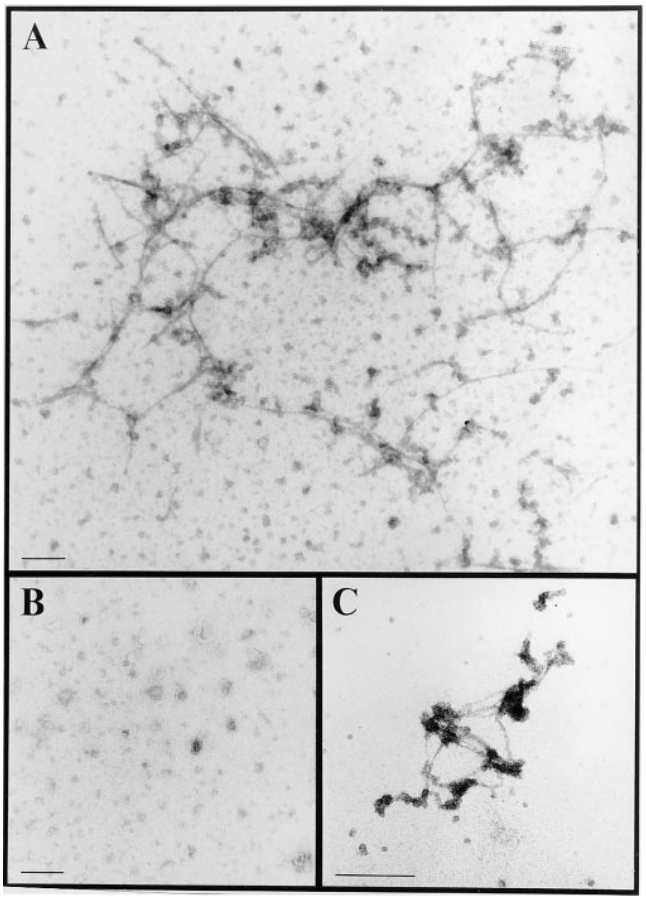

Figure 12. Identification of bundles of filamentous networks in HSP fraction, and the detergent insoluble component of this fraction, from adipocytes. The HSP fraction from basal adipocytes was subjected to velocity sedimentation analysis through sucrose gradients as described in Fig. 9. Fractions enriched in GLUT4 (fractions 5–7) or IRS-1/PI 3-kinase (fractions 8–10) were pooled and made isotonic by diluting in buffer (20 mM Hepes, pH 7.4, 1 mM EDTA) and subjected to high speed centrifugation. The resultant pellet from this step was resuspended in PBS and subsequently fixed in 2% paraformaldehyde/PBS, and subjected to electron microscopy analysis. Electron micrographs of paraformaldehyde-fixed samples obtained from gradient fractions enriched in (*A*) IRS-1/PI 3-kinase and (*B*) GLUT4 are shown. In separate experiments, (*C*) the T_x-100–insoluble component of the HSP fraction was prepared and fixed in 2% paraformaldehyde/PBS and subjected to electron microscopy analysis. Bars, 200 nm.

We have attempted to immunolocalize both IRS-1 and PI 3-kinase in adipocytes and CHO/IR/IRS-1 cells. However, these analyzes have been largely confounded by the lack of specificity of available antibodies. Whereas IRS-1 appears to be localized to the cytosol in CHO cells (Sun et al., 1992), this may reflect staining of the rather large cytosolic pool of IRS-1 that is evident in these cells (Fig. 11). We have failed to observe an effect of agents known to disrupt either microtubules or microfilaments on the distribution of IRS-1 or PI 3-kinase in 3T3-L1 adipocytes (data not shown). Furthermore, we do not observe any effect of these agents on insulin action. Hence, the nature of this putative cytoskeletal attachment remains to be determined.

If IRS-1 and PI 3-kinase are associated with the cytoskeleton in adipocytes, how then do they gain access to their regulatory intermediates which, in this case, correspond to the insulin receptor and the inner leaflet of a lipid bilayer, respectively? As described earlier, a large proportion of the available IRS-1 pool is rapidly phosphorylated subsequent to insulin addition (Madoff et al., 1988) and this occurs under conditions where internalization of the insulin receptor is inhibited (Heller-Harrison et al., 1995). One model that would account for these findings, is that IRS-1 and PI 3-kinase may be tethered to elements of the cytoskeleton that directly underlie the plasma membrane of the cell. This structure, which is composed primarily of actin filaments, has been documented in other cell types and has been referred to as a membrane skeleton (Mays et al., 1994). This membrane skeleton may serve several important functions in adipocytes. First, by preassembling the proteins that comprise the insulin-signaling cascade onto a cytoskeletal scaffold, this may ensure the correct juxtaposition of different enzymes in this cascade enabling them to interact in a very robust manner. In this regard, it is noteworthy that both IRS-1 and PI 3-kinase are found in the cytoskeletal fraction in both nonstimulated adipocytes and CHO/IR/IRS-1 cells (Figs. 9 and 10). Indeed, it will be of interest to determine if other downstream signaling proteins are found at this location as well. Second, the cytoskeleton may provide a barrier through which recycling vesicles have to transit en route to the cell surface. Several studies have ascertained that receptor-mediated endocytosis and the trafficking of intracellular secretory vesicles to the cell surface, in a variety of cell types, requires an intact cytoskeleton (Mays et al., 1994). Thus, by regulating the integrity of this barrier, it may be possible to regulate the recycling rate of certain membranes. In this respect, the PI 3-kinase inhibitor, wortmannin, has been shown to inhibit the insulin-regulated exocytosis of a number of membrane-associated proteins to the cell surface in adipocytes, including the transferrin receptor and Rab4, in addition to GLUT4 (Shepherd et al., 1995; Cormont et al., 1996). Finally, by regulating the association of integral signaling molecules with this cytoskeletal scaffold, it may be possible to regulate hormonal responsiveness. Of note in this regard, and consistent with recent studies (Heller-Harrison et al., 1995), is the insulin-dependent release of IRS-1 from the HSP (Figs. 6, 7, and 9). It appears, from these studies, that tyrosyl-phosphorylated IRS-1 is tightly retained within the HSP, thus providing further evidence that the insulin-dependent release of IRS-1 from this fraction may correspond to serine phosphorylation of IRS-1. Temporal differences in the phosphorylation of IRS-1 on tyrosine versus serine residues in response to insulin have been reported, with serine phosphorylated IRS-1 predominating under prolonged insulin-stimulated conditions (Sun et al., 1992). In fact, serine phosphorylation of IRS-1 correlates with reduced insulin signaling, as evident in cells treated with okadaic acid (Tanti et al., 1994) or tumor necrosis factor-α (Hotamisligil et al., 1996). Hence, the release of IRS-1 from its cytoskeletal anchor, an event that may be triggered by serine phosphorylation, may rapidly terminate the insulin signal simply by preventing access of IRS-1 to the IR.

In conclusion, these studies point to an important role for the subcellular localization of signaling intermediates in regulating specific biological events. Our data raise the

intriguing possibility that IRS-1 and one of its downstream effectors, PI 3-kinase, are localized to the cytoskeleton. It is within this context that both of these proteins are activated by the IR, suggesting that this scaffold must directly appose the plasma membrane. This raises the question, which represents the major impetus for these studies, as to why the PDGF receptor, which is also enriched in the plasma membrane of adipocytes (Fig. 4 *B*) and binds PI 3-kinase in response to PDGF stimulation (Fig. 3), is incapable of regulating glucose transport (Fig. 1). One possibility is that the attachment of PI 3-kinase to the cytoskeleton affords access not only to the lipid bilayer, but also to the relevant downstream target(s) of the 3′-phosphorylated phosphoinositide products of this enzyme, that may also be localized to this site. The other possibility is that the PDGF receptor may be sequestered within a microdomain of the plasma membrane that prevents its access to these downstream targets and/or to the relevant lipid substrates which result in the generation of 3′-phosphorylated phosphoinositides. In support of the latter, it has been shown that, despite the recruitment of a significant amount of PI 3-kinase to the plasma membrane in PDGF-treated adipocytes (Fig. 3), PDGF does not significantly stimulate the production of 3′ phosphorylated phospholipids (Conricode, 1995), nor does it activate downstream targets such as Akt/PKB, with the same potency as insulin (Tanti et al., 1997). Such sequestration mechanisms may be important in terminally differentiated cells to subvert the inappropriate activation of growth factor receptors.

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