Insulin receptor substrate (IRS) proteins are major substrates of the insulin receptor (IR). IRS-1 associates with an insoluble multiprotein complex, possibly the cytoskeleton, in adipocytes. This localization may facilitate interaction with the IR at the cell surface. In the present study, we examined the hypothesis that the release of IRS proteins from this location may be a mechanism for insulin desensitization. We show that a second IRS protein, IRS-2, is associated with a multiprotein complex in adipocytes with similar characteristics to the IRS-1 complex. Insulin treatment (15–60 min) caused the release of IRS-1 and IRS-2 from this complex (high speed pellet; HSP) into the cytosol, whereas the level of tyrosyl-phosphorylated IRS proteins remained constant. Chronic insulin treatment resulted in a dramatic reduction in IRS-1 and IRS-2 in the HSP, eventually (>2 h) leading to IRS protein degradation and decreased levels of tyrosyl-phosphorylated IRS proteins. Okadaic acid, which rapidly induces insulin resistance in adipocytes independently of IR function, caused an almost quantitative release of IRS-1 into the cytosol concomitant with a significant reduction in tyrosyl-phosphorylated IRS proteins. Platelet-derived growth factor, a factor known to compromise insulin signaling, caused a more moderate release of IRS proteins from the HSP. Collectively, these results suggest that the assembly of IRS-1/IRS-2 into a multiprotein complex facilitates coupling to the IR and that the regulated release from this location may represent a novel mechanism of insulin resistance.

The insulin receptor (IR) is a member of the tyrosine kinase growth factor receptor family. One property that distinguishes the IR from other growth factor receptors is its ability to induce the tyrosine phosphorylation of a family of intracellular signaling molecules referred to as insulin receptor substrate (IRS) proteins. IRS proteins contain a pleckstrin homology domain and a phosphotyrosine binding domain, both of which are required for the efficient tyrosine phosphorylation of these proteins by the activated insulin receptor tyrosine kinase. The first member of the IRS family to be identified, IRS-1, encodes a 160-kDa protein that is highly expressed in physiologically insulin-responsive tissues such as adipocytes and muscle cells and has been implicated in the control of a number of insulin-sensitive metabolic pathways including glucose transport, lipid deposition, and glycogen synthesis. In response to insulin, the phosphorylation of multiple tyrosine residues within the C terminus of IRS-1 by the IR leads to the generation of highly specific binding sites for a number of Src homology 2 domain-containing downstream signaling molecules such as phosphatidylinositide 3-kinase (PI 3-kinase), Syp, Nck, Fyn, and Grb-2. PI 3-kinase appears to be a central insulin-signaling molecule, because inhibition of its activity by either pharmacological agents or dominant-negative mutants profoundly abrogates several biological responses to this hormone. IRS-1 is also highly phosphorylated on serine and threonine (Ser/Thr) residues, and insulin acutely stimulates a further increase in IRS-1 Ser/Thr phosphorylation. The signaling function of IRS-1 Ser/Thr phosphorylation is unknown, although this may regulate the docking of other types of signaling molecules such as 14-3-3 proteins.

Defects within insulin signaling pathways comprise a major locus for the development of insulin resistance in disease states such as non-insulin-dependent diabetes mellitus. Certain forms of insulin resistance, such as that induced by tumor necrosis factor-α, okadaic acid, or chronic insulin treatment, may be due to uncoupling of the IR activity toward IRS-1. While the molecular basis for this defect is unclear, a common observation in cells subjected to these conditions is that IRS-1 becomes hyperphosphorylated on serine and threonine residues and various intracellular Ser/Thr kinases, including glycogen synthase kinase-3, protein kinase C-α, mitogen-activated protein kinase, and protein kinase B/Akt, have been implicated in mediating this effect. The decline in insulin sensitivity invoked by tumor necrosis factor-α has recently been attributed to a dominant negative effect exerted by hyperphosphorylated IRS-1 upon the IR intrinsic tyrosine kinase. However, neither okadaic acid nor chronic insulin treatment appear to disrupt IR tyrosine kinase activity in response to acute insulin activation. These latter observations suggest that other mechanisms may operate to induce insulin resistance.

We have recently provided evidence to suggest that IRS-1 is enriched in a cytoskeletal fraction in adipocytes that is insoluble in a range of nonionic detergents. This accounts for early reports suggesting that IRS-1 is bound to membranes, because the cytoskeletal fraction co-fractionates with microsomal membranes during ultracentrifugation. The anchoring of IRS-1 to the cytoskeleton may be of particular im-

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† The first two authors contributed equally to this work.

§ To whom correspondence should be addressed: Tel.: 61 7 3365 4986; Fax: 61 7 3365 4430; E-mail: D.James@cmcb.uq.edu.au.

1 The abbreviations used are: IR, insulin receptor; IRS, insulin receptor substrate; PI, phosphatidylinositol; PDGF, platelet-derived growth factor; CHO, Chinese hamster ovary; HSP, high speed pellet; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
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portance to the efficacy of insulin signaling by providing a platform for localizing IRS-1 within proximity to the insulin receptor. This arrangement may also provide a robust link between IRS-1 and downstream signaling proteins such as PI 3-kinase, which also appears to associate with this insoluble fraction (29). One functional consequence of this spatial localization is that it may create a unique site for the generation of specific signals required for insulin action. Consistent with the latter notion, platelet-derived growth factor (PDGF) also activates PI 3-kinase in adipocytes but has no significant effect on PI 3-kinase-dependent functions in these cells, including glucose transport and glycogen synthesis (32).

It has previously been reported that IRS-1 exists in at least two distinct pools in adipocytes: the cytoskeletal component and the cytosol (31, 33). Furthermore, short term insulin treatment triggers the release of IRS-1 from the cytoskeletal fraction into the cytosol (31). It has been argued that the cytoskeletal component represents the functional pool of IRS-1, because most of the tyrosyl-phosphorylated IRS-1 is found in this pool, and there is a net increase in PI 3-kinase in this fraction in response to insulin (34). Hence, it may be postulated that the deactivation of IRS-1 corresponds to its translocation from this fraction into the cytosol. This model raises the possibility that the cytosolic pool of IRS-1 is nonfunctional presumably due to its inaccessible to the IR. Therefore, inappropriate accumulation of IRS-1 in the cytosol may disengage this protein from the receptor, resulting in a state of insulin resistance. In the present study, we have tested the notion that the intracellular location of IRS-1 can be modified under conditions that normally cause insulin resistance and/or alter insulin signaling potential. In addition, we have extended this hypothesis to include the IRS-1 homologue, IRS-2, which is also expressed in 3T3-L1 adipocytes. Our results show that a significant proportion of IRS-1 and IRS-2 are found in a detergent-resistant insoluble fraction that has properties analogous to the cytoskeleton. Moreover, IRS proteins translocate from this location into the cytosol following exposure of adipocytes to chronic insulin treatment or okadaic acid or PDGF. These data suggest that the intracellular location of IRS proteins is regulated in a way that may influence insulin action.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—All tissue culture media was purchased from Life Technologies, Inc., except fetal calf serum, which was obtained from Trace Biosciences (Clayton, Australia). Insulin was obtained from Calbiochem, and PDGF-B was from Life Technologies, Inc. Bovine serum albumin was purchased from ICN (Costa Mesa, CA). Unless specified, all other reagents were from Sigma. The GLUT4 polyclonal antibody (R280) was raised against a synthetic peptide as described previously (35). The anti-phosphotyrosine monoclonal antibody (4G10) was kindly provided by Dr. B. Druker (Oregon Health Sciences University, Portland, OR), and polyclonal antibodies raised against Akt-2 were provided by Dr. M. Birnbaum (Howard Hughes Medical Institute, Philadelphia, PA). All other antibodies used in this study were purchased from the following sources: anti-IRS-1 polyclonal antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-p85 and anti-IRS-2 polyclonal antibodies from Upstate Biotechnology Inc. (Lake Placid, NY); anti-hemagglutinin (HA) monoclonal antibody from Babco (Richmond, CA); peroxidase-coupled secondary antibodies from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom).

Construction of HA-IRS-1—Full-length mouse IRS-1 cDNA in pBluescriptSK, generously provided by Drs. S. Keller and G. Lienhard (Dartmouth Medical School, Hanover, NH), was used as template in a polymerase chain reaction to generate a construct encoding IRS-1 tagged at the C terminus with the HA epitope (HA-IRS-1). In this reaction, the forward primer consisted of the pBluescript sequencing primer, T7, whereas the reverse primer was comprised of the following sequence: 5'-CTCGGCTGACTAAAGCTTACCTGGAAGACTCATGATGTTAAGCTTGAGCTTCTCAGGCTCTTGGCTCTTGGAAGCTGATCTCGGCC-3' (Pacific Oligos, Lismore, Australia). A DNA fragment encoding the entire sequence of HA-IRS-1 was then amplified using standard polymerase chain reaction protocols. The amplified product was isolated, digested with SalI, and subcloned into SalI sites of a eukaryotic expression vector, pMEX (36). Clones carrying the insert in the desired orientation were verified by restriction mapping.

Cell Culture and Treatment—3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described previously (37). Cells were serum-starved in Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin and 2 mM glutamine for 2 h at 37 °C and subsequently incubated with insulin (1 μM) for 0.25 h (acute) or for 0.25, 1, or 4 h (chronic) or with PDGF (50 ng/ml) for 1 h at 37 °C. Where indicated, cells were incubated with wortmannin (100 nM) for 15 min before addition of insulin (1 μM) or wortmannin for 1 h at 37 °C. Medium was replaced with medium containing fresh wortmannin (100 nM) and insulin (1 μM) 30 min prior to harvesting cells. In other experiments, cells were incubated with okadaic acid (2.5–5.0 μM) for 30 min, during which insulin (1 μM) was added to the incubation medium for the last 15 min. In glucose uptake assays, 3T3-L1 adipocytes were serum-starved in Krebs-Ringer phosphate buffer (2.5 mM Hepes (pH 7.4), 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 10 mM CaCl2, 0.4 mM NaH2PO4, 0.6 mM NaHCO3, KRP, containing 0.1% bovine serum albumin and 3.0 mM sodium pyruvate, for 2 h at 37 °C. Cells were then incubated with PDGF (50 ng/ml) for 1 h and subsequently incubated with insulin (1 μM) for a further 15 min.

CHO cells overexpressing the insulin receptor (CHO-IR) were kindly donated by Dr. M. White (Harvard Medical School, Boston, MA). Both CHO-IR and CHO cells were maintained in culture as described previously (29). Prior to transfection, cells were seeded in 60-mm dishes and grown for a further 24 h to achieve 60–80% confluence. Transient transfections were performed by incubating cells in medium (Dulbecco's modified Eagle's medium, nonessential amino acids, 2 mM t-glutamine) containing 6 μg of HA-IRS-1/pMEX and 30 μl of Lipofectamine reagent for 5 h at 37 °C. Transfection medium was then replaced with CHO cell culture media, and cells were grown to 100% confluence. Transfected cells were subsequently serum-starved and exposed to chronic insulin treatment as described above for adipocytes. In preliminary experiments, immunofluorescence studies of CHO-IR cells transfected with HA-IRS-1 cDNA showed that at least 20–30% of cells expressed full-length HA-IRS-1 protein.

Cell Fractionation—After incubation with the appropriate agents, 3T3-L1 adipocytes were washed three times with ice-cold HES buffer (20 mM Hepes (pH 7.4), 1 mM EDTA, 250 mM sucrose) and homogenized in the same buffer supplemented with phosphatase and protease inhibitors (2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM tetra-sodium pyrophosphate, 1 mM ammonium molybdate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 250 μM phenylmethylsulfonyl fluoride). Subcellular fractions were isolated by differential centrifugation as previously detailed (37). All procedures were performed at 4 °C. Briefly, cell homogenates were centrifuged at 13,000 × g for 20 min. The resulting pellet was then resuspended in HES buffer and layered onto a 1.12 M sucrose cushion as described by Piper et al. (37). After centrifugation at 77,000 × g for 1 h, the plasma membrane fraction was collected from the 1.12 M sucrose interface. The supernatant from the 13,000 × g centrifugation step was subjected to centrifugation at 30,000 × g for 30 min to pellet the high density microsomal fraction. The resultant supernatant was subjected to further centrifugation at 175,000 × g for 75 min to obtain the high speed pellet (HSP). The supernatant from this centrifugation step was designated the cytosol fraction. The membrane pellets were solubilized with 1% SDS in PBS. In some experiments, the HSP fraction from 3T3-L1 adipocytes was resuspended and incubated in HES containing 1% Triton X-100 (Pierce) for 1 h on ice. Insoluble material was collected by centrifugation at 175,000 × g for 75 min at 4 °C, and the resultant pellet was solubilized in 1% SDS in PBS.

CHO-IR cells overexpressing HA-IRS-1 were subjected to chronic insulin treatment, and the HSP from these cells was obtained as described previously (29) with some modifications. All steps were performed at 4 °C. Cells were washed in ice-cold HES and then homogenized in HES buffer containing phosphatase and protease inhibitors by passage through a 22-gauge needle. The homogenate was subjected to centrifugation at 17,500 × g for 15 min to remove high density microsomes, plasma membranes, mitochondria/nuclei, and cell debris. The supernatant was then centrifuged at 175,000 × g for 75 min. The resultant supernatant was designated the cytosol. The pellet (HSP), was solubilized in 1% SDS in PBS.

Immunoblotting and Densitometry Analysis—The amount of protein present in all samples was determined using BCA reagent (Pierce). Samples were subjected to SDS-PAGE (38) and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp., Bedford,
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RESULTS

Subcellular Distribution of IRS-1 Versus IRS-2 in 3T3-L1 Adipocytes—In basal adipocytes, IRS-1 is enriched in a microsomal fraction, often referred to as the low density microsomes (31, 33, 40). In addition to intracellular membranes that contain the insulin-regulatable glucose transporter, GLUT4, the low density microsomes fraction also contains cytoskeleton and other large protein complexes. In fact, >60% of the protein in this fraction is insoluble in nonionic detergents, so we now refer to this fraction as the high speed pellet, or HSP (41). In contrast to membrane proteins such as GLUT4, IRS-1 remains insoluble following treatment of the HSP with nonionic detergents, and it does not have a buoyant density that enables it to float up through a 50% sucrose solution (29). Based on these data, we proposed that IRS-1 is either attached to the cytoskeleton or found in a large protein complex that enables it to be pelleted during high speed centrifugation.

In certain cellular contexts, IRS-2 may functionally substitute for IRS-1 in mediating insulin action (42). In 3T3-L1 adipocytes, the subcellular distribution of IRS-2 is indistinguishable from IRS-1 (Fig. 1A). Consistent with recent studies (34, 43), we observed a significant amount (60–80% of the total) of both IRS-1 and IRS-2 in the HSP fraction in basal adipocytes. Low levels were found in the plasma membrane and high density microsome fractions, possibly due to contamination of these fractions with HSP-derived material. Significant levels (20–40%) of both IRS-1 and IRS-2 were found in the cytosol under basal conditions. Consistent with previous studies (31, 34), both IRS-1 and IRS-2 were released from the HSP into the cytosol upon acute treatment of the cells with insulin (Fig. 1A). The HSP pool of IRS-2 exhibited biochemical properties to that of IRS-1, in that it remained insoluble following treatment with the nonionic detergent, Triton X-100 (Fig. 1B). In addition, flotation analysis of the HSP as described previously (29) showed that, in contrast to membrane-associated proteins but identical to IRS-1, IRS-2 does not float up through a 50% sucrose solution (data not shown). These data are consistent with a model where the HSP pool of both IRS-1 and IRS-2 is not membrane-associated.

Effects of Chronic Insulin Treatment on the Subcellular Distribution of IRS Proteins—As depicted in Fig. 1A and described in more recent studies (34), both IRS-1 and IRS-2 undergo acute insulin-stimulated release from the HSP into the cytosol. With extended insulin treatment (15–60 min), there was significant loss of immunoreactive IRS-1 and IRS-2 from the HSP (>80%), but the amount of tyrosine-phosphorylated IRS proteins remained fairly constant during this time (Fig. 2, A and B). This suggests that a relatively small component of the total IRS protein pool is tyrosine-phosphorylated in response to insulin at steady state and that this pool of IRS proteins is maintained despite a marked loss of immunoreactive protein from the HSP fraction following prolonged exposure to insulin. The amount of IRS proteins in the HSP continued to decline in response to long term exposure to insulin, and it was not until the cells had been incubated in the presence of insulin for up to 4 h before there was a significant decrease in tyrosine-phosphorylated IRS proteins in the HSP fraction (Fig. 2, A and B). This corresponded to the time of onset of insulin resistance as determined by the amount of the insulin-regulatable glucose transporter, GLUT4, in the plasma membrane (Fig. 2D), and the amount of immunoreactive p85 in the HSP fraction that also declined at the 4-h time point (Fig. 2A). A similar time course of insulin-induced insulin resistance in 3T3-L1 adipocytes has been reported by other groups (21, 24).

Commensurate with the loss of IRS-1 and IRS-2 from the HSP in response to insulin, we observed a significant increase in the cytosolic fraction (Fig. 2C). This increase in cytosolic IRS proteins peaked after 1 h of insulin treatment and declined thereafter. We did not observe a corresponding increase in the level of either IRS-1 or IRS-2 in any other fraction (data not shown), suggesting that this loss reflects degradation of the IRS proteins. The rate of decline in cytosolic IRS-1 was considerably more rapid than that of cytosolic IRS-2, suggesting that the degradation of the two IRS isoforms following chronic insulin exposure may be controlled by distinct mechanisms. The release of IRS-1 and IRS-2 from the HSP in response to insulin was accompanied by a significant decrease in the electrophoretic mobility of both proteins (Fig. 2A). This was quite evident in the HSP fraction after only 15 min of incubation with insulin. Interestingly, there appeared to be a stepwise decrease...
in the mobility of both IRS-1 and IRS-2 with time: this was most clearly observed in the cytosol (compare 0.25- versus 1-h insulin exposure).

To verify the above findings in a heterologous system, CHO cells overexpressing the IR (CHO-IR) were transfected with a hemagglutinin-tagged IRS-1 cDNA (HA-IRS-1), and replicated the above experimental regimen. When expressed in CHO-IR cells, the HA-IRS-1 protein was tyrosyl-phosphorylated in response to insulin, suggesting that it forms a competent IR substrate under these conditions (data not shown). As indicated in Fig. 3A, an anti-HA antibody immunolabeled a protein that corresponded to a molecular mass of 170 kDa in CHO cells transfected with HA-IRS-1, whereas no specific band was detected in control cells (not shown). HA-IRS-1 was distributed between the HSP fraction and the cytosol in CHO cells, consistent with the distribution of IRS-1 and IRS-2 in adipocytes.

Insulin treatment caused translocation of HA-IRS-1 from the HSP fraction into the cytosol in CHO-IR cells, with a time course comparable with that observed in adipocytes (Fig. 3B). After 2 h of insulin treatment, the level of HA-IRS-1 in the HSP was reduced to ~20% of that found under basal conditions. Initially, there was an increase in HA-IRS-1 in the cytosol in response to insulin that was presumably derived from the HSP. However, as was the case in adipocytes for both IRS-1 and IRS-2, after 1 h of prolonged insulin treatment (Fig. 2C), the level of HA-IRS-1 in the cytosol declined, presumably indicative of net loss of the protein from the cell. This was not due to nonspecific degradation of the recombinant protein, because in cells maintained at basal conditions for the 4-h time period, levels of HA-IRS-1 were not significantly altered (data not shown). These data suggest that the targeting of IRS-1 to an insoluble intracellular location as well as its regulated release from this site, can be reconstituted in other cell types that are not normally considered to be bona fide insulin-sensitive cells. Whereas chronic insulin treatment caused a stepwise decrease in the mobility of IRS-1 in the HSP fraction isolated from 3T3-L1 adipocytes (Fig. 2A), no such change in the mobility of HA-IRS-1 could be detected in the HSP isolated from CHO-IR cells (Fig. 3A). This did not appear to be due to an inability to detect a mobility shift of HA-IRS-1, because a shift was observed in the mobility of HA-IRS-1 present in the cytosolic pool.

Effects of Wortmannin on the Insulin-dependent Release of IRS Proteins from the HSP—To test the role of PI 3-kinase or of signaling proteins downstream of this enzyme in the insulin-dependent release of IRS-1 and IRS-2 from the HSP, we looked at the effects of the PI 3-kinase inhibitor, wortmannin, on this process. Wortmannin at a concentration of 100 nM had no significant effect on the insulin-dependent release of IRS-1 or IRS-2 from the HSP (Fig. 4A). The efficacy of wortmannin inhibition during these treatments was confirmed by the absence of an electrophoretic shift in cytosolic Akt-2, a PI 3-kinase-dependent serine kinase (Fig. 4B) (44). Similar data were obtained using an alternate PI 3-kinase inhibitor, LY294002 (not shown). While we did not observe a significant effect of wortmannin on the insulin-stimulated release of IRS proteins in six experiments, in three of these studies we observed a modest increase in the amount of IRS-1 and IRS-2 associated with the HSP in cells treated with wortmannin alone (Fig. 4A).

Nevertheless, while these data suggest that the association of IRS proteins with the HSP may be regulated by a wortmannin-sensitive factor in the basal state, this is not the mechanism for the release of IRS proteins in response to insulin.
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Effects of Okadaic Acid on the Subcellular Distribution of IRS Proteins—Okadaic acid, a serine/threonine phosphatase inhibitor, induces insulin resistance in both adipocytes and muscle cells and potently inhibits insulin-regulated glucose transport by blocking the translocation of GLUT4 (20, 23, 45, 46). Consistent with these studies, we also observed a substantial reduction in the level of insulin-stimulated glucose uptake in 3T3-L1 adipocytes in the presence of 2.5–5.0 μM okadaic acid (data not shown). In further agreement with previous studies (23), okadaic acid induced a significant shift in the electrophoretic mobility of IRS-1 (Fig. 5). Okadaic acid also caused the release of >90% of IRS-1 from the HSP. This was accompanied by a large increase in IRS-1 in the cytosol fraction. A similar distribution of IRS-1 was observed in cells treated with okadaic acid plus insulin (Fig. 5). Consistent with the hypothesis that the HSP pool of IRS-1 interacts with the IR, we observed no detectable insulin-stimulated tyrosine phosphorylation of IRS-1 in the cytosol after treatment with okadaic acid, despite a considerable proportion of the protein in this fraction (Fig. 5). Insulin-stimulated IRS-1 tyrosine phosphorylation in the HSP fraction after okadaic acid treatment was also significantly decreased and coincided with the large decrease in the IRS-1 protein in this fraction. This decrease in IRS-1 tyrosine phosphorylation did not appear to be due to a defect in the IR tyrosine kinase activity, because insulin-dependent tyrosine phosphorylation of the IR β-subunit, which is enriched in the plasma membrane fraction (PM), was unaffected by okadaic acid (Fig. 5).

The okadaic acid-induced block in IRS tyrosyl phosphorylation was accompanied by a decrease in the recruitment of the p85 subunit of PI 3-kinase to the HSP fraction (Fig. 5). While insulin alone stimulated recruitment of p85 to the HSP, this effect was almost entirely abolished in the presence of okadaic acid. It is noteworthy that okadaic acid did not significantly alter the levels of p85 associated with the HSP fraction in the basal state. This suggests that the presence of PI 3-kinase in the adipocyte HSP fraction under basal conditions is probably not mediated by a direct interaction with IRS-1, and the effects of okadaic acid on the organization of proteins in this fraction are somewhat specific.

Effects of PDGF on the Subcellular Distribution of IRS Proteins—Thus far, we have shown that chronic insulin treatment and okadaic acid independently trigger the release of IRS proteins from the adipocyte HSP fraction into the cytosol (Figs. 2 and 5). Furthermore, both treatments cause a distinct reduction in the electrophoretic mobility of IRS proteins, both within the HSP fraction and the cytosol. PDGF has previously been shown to induce a similar change in the electrophoretic mobility of IRS-1 (47). In addition, decreases in insulin-stimulated tyrosyl phosphorylation of IRS-1 and recruitment of PI 3-kinase following PDGF treatment of adipocytes has been reported (48). We examined the effect of a similar PDGF treatment protocol on the subcellular distribution of IRS proteins in 3T3-L1 adipocytes. Extended incubation with PDGF (1 h), led to a significant dissociation of IRS proteins from the HSP (up to 50%), with a concomitant increase in the cytosolic component (Fig. 6A). However, the magnitude of this effect was not as great as that observed using chronic insulin treatment (Fig. 6A). In addition, and unlike the effects of insulin, we did not observe any detectable electrophoretic shift in IRS-1 or IRS-2 with PDGF in the HSP, although a noticeable decrease was observed in the cytosolic fraction. Furthermore, PDGF treatment of adipocytes did not significantly impair insulin-stimulated glucose uptake in adipocytes at maximal or submaximal concentrations of insulin (Fig. 6B). These data suggest that the moderate loss of IRS proteins from the HSP induced by PDGF does not significantly alter insulin sensitivity.

DISCUSSION

The strength, duration, and specificity of growth factor signaling may be accomplished, at least in part, by the spatial compartmentalization of the cognate signal transduction machinery. In accordance with this notion, we and others have previously reported that a cohort of insulin-regulatable signaling proteins, including IRS-1, associate with a low density insoluble fraction in basal adipocytes (29, 31, 33, 40) that we refer to as the HSP. Biochemical analyses of the HSP indicates that IRS-1 does not associate with membranes in this fraction.
the cytoskeletal localization of IRS proteins that is present in the HSP may be required for this to occur efficiently. Considerable evidence suggests that IRS-1 encounters the IR at the plasma membrane, so we have proposed that this HSP pool of IRS-1 represents a cytoskeleton that decorates the underside of the plasma membrane. First, immunofluorescence localization of tyrosyl-phosphorylated IRS-1 in CHO cells indicates that at early times (~1 min), after insulin addition there is cell surface labeling, followed by increased cytosolic staining at later times (51). Second, inhibition of endocytosis by incubation at 4 °C (31), potassium depletion (52), or use of a mutant dynamin allele has little effect on insulin-stimulated tyrosine phosphorylation of either IR or IRS-1 (53). Finally, microinjection of a fusion protein that specifically interacts with the phosphatidylinositol product of PI 3-kinase activity, PI 3,4,5-trisphosphate, into adipocytes showed pronounced labeling of the plasma membrane within 1 min of insulin stimulation (54). Hence, the localization of IRS-1 and IRS-2 to the cytoskeleton underlying the plasma membrane may allow an efficient interaction between the pleckstrin homology and phosphotyrosine binding domains of these proteins with the intracellular tail of the IR and thus rapidly promotes tyrosine phosphorylation of these proteins in response to insulin. Furthermore, the coordination of these signaling events with the recruitment of PI 3-kinase may ensure the correct localization of this enzyme to its membrane-bound substrate(s), located at the cell surface.

It has previously been shown that with short term insulin treatment, IRS-1 translocates from the HSP into the cytosol in 3T3-L1 adipocytes (31). One possibility is that this translocation event may be necessary to propagate the insulin signal by allowing the IRS-signaling complex access to the relevant downstream targets. This does not appear to be the case, at least for PI 3-kinase, given that downstream targets of this enzyme are recruited to the cell surface (54). Alternatively, this translocation event may act as the “off” switch for insulin action. This implies that the inappropriate release of IRS proteins into the cytosol may disengage IR and IRS proteins, resulting in desensitization and/or insulin resistance. In support of this model, we have shown that exposure of adipocytes to conditions known to cause insulin resistance, namely chronic insulin or okadaic acid treatment, also stimulate the release of IRS proteins from the HSP into the cytosol (Figs. 2 and 5, respectively).

PDGF treatment caused a relatively modest decline in IRS levels in the HSP (Fig. 6A), whereas the loss with okadaic acid treatment was almost quantitative (Fig. 5). This is an important distinction to make, because preincubation of adipocytes with PDGF for 60 min prior to insulin treatment does not impair insulin-stimulated glucose transport (Fig. 6B) (48), while okadaic acid potently inhibits this process (20, 23, 45, 46). Similarly, the onset of insulin resistance in cells exposed to chronic insulin treatment coincides with the loss of more than 90% of IRS protein from the HSP (Fig. 2). These data suggest that there is a nonlinear relationship between the level of IRS protein associated with the HSP and the onset of insulin resistance and that a small amount of IRS proteins associated with the HSP is sufficient to evoke a full biological response to insulin. We propose that a competent level of IRS proteins is maintained in the HSP in the presence of short term PDGF treatment and during several hours of insulin treatment. However, this threshold cannot be maintained in response to longer insulin treatment or okadaic acid.

In each of the three experimental regimens used to challenge the localization of IRS proteins, the function of the insulin receptor appears to be retained, but the insulin-regulated tyrosyl phosphorylation of IRS proteins is decreased (20, 21, 24,
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