Different Subcellular Distribution and Regulation of Expression of Insulin Receptor Substrate (IRS)-3 from Those of IRS-1 and IRS-2*

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Adipocytes contain three major substrate proteins of the insulin receptor, termed IRS-1, IRS-2, and IRS-3. We demonstrated that IRS-1 and IRS-2 are located mainly in the low density microsome (LDM) fraction and are tyrosine phosphorylated in response to insulin stimulation, leading to phosphatidylinositol (PI) 3-kinase activation. In contrast, IRS-3 is located mainly in the plasma membrane (PM) fraction and contributes to PI 3-kinase activation in the PM fraction. The different cellular localizations of IRS proteins may account for the mechanism of insulin resistance induced by a high fat diet, considering that PI 3-kinase activation in the LDM fraction is reportedly essential for the translocation of GLUT4 in adipocytes. High fat feeding in rats increased both protein and mRNA levels of IRS-3 but decreased those of IRS-1 and IRS-2 in epididymal adipocytes. As a result, selective impairment of insulin-induced PI 3-kinase activation was observed in the LDM fraction, whereas PI 3-kinase activation was conserved in the PM fraction. This is the first report showing that different IRS proteins function in different subcellular compartments, which may contribute to determining the insulin sensitivity in adipocytes.

Insulin induces numerous cell activities in adipose tissue, which include cell proliferation and differentiation, stimulation of glucose uptake, inhibition of lipolysis, translocation of various membrane proteins such as transferrin receptor and insulin-like growth factor II receptor, and synthesis and/or secretion of leptin (1–3). Although it remains unclear which step in the insulin signaling pathway is related to each of these individual activities, it seems certain that insulin-stimulated phosphatidylinositol 3-kinase (PI 3-kinase) activation plays a critical role in the translocation of GLUT4 from intracellular vesicles to the cell surface (4–6). In addition, Yang et al. (7) suggested that PI 3-kinase activation in the intracellular compartment, but not on the plasma membrane, is necessary for the efficient translocation of GLUT4 (7).

In adipocytes it has been reported that not only IRS-1 and IRS-2, but also a protein of approximately 60 kDa referred to as pp60, are major substrates that are tyrosine phosphorylated by the insulin receptor (8–10). Recently, pp60 was cloned and termed IRS-3 in rat and mouse adipocytes, and its structure was shown to have several similarities to IRS-1 and IRS-2 (11, 12). Sequence alignment of IRS-3 with the other members of the IRS family revealed that these IRS proteins contain pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains that are highly conserved (11, 12). In addition, there is conservation of many tyrosine phosphorylation motifs responsible for interactions with downstream signaling molecules containing SH2 domains, including PI 3-kinase. However, IRS-3 is far smaller than IRS-1/2 and has some regions that have no homology to IRS-1/2. Therefore, IRS-3 may have some unique role in insulin signaling and glucose metabolism. IRS-1 and IRS-2 have been shown to participate in the insulin signaling pathway whereby insulin stimulates translocation of GLUT4 in adipose cells (13, 14).

The aim of this study was to clarify the unique role of IRS-3 compared with IRS-1 and IRS-2. Our data suggest that different IRS proteins may be important in the activation of SH2-containing proteins including PI 3-kinase in different subcellular compartments, which may induce different cellular activities. In addition, we investigated how the expression levels of IRS-3 as well as IRS-1 and IRS-2 are regulated in high fat-fed rat adipocytes, in which insulin resistance is present. The different regulation of IRS-3 from that of IRS-1 and IRS-2 would induce a different degree of PI 3-kinase activation in the different subcellular locations, which may be involved in the pathogenesis of insulin resistance. This is the first paper showing the different role of IRS-3 and also suggesting the possibility that the difference in regulation of expression of IRS proteins may affect insulin sensitivity in fat cells.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, fraction V, was purchased from Intergen (Purchase, NY). Collagenase and PI were purchased from Sigma (St. Louis, MO). Insulin was purchased from Novo Nordisk (Denmark). [γ-32P]ATP was purchased from ICN (Costa Mesa, CA). Aluminum-backed silica gel thin layer chromatographic plates were purchased from Merck (Darmstadt, Germany). Protein A-Sepharose 6MB was purchased from Amersham Pharmacia Biotech (U. K). All other chemicals were purchased from Wako (Osaka, Japan). Animals—Male Sprague-Dawley rats, 5 weeks old and 110–140 g, were purchased from Tokyo Experimental Animals (Tokyo, Japan) and housed under controlled light (12/12 h) and temperature conditions with free access to food and water.

After a 2–3-day acclimatization period, the rats were divided into a...
were collected as a pellet; however, in this experiment, we did not use sulfonyl fluoride) and kept on ice for 30 min. The homogenates were adjusted to 1 mg/ml. IRS-1, IRS-2, IRS-3, and tyrosine-phosphorylated bovine serum albumin as the standard, and the concentrations were determined by BCA protein assay using the methods of Robobbell (16).

Isolated adipocytes were preincubated in 1% bovine serum albumin and Krebs-Ringer bicarbonate buffer for 15 min and then stimulated with 10^{-7} M insulin for 5 min. After insulin stimulation, adipocytes were washed three times with HES buffer (20 mM Hepes, pH 7.5, 1 mM EDTA, 255 mM sucrose, 10 mM NaF, 1 mM sodium pyrophosphate) and used for PI 3-kinase assay or Western blotting immediately or after cell fractionation.

**Cell Fractionation**—Isolated adipocytes incubated with or without insulin were homogenized and fractionated as described previously by Simpson et al. (17) with several modifications. One volume of HES buffer with 1 mM vanadate, 1 \mu M aprotinin, 1 \mu M leupeptin, and 1 mM phenylmethylsulfonyl fluoride was added to isolated adipocytes, and the mixture was homogenized by 10 strokes in a Potter-Elvehjem Teflon pestle homogenizer. The homogenates were centrifuged at 16,000 \times g for 15 min at 4 °C, the solidified fat cake was removed carefully, and the supernatant was resuspended and recentrifuged once.

The initial pellet was resuspended and recentrifuged once before being resuspended in 5 ml of buffer, applied to a 1.12 M sucrose cushion containing 20 mM Tris-HCl and 1 mM EDTA, and centrifuged at 101,000 \times g for 70 min. The mitochondria, nuclei, and cell debris were collected as a pellet; however, in this experiment, we did not analyze this fraction further. The plasma membranes (PM), collected at the interface, were resuspended in 50 ml of buffer and centrifuged at 48,000 \times g for 45 min. The pellet was resuspended at approximately 2 mg of protein/ml.

The initial supernatant was centrifuged at 48,000 \times g for 20 min, yielding a pellet of high density microsomal membranes (HDM). The supernatant was then recentrifuged at 212,000 \times g for 70 min, yielding a second pellet of low density microsomal membranes (LDM), and the remaining supernatant was condensed by Centricon-30 and used as cytosol. All pellets were resuspended in 1–10 ml of buffer and repelleted at final resuspension at 1 mg of protein/ml.

**PI 3-Kinase Assay**—Isolated adipocytes were prepared, and some were incubated with insulin as described above. Subcellular fractions were separated as described above, and precipitated fractions were resuspended in ice-cold buffer A (50 mM Hepes, pH 7.5, 137 mM NaCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 2 mM Na$_2$VO$_4$, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2 \mu M aprotinin, 5 \mu M leupeptin, and 34 \mu M phenylmethylsulfonyl fluoride). Protein concentrations were determined by BCA protein assay using bovine serum albumin as the standard. IRS-1, IRS-2, IRS-3, and tyrrosine-phosphorylated proteins were immunoprecipitated from aliquots of the supernatant containing 1 mg of protein with anti-IRS-1, anti-IRS-2, anti-IRS-3, or 4G10 antibodies, respectively, followed by protein A-Sepharose 6MB. The assays of PI 3-kinase activities in the precipitates were performed as described previously (15).

**Western Blotting**—Isolated adipocytes were prepared, and some were incubated with insulin as described above. Subcellular fractions were separated as described above, and precipitated fractions were resuspended in ice-cold buffer B. The protein concentration was determined by BCA protein assay using bovine serum albumin as the standard, and the concentrations were adjusted to 1 mg/ml. IRS-1, IRS-2, IRS-3, and tyrrosine-phosphorylated proteins were immunoprecipitated from each supernatant containing 1 mg of protein with 2 \mu M anti-IRS-1, anti-IRS-2, or 4G10 antibodies, respectively, followed by protein A-Sepharose 4FF. After centrifugation, the supernatant was removed and used subsequently for another two immunoprecipitations. Immunoprecipitates were boiled as described under “Experimental Procedures” and subjected to SDS-PAGE. Immunoblotting was performed with anti-IRS-3 antibody to detect IRS-3 (upper panel). The bar graph represents the amount of IRS-3 in the first (1), second (2), and third (3) immunoprecipitates. Panel B, a cell lysate of isolated adipocytes was prepared and 1 mg of cell lysate was immunoprecipitated with anti-IRS-1 and anti-IRS-2 antibody. Immunoprecipitates were subjected to SDS-PAGE, transferred to PVDF membrane, and immunooblotted with anti-IRS-1 antibody.

**RESULTS**

**Efficiency of Immunodepletion of Anti-IRS-3 Antibody and Immunospecificity of Anti-IRS-1/2 Antibody**—To determine the efficiency of immunoprecipitation with anti-IRS-3 antibody, the amount of IRS-3 in the supernatant after immunoprecipitation was determined. As shown in Fig. 1, IRS-3 in the supernatant after immunoprecipitation was only 6% of that in the first. These results indicate that the anti-IRS-3 antibody used in this study immunodepleted IRS-3 from the cell lysate effectively.

The specificity of anti-IRS-1 and anti-IRS-2 antibody was demonstrated. As shown in Fig. 1B, IRS-1 was detected in anti-IRS-1 immunoprecipitates, whereas IRS-1 was not detected in anti-IRS-2 immunoprecipitates by anti-IRS-1 immu-

![FIG. 1. Efficiency of immunodepletion of anti-IRS-3 antibody and immunospecificity of anti-IRS-1/2 antibody. Panel A, a cell lysate of isolated adipocytes was prepared as described under “Experimental Procedures.” 1 mg of cell lysate was incubated for 2 h at 4 °C with anti-IRS-3 antibody that chemically cross-linked with protein A-Sepharose 4FF. After centrifugation, the supernatant was removed and used subsequently for another two immunoprecipitations. Immunoprecipitates were boiled as described under “Experimental Procedures” and subjected to SDS-PAGE. Immunoblotting was performed with anti-IRS-3 antibody to detect IRS-3 (upper panel). The bar graph represents the amount of IRS-3 in the first (1), second (2), and third (3) immunoprecipitates. Panel B, a cell lysate of isolated adipocytes was prepared and 1 mg of cell lysate was immunoprecipitated with anti-IRS-1 and anti-IRS-2 antibody. Immunoprecipitates were subjected to SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-IRS-1 antibody.](image-url)
HDM, and cytosol (incubated with or without insulin for 5 min at 37 °C, and PM, LDM, and cytosol (Cyt) were prepared as described. Aliquots of each fraction (1 mg) were incubated with anti-IRS-1 antibody and subsequently precipitated by adding protein A-Sepharose 4FF. Immunoprecipitates were subjected to SDS-PAGE and transferred to PVDF membrane. Immunoblotting was performed with anti-IRS-1, anti-phosphotyrosine antibody (4G10), and anti-p85 antibody to measure IRS-1 protein (panel A), tyrosine phosphorylation of IRS-1 (panel B), and p85α associated with IRS-1 (panel C), respectively. In addition, PI 3-kinase activity in the anti-IRS-1 immunoprecipitate from each fraction was assayed as described under “Experimental Procedures” (panel D).

Fig. 2. Subcellular localization of IRS-1 in isolated adipocytes. The epididymal fat pad was excised, and adipocytes were isolated as described under “Experimental Procedures.” Isolated adipocytes were incubated with or without insulin for 5 min at 37 °C, and PM, LDM, and cytosol (Cyt) were prepared as described. Aliquots of each fraction (1 mg) were incubated with anti-IRS-1 antibody and subsequently precipitated by adding protein A-Sepharose 4FF. Immunoprecipitates were subjected to SDS-PAGE and transferred to PVDF membrane. Immunoblotting was performed with anti-IRS-1, anti-phosphotyrosine antibody (4G10), and anti-p85 antibody to measure IRS-1 protein (panel A), tyrosine phosphorylation of IRS-1 (panel B), and p85α associated with IRS-1 (panel C), respectively. In addition, PI 3-kinase activity in the anti-IRS-1 immunoprecipitate from each fraction was assayed as described under “Experimental Procedures” (panel D).

Fig. 3. Subcellular localization of IRS-2 in isolated adipocytes. The epididymal fat pad was excised, and adipocytes were isolated as described under “Experimental Procedures.” Isolated adipocytes were incubated with or without insulin for 5 min at 37 °C, and PM, LDM, and cytosol (Cyt) were prepared as described. Aliquots of each fraction (1 mg) were incubated with anti-IRS-2 antibody and subsequently precipitated by adding protein A-Sepharose 4FF. Immunoprecipitates were subjected to SDS-PAGE and transferred to PVDF membrane. Immunoblotting was performed with anti-IRS-2, anti-phosphotyrosine antibody (4G10), and anti-p85 antibody to measure IRS-2 protein (panel A), tyrosine phosphorylation of IRS-2 (panel B), and p85α associated with IRS-2 (panel C), respectively. In addition, PI 3-kinase activity in the anti-IRS-2 immunoprecipitate from each fraction was assayed as described under “Experimental Procedures” (panel D).

Fig. 4. Subcellular localization of IRS-3 in isolated adipocytes. The epididymal fat pad was excised, and adipocytes were isolated as described under “Experimental Procedures.” Isolated adipocytes were incubated with or without insulin for 5 min at 37 °C, and PM, LDM, and cytosol (Cyt) were prepared as described. Aliquots of each fraction (1 mg) were incubated with anti-IRS-3 antibody and subsequently precipitated by adding protein A-Sepharose 4FF. Immunoprecipitates were subjected to SDS-PAGE and transferred to PVDF membrane. Immunoblotting was performed with anti-IRS-3, anti-phosphotyrosine antibody (4G10), and anti-p85 antibody to measure IRS-3 protein (top panel), tyrosine phosphorylation of IRS-3 (second panel from top), and p85α associated with IRS-3 (third panel from top). In addition, PI 3-kinase activity in the anti-IRS-3 immunoprecipitate from each fraction was assayed as described under “Experimental Procedures” (bottom panel).

Similar results were obtained regarding the amount of p85α, a regulatory subunit of PI 3-kinase, associated with each of the IRS proteins, when stimulated with insulin. In the insulin-stimulated condition, p85α associated with IRS-1 and IRS-2 was detected predominantly in the LDM sample (Figs. 2C and 3C, respectively), whereas that associated with IRS-3 was detected mainly in the PM, but a smaller amount was also detectable in the LDM sample (Fig. 4, second panel from bottom).

PI 3-Kinase Activity Associated with IRS-1, IRS-2, and IRS-3 in Each Fraction of Isolated Adipocytes—The subcellular localization of PI 3-kinase activity in anti-IRS-1, anti-IRS-2, and anti-IRS-3 antibody immunoprecipitates from isolated adipocytes was investigated (Figs. 2D, 3D, and 4, bottom panel, respectively). Enhancement of the kinase activity by insulin stimulation was observed irrespective of the fraction. In anti-IRS-1 and anti-IRS-2 antibody immunoprecipitates, the major-
ity of PI 3-kinase activity was detected in LDM, and the activity in the PM samples was less than 10% of that in the LDM samples. In contrast, PI 3-kinase activity associated with IRS-3 was detected mainly in the PM, and a much lower activity was detected in the LDM samples.

Taking the different total amounts of protein obtained from each of the subcellular fractions into consideration, the distribution of each IRS protein, the tyrosine phosphorylation level, and PI 3-kinase protein and activity associated with each IRS protein were calculated in whole PM, LDM, HDM, and cytosol fractions and are summarized in Table I. Briefly, 74% and 69% of the PI 3-kinase activity associated with IRS-1 and IRS-2, respectively, were detected in the LDM fraction in the insulin-stimulated condition, whereas 77% of that associated with IRS-3 was detected in the PM fraction.

High Fat Diet Feeding Up-regulates IRS-3 but Down-regulates IRS-1 and IRS-2—It is well known that a high fat diet induces the enlargement of adipocytes and also insulin resistance in glucose uptake. A diet rich in fat or a normal diet was administered to rats for 2 weeks, and epididymal fat cells were used to investigate the regulation of expression levels of IRS-1, IRS-2, and IRS-3. An RNase protection assay and immunoblotting were performed to measure the amounts of mRNA and protein, respectively. The high fat diet decreased mRNA levels of IRS-1 and IRS-2 by 24 and 27%, respectively (Fig. 5A and B), respectively, compared with the controls, whereas the mRNA level of IRS-3 was up-regulated significantly by 49% (Fig. 5C). Similar regulation was observed regarding the protein levels. IRS-1 and IRS-2 proteins in high fat-fed rat adipocytes were revealed to be decreased by 27 and 52%, respectively, compared with the controls (Fig. 5, D and E), whereas IRS-3 protein level was increased by 282% (Fig. 5F).

These results indicate that the expression level of IRS-3 is regulated differently from that of IRS-1 and IRS-2 and that these different regulations are likely to act at the transcriptional level.

Effects of High Fat Diet on IRS Protein Content in PM and LDM Fractions in Adipocytes—LDM and PM fractions were prepared from adipocytes of high fat-fed rats and control rats. The amount of IRS-1 in the PM of normal diet rats was 11% of that in LDM (Fig. 6A). The amount of IRS-1 in the adipocytes of high fat-fed rats in both PM and LDM was decreased by 31 and 74%, respectively, compared with the controls. Thus, the effect of a high fat diet on IRS-1 protein amount was more pronounced in LDM.

IRS-2 expression was also dominant in the LDM in adipocytes of normal diet rats. The high fat diet reduced the IRS-2 protein level in both PM and LDM compared with normal diet by 24 and 59%, respectively. Taken together, in high fat-fed rat adipocytes, the decreases in IRS-1 and IRS-2 content were more marked in the LDM fraction than those in the PM fraction. In contrast, the amount of IRS-3 protein was demonstrated to be increased similarly in both PM (by 62%) and LDM fractions (by 26%).

Effect of High Fat Diet on PI 3-Kinase Activity Associated with Anti-IRS-1, Anti-IRS-2, Anti-IRS-3, and Anti-phosphotyrosine Immunoprecipitates in PM and LDM—The PI 3-kinase activity associated with anti-IRS-1, anti-IRS-2, and anti-IRS-3 immunoprecipitates was determined. The high fat diet decreased the PI 3-kinase activity associated with IRS-1 and IRS-2 after insulin stimulation in the LDM by 46 and 43%, respectively. In contrast, the PI 3-kinase activity associated with IRS-3 after insulin stimulation was significantly increased in both LDM and PM fractions by high fat diet.

Panel C of Fig. 7 demonstrates the PI 3-kinase activity associated with anti-IRS-3 immunoprecipitates. The high fat diet increased PI 3-kinase activity associated with anti-IRS-3 immunoprecipitates in the basal state (PM, 1.7-fold; LDM, 1.9-fold). Insulin-induced PI 3-kinase activation associated with anti-IRS-3 immunoprecipitates was also increased by a high fat diet in both PM and LDM (PM, 1.5-fold; LDM, 2.4-fold).

PI 3-kinase activity associated with anti-phosphotyrosine immunoprecipitates may represent the increment of those (associated with) anti-IRS-1, anti-IRS-2, and anti-IRS-3 immunoprecipitates. Fig. 7D demonstrates that insulin-induced PI 3-kinase activation associated with anti-phosphotyrosine antibody was decreased in both PM and LDM in high fat-fed rats by 10 and 40%, respectively.

**DISCUSSION**

Insulin exerts numerous cellular activities in various cells. Insulin signaling is initiated by the binding of insulin to its specific receptor on the cell surface. By the activated insulin receptor tyrosine kinase, several substrates reportedly are phosphorylated on their tyrosine residues. In rat adipocytes, not only IRS-1 and IRS-2, but also pp60, were found to be major substrates of the insulin receptor (1–3). Recently, pp60 was cloned from rat and mouse adipocytes and termed IRS-3 (11, 12). IRS-3 was shown to contain an amino-terminal PH domain, followed by a PTB domain, and these domains are highly homologous (about 50% identical amino acids) to those of IRS-1 and IRS-2 (11, 12). In addition, there is conservation of many tyrosine phosphorylation motifs responsible for interactions with downstream signaling molecules containing SH2 domains including PI 3-kinase, SHP2, and Grb-2 (12). Thus, to date no...
functional difference of IRS-3 from IRS-1 and IRS-2 has been reported.

IRS-3 mRNA is expressed abundantly in adipocytes and hepatocytes but is also highly expressed in the first part of embryonic life, when IRS-1 mRNA is barely detected (11). These results suggest that IRS-3 may be responsible not only for the regulation of metabolic functions in adipocytes and hepatocytes but also for differentiation and/or proliferation.

In this study we used specific antibodies for IRS-1, IRS-2, and IRS-3 and determined the subcellular localization of these proteins and PI 3-kinase activity mediated by each of these insulin receptor substrates. Insulin-stimulated PI 3-kinase activity associated with IRS-1 and IRS-2 was located mainly in the LDM (74 and 69% of total activity in cells, respectively), whereas it was barely detected in plasma membrane (19 and 15% of total activity in cells, respectively) (Table I). In fact, IRS-1 and IRS-2 proteins were barely detected in PM. Because increased amounts of IRS-1 and IRS-2 in the LDM were not detected in our experimental condition, PI 3-kinase activation associated with IRS-1 and IRS-2 in LDM resulted from an increase in the PI 3-kinase associated with the YM motif in IRS-1 and IRS-2 phosphorylated on tyrosine residues by insulin.

Heller-Harrison et al. (18) reported that insulin action on 3T3-L1 adipocytes progressively decreased the amount of IRS-1 protein associated with the LDM fraction. Although we could not observe a similar decrease in IRS-1 in the LDM of isolated rat adipocytes, we speculate that this contradiction may be caused by differences of the cell types and/or experimental conditions such as serum starvation.

In contrast to the case of IRS-1 and IRS-2, IRS-3 protein was detected in both PM and LDM, irrespective of stimulation with insulin. As reflected by the subcellular distribution of IRS-3, 77% of the PI 3-kinase activity associated with IRS-3 in the whole cell was detected in the PM fraction, and only 12% was in the LDM fraction (Table I). Thus, only IRS-3 efficiently contributes to the insulin-induced PI 3-kinase activation on the plasma membrane in adipocytes.

Although it remains unclear how the subcellular distributions of these signaling molecules are determined, it seems that the PH domain plays an important role in anchoring the protein to the membrane via its association with phospholipid (19, 20). On the other hand, the PTB domain of IRS proteins is reportedly essential for association with the insulin receptor (21). Therefore, the NH2-terminal portion containing the PH domain and PTB domain could have some role in determining the location of IRS proteins. However, because these portions are highly conserved between IRS-3 and IRS-1/2, it is quite unlikely that these portions contribute to their different subcellular distributions. Because there is no extended homology in the portion outside the PH and PTB domains between IRS-3 and either IRS-1 or IRS-2, it seems reasonable to consider that there is a region other than PH and PTB domains responsible for the different subcellular distributions. Further study is necessary to clarify this issue.

Insulin induces numerous cell activities in adipose tissue,
which include cell proliferation and differentiation, stimulation of glucose and amino acid uptake, inhibition of lipolysis and translocation of various membrane proteins such as transferrin receptor and insulin-like growth factor II receptor, and synthesis and/or secretion of leptin (1–3). Among them, insulin-stimulated PI 3-kinase activation plays a critical role in the translocation of GLUT4 from intracellular vesicles to the cell surface (4–6). In addition, Yang et al. (7) suggested that PI 3-kinase activation in the intracellular compartment, but not on the PM, is necessary for the translocation of GLUT4, because treatment with epidermal growth factor or platelet-derived growth factor, which stimulate PI 3-kinase activity in the whole cell as strongly as insulin, failed to induce translocation of the glucose transporter to the cell surface as fully as that with insulin (7).

Assuming that this is the case, PI 3-kinase activation on the plasma membrane by IRS-3 may not contribute to insulin-induced GLUT4 translocation, but one in LDM by IRS-1 and IRS-2 may be much more important to contribute to insulin-induced GLUT4 translocation.

A high fat diet is one of the major causes inducing insulin resistance with respect to the insulin-induced translocation of GLUT4 to the cell surface (22–26). We demonstrated that high fat diet up-regulates IRS-3 but down-regulates IRS-1 and IRS-2. As reflected by these altered expression levels, PI 3-kinase activity associated with anti-IRS-1, anti-IRS-2, anti-IRS-3, and anti-phosphotyrosine antibody is represented in panels A, B, C, and D, respectively.

Fig. 6. Effect of high fat diet on protein levels of IRS-1, IRS-2, and IRS-3 in plasma membrane and low density microsomes of isolated adipocytes. Aliquots of fractionated homogenate were immunoprecipitated with anti-IRS-1, anti-IRS-2, and anti-IRS-3 antibodies and subsequently with protein A-Sepharose 4FF. Immunoprecipitates were subjected to SDS-PAGE, transferred to PVDF membrane, and immunoblotted with specific antibodies using an ECL plus kit. The quantitation was performed with a Bio-Rad PhosphorImager with Screen-CH. The bar graph represents quantitation of the representative results of three independent experiments. Results are presented as % of each protein level in LDM of normal diet rats. Protein levels of IRS-1, IRS-2, and IRS-3 are represented in panels A, B, and C, respectively.

Fig. 7. Effect of high fat diet on PI 3-kinase activity associated with anti-IRS-1, anti-IRS-2, anti-IRS-3, and anti-phosphotyrosine immunoprecipitates in plasma membrane and low density microsomes of isolated adipocytes. Aliquots of fractionated homogenate were immunoprecipitated with anti-IRS-1, anti-IRS-2, and anti-IRS-3 antibodies and subsequently with protein A-Sepharose 6MB. PI 3-kinase assay for each immunoprecipitate was performed as described under “Experimental Procedures.” The quantitation was performed with a Bio-Rad PhosphorImager with Screen-BI. The bar graph represents quantitation of the representative results of three independent experiments. Results are presented as the percent of each insulin-stimulated PI 3-kinase activity in LDM of normal diet rats. PI 3-kinase activity associated with anti-IRS-1, anti-IRS-2, anti-IRS-3, and anti-phosphotyrosine antibody is represented in panels A, B, C, and D, respectively.
Because IRS-3 and IRS-1/2 are located mainly in the PM and LDM, respectively, as a consequence of their altered expression levels, insulin-induced PI 3-kinase activation is impaired markedly in the LDM, whereas that on the PM is maintained. We speculate that this abnormality in the portion in the cell where PI 3-kinase activation occurs may be one of the mechanisms causing insulin resistance in high fat diet-induced insulin resistance, in terms of glucose uptake.

Although the role of PI 3-kinase activation on the PM induced by IRS-3 in adipocytes remains unclear, several possibilities can be raised. Treatment of adipocytes with platelet-derived growth factor, epidermal growth factor, or fibroblast growth factor reportedly induces a decrease in the number of developing fat cells and the activity of glycerol-3-phosphate dehydrogenase, a marker of adipocyte differentiation (27). In addition, this inhibitory action regarding the adipocyte differentiation was associated with markedly potent stimulation of cell proliferation. The inhibitory effect of platelet-derived growth factor and epidermal growth factor on adipocyte differentiation was suggested to be induced by the reduction of peroxisome proliferator-activated receptor γ1 transcriptional activity caused by phosphorylation of peroxisome proliferator-activated receptor γ1 by mitogen-activated protein kinase (28). 

Taking these previous reports into consideration, it can be speculated that IRS-3 phosphorylated mainly on the plasma membrane, similarly to the receptors of epidermal growth factor or platelet-derived growth factor, may have a role promoting cell proliferation and inhibiting adipocyte differentiation. In addition to the functions of adipocytes discussed above, fat cells have more specific functions that are affected by insulin stimulation. It would be of great interest to clarify which IRS protein transduces the signal inducing the individual insulin-induced cell activity. Further study of this issue is needed.

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