The 60 kDa Insulin Receptor Substrate Functions Like an IRS Protein ($pp60^{IRS3}$) in Adipose Cells†

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Received December 17, 1996; Revised Manuscript Received April 24, 1997⊗

ABSTRACT: The 60 kDa insulin receptor substrate in rat adipocytes that binds to the PI-3 kinase displays several functional characteristics in common with the IRS proteins; so we propose the name $pp60^{IRS3}$ to distinguish it from other tyrosine phosphorylated proteins of similar size. During insulin stimulation, p85 associated with $pp60^{IRS3}$ more rapidly than with IRS-1 or IRS-2. In mice lacking IRS-1, p85 associated more strongly with $pp60^{IRS3}$ than with IRS-2, suggesting that $pp60^{IRS3}$ provides an alternate pathway in these cells. Synthetic peptides containing two phosphorylated YMPM motifs displace $pp60^{IRS3}$ and IRS-1 from αp85 immune complexes, suggesting that $pp60^{IRS3}$, like IRS-1, engages both SH2 domains in p85. Moreover, $pp60^{IRS3}$ binds to immobilized peptides containing a phosphorylated NPXY motif, suggesting that it contains a PTB domain with similar specificity to that in IRS-1. The cloning of $pp60^{IRS3}$ will reveal a new member of the IRS protein family which mediates insulin receptor signals in a narrow range of tissues.

Insulin-stimulated phosphorylation of IRS-1 occurs on multiple tyrosine residues which mediate the association and activation of signaling proteins that contain Src homology-2 (SH2) domains. These SH2 proteins, including PI-3 kinase, SHP-2, and several smaller adapter molecules (Grb-2, Crk, and nck), couple the insulin receptor substrates to various downstream signaling pathways which mediate metabolic responses, cell growth, survival, and differentiation (Myers et al., 1994; Beitner-Johnson & LeRoith, 1995; Skolnik et al., 1993). Without IRS-1, mice are smaller than expected and display mild hyperinsulinemia, but never develop NIDDM (Araki et al., 1994); however, skeletal muscle displays an 80% reduction in many insulin-stimulated responses, including glucose uptake, glycogen synthesis, and protein synthesis (Yamauchi et al., 1996). Whereas IRS-2 apparently compensates for the absence of IRS-1 in hepatocytes, this is not the case in skeletal muscle or adipocytes (Yamauchi et al., 1996; Patti et al., 1995).

The identification of physiologically relevant proteins that are phosphorylated by the insulin receptor is difficult, because these proteins occur at low concentrations in cells and have limited amino acid sequence identities (Sun et al., 1995). However, the alignment of IRS-1 and IRS-2 and the related molecules Gab1 and $p62^{dok}$ reveals some general features expected for this class of signaling molecule (Carpino et al., 1997; Yamanashi & Baltimore, 1997; Holgado-Madruga et al., 1996; Sun et al., 1995). Each of these insulin receptor substrates contain a pleckstrin homology (PH) domain at the NH_2-terminus. In addition, IRS-1 and IRS-2 contain a phosphotyrosine binding (PTB) domain which interacts with a phosphorylated NPXY motif in the receptors for insulin, IGF-1 and IL-4 (Sawka-Verhelle et al., 1996; Zhou et al., 1996; Wolf et al., 1995; O'Neill et al., 1994). Both of these domains couple the IRS proteins to the insulin receptor, although the PH domain mediates the most sensitive interaction (Yenush et al., 1996). Moreover, Gab1 does not contain a PTB domain and $p62^{dok}$ contains only a partial PTB domain suggesting that they rely on the PH domain or other regions for interaction with membrane receptors. Recently, IRS-2 was shown to contain a novel interaction domain which associates with the phosphorylated regulatory loop in the insulin receptor β-subunit (Sawka-Verhelle et al., 1996).

A 60 kDa insulin receptor substrate called pp60 has been described in rat adipocytes but has been difficult to characterize, owing to its low expression levels (Lavan & Lienhard, 1993; Kanai et al., 1993; Zhang-Sun et al., 1996). Here, we propose the name $pp60^{IRS3}$ because our results suggest that it is functionally similar to IRS-1 and IRS-2. In adipocytes, $pp60^{IRS3}$ bound more rapidly than IRS-1 or IRS-2 to p85 during insulin stimulation, suggesting that it could be a principal regulator of the PI-3 kinase. Biochemical experiments indicate that it contains a PTB domain and binds to both SH2 domain in p85. The recent cloning of cDNA encoding pp60 confirms our expectations and reveals a new member of the IRS protein family (Lavan, 1997).

MATERIALS AND METHODS

Materials. Bovine serum albumin (fraction V) for immunoblotting was purchased from Arnel, and fatty acid-free bovine serum albumin (fraction V) was purchased from Miles. Collagenase D, adenosine, and human recombinant insulin were from Boehringer Mannheim, Inc.; immobilon

† This research was supported by DK38712 (M.F.W.) and DK33201 (C.R.K.). J.S.-H. was supported by the American Heart Association, Indiana affiliate, and M.E.P. was supported by the Mary K. Iacocca Fellowship.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

S0006-2960(96)03097-8 CCC: $14.00

P was purchased from Millipore; Glutathione-Sepharose 4B, protein A-Sepharose, and pGEX were from Pharmacia LKB Biotechnology; Affi-gel 15 and chemicals for electrophoresis were from Bio-Rad Laboratories; [^{125}I]protein A was purchased from ICN; sodium nembutal solution was purchased from Abbott Laboratories. Male Sprague−Dawley rats were purchased from Taconic Farms and fasted overnight before use. Peptides were synthesized by Dr. Charles Dahl (Harvard Medical School, Boston, MA), on a Milligen/Biosearch 9600 synthesizer, and each peptide was purified by reverse-phase high-pressure liquid chromatography and confirmed by amino acid analysis.

Polyclonal antibodies against the IRS proteins were made as previously described; αIRS-1 was raised against a synthetic peptide containing residues 1221−1234 of rat IRS-1 (Sun et al., 1991); αIRS-2 was made using a GST-fusion protein containing residues 619−746 of mouse IRS-2 (Sun et al., 1995). Antiphosphotyrosine antibody (αPY) was prepared with phosphotyramine coupled to keyhole limpet hemocyanin (White & Backer, 1991); 4G10 was purchased from UBI. A broad specificity polyclonal antibody against most p85 isoforms (αp85) was prepared in rabbits immunized with a glutathione S-transferase fusion protein containing the NH_2-terminal SH2 domain of p85α (Backer et al., 1992); a specific antibody against p85α (αp85^{NT}) was raised against a peptide containing residues 146−161 of mouse p85α coupled to keyhole limpet hemocyanin (Backer et al., 1993). The αp110 antibody was from Santa Cruz (catalog no. sc-603).

Isolation of Rat and Mouse Adipocytes and Preparation of Cell Lysates. Adipocytes were isolated from male Sprague−Dawley rats (280−300 g) fasted overnight as previously described (Cushman & Wardzala, 1980). IRS1$^{-/-}$ and wild-type mice were identified by genotype as previously described (Patti et al., 1995a). Following a 6 h fast, the mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (115 mg/kg) and adipocytes were isolated as described below. Tissue from rat or mouse epididymal fat was collected in Krebs Ringer bicarbonate containing 10 mM HEPES, 2.5% fatty acid-free BSA, and 200 nM adenosine (buffer A). Fat pads (1 g wet weight) were minced and incubated with 2 mL of buffer A containing 1 mg of collagenase/mL for 40 min at 37 °C in a shaker bath (10 rpm/6 s). The digested tissue was passed through a 50 μ nylon screen and rinsed several times with buffer A to remove the collagenase, then rinsed once with BSA-free buffer A. The isolated adipocytes were suspended in 10 mL of Krebs Ringers bicarbonate by gentle rotation at 37 °C and treated without or with 80 nM insulin for 5 min. The packed adipocytes were lysed at room temperature by vortexing in 15 mL of 50 mM HEPES (pH 7.4), 150 mM sodium chloride, 4 mM EDTA, 20 mM sodium flouride, 20 mM sodium pyrosphosphate, 4 mM sodium orthovanadate, 4 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 20% glycerol, and 2% NP-40 (lysis buffer). Fat and insoluble materials were removed by centrifugation at 12 000 rpm for 30 min at 0 °C in a Ti70 rotor (Beckman).

Preparation of Testis Lysates. Male Sprague−Dawley rats (280−300 g) were fasted overnight and anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The rats were treated without or with insulin (3.5 mg/kg) via the inferior vena cava, and 3 min later both testis were removed, minced in 15 mL of lysis buffer (66 μg/mL), and homongenized with a polytron using three intervals of 10 s each. The homogenates were clarified by centrifugation at 12 000 rpm for 30 min at 4 °C in a Ti70 rotor (Beckman).

Immunoprecipitations, Gel Electrophoresis, and Immunoblotting. Adipocyte lysates (5 mL) were incubated with 5 μL of appropriate antibody for 4 h at 4 °C. The immune complexes were collected on 40 μL of protein A-Sepharose, washed twice with 1 mL of cold lysis buffer, and the proteins were eluted with Laemmli sample buffer as previously described (Ballotti et al., 1986). The precipitated proteins were resolved by reducing SDS−PAGE on 10% acrylamide gels, and transferred to Immobilon P at 110 V for 2 h in Towbin buffer containing 0.02% SDS and 10% (vol/vol) methanol. Proteins were detected by immunoblotting. In all the experiments, membranes were incubated for 1 h with the appropriate antibody at 1:300 dilution, washed, and incubated with [^{125}I]protein A diluted in 20 mL of TTBS containing 2.5% BSA, as previously described (Ballotti et al., 1986; Pons et al., 1995). The immunoblots were analyzed on a Molecular Dynamics Phosphorimager.

IRS-1 Phosphopeptide Competition Studies. Lysates (17 mL) from insulin-stimulated rat adipocytes prepared as described above were incubated with 170 μL of αp85^{NT} antibody (Pons et al., 1995) for 30 min at 4 °C, divided into 1 mL aliquots, and then incubated at 4 °C for 14 h with synthetic YMXM-containing peptides between 1.0 nM to 100 μM. The immune complexes were collected on 40 μL protein A-Sepharose, resolved on a 10% gel, and analyzed by immunoblotting with αPY (Ballotti et al., 1986).

NPXY Phosphopeptide Binding Studies. Synthetic peptides (5 mg) containing phosphorylated or unphosphorylated NPXY motifs were immobilized overnight on 0.5 mL of Affi-Gel 15 suspended in 5 mL of 100 mM HEPES (pH 7.4). Unreacted sites were blocked with glycine, and the Affi-Gel was washed extensively before use with PBS as suggested in the manufacturers' instructions. Basal and insulin-stimulated rat adipocyte lysates were made as described above, except that 0.05% NP-40 was included in the lysis buffer to enhance binding (Wolf et al., 1995). Cell lysate (10 mL) was mixed with each immobilized peptide and continuously mixed by rotation for 14 h at 4 °C. The complexes were washed twice with PBS, then eluted with 100 μL of Laemmeli sample buffer, resolved on a 10% gel, transferred to immobilon P and analyzed by immunoblotting with αPY.

RESULTS

Insulin Stimulates pp60^{IRS3} Phosphorylation in Isolated Adipocytes. The effect of 80 nM insulin for 5 min on tyrosine phosphorylation in isolated primary rat adipocytes was investigated by immunoblotting cell lysates with antiphosphotyrosine antibodies (αPY). As previously shown, several proteins were tyrosine phosphorylated in response to insulin treatment, including the IRS proteins and the insulin receptor β-subunit; a 60 kDa protein was also detected in insulin-stimulated adipocytes, which is commonly referred to as pp60 (Figure 1A). On The basis of the results in this paper, we designate this protein pp60^{IRS3} to emphasize its functional similarity to IRS-1 and IRS-2.

During insulin stimulation, IRS-1 and IRS-2 and pp60^{IRS3} coimmunoprecipitated with a broad specificity antibody

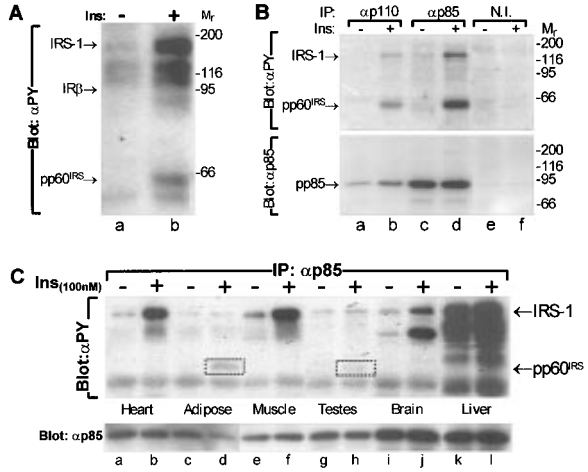

FIGURE 1: Insulin stimulates pp60^{IRS3} phosphorylation in primary rat adipocytes. Isolated rat adipocytes (from 1 g wet weight tissue) were incubated without (−) or with (+) 80 nM insulin for 5 min. Lysates were separated directly by SDS−PAGE (panel A) or immunoprecipitated (panel B) with αp110 (lanes a and b), p85 (lanes c and d), or nonimmune serum (lanes e and f). The immunoprecipates were resolved by SDS−PAGE and protein in two parallel blots was detected by immunoblotting with αPY or αp85. An equal portion of tissues (based on wet tissue weight) from control (−) or insulin-stimulated (+) rats was homogenized, clarified by centrifugation and incubated with αp85^{PAN}. The immunoprecipitates were resolved by SDS−PAGE, transferred to Immobilon P and immunoblotted with αPY (panel C).

against p85 as previously described (Figure 1B) (Zhang-Sun et al., 1996; Kanai et al., 1993; Lavan & Lienhard, 1993). Similar results were obtained with the p85α-specific antibody (αp85^{NT}), alleviating concerns that pp60^{IRS3} is one of hte small p85 homologs (data not shown, also see Figure 5). Moreover, antibodies against p110α immunoprecipitated both pp60^{IRS3} and IRS-1/IRS-2 from insulin-stimulated rat adipocytes, suggesting that both proteins regulate the PI-3 kinase during insulin stimulation (Figure 1B).

Insulin-stimulated tyrosine phosphorylation of pp60^{IRS3}, IRS-1, and IRS-2 was tested in various rat tissues (Figure 1C). IRS-proteins migrating near 185 kDa were immunoprecipitated with αp85 from lysates of insulin-stimulated heart, skeletal muscle, brain, and liver; however, pp60^{IRS3} was absent from these tissues. The insulin receptor was detected in αp85 immunoprecipitates from rat liver, but this interaction is attributed to a weak but stable complex between the insulin receptor and the IRS proteins, as previously discussed (Backer et al., 1993). The pp60^{IRS3} was detected by αp85 immunoprecipitation from adipose and testis of insulin-stimulated rats. The IRS proteins were weakly detected in these tissues, consistent with the short time of *in vivo* insulin stimulation used in this experiment (see Figure 2).

pp60^{IRS3} Is Rapidly Phosphorylated during Insulin Stimulation. Isolated rat adipocytes were stimulated with 80 nM insulin for various time intervals, and the cells were lysed promptly and immunoprecipitated with αp85. Immunoblotting with αPY revealed that pp60^{IRS3} in the p85 complex was tyrosine-phosphorylated maximally during the first minute of insulin treatment. In contrast, the association between p85 and tyrosine phosphorylated IRS-1 reached a maximum after 5 min (Figure 2). This time course suggests that pp60^{IRS3} is the first substrate encountered by PI-3 kinase during insulin stimulation of rat adipocytes.

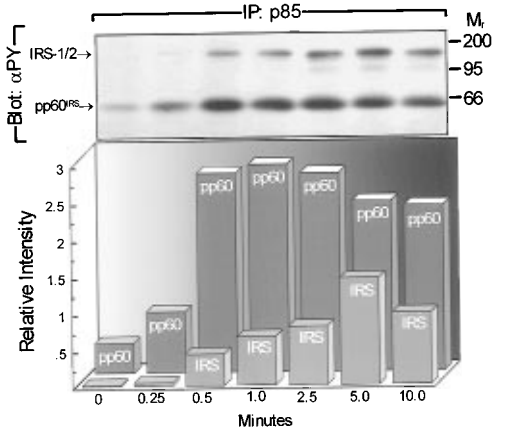

FIGURE 2: Time course of the association of p85 with pp60^{IRS3} and IRS-1. Isolated rat adipocytes from 4 g of wet tissue were divided equally into 14 aliquots and incubated at 37 °C without insulin or with 80 nM insulin for the indicated time intervals: no insulin (lane a), 15 s (lane b), 30 s (lane c), 1.0 min (lane d), 2.5 min (lane e), 5 min (lane f), 10 min (lane g). The cells were rapidly lysed and immunoprecipitated with a αp85^{PAN}. Immunoprecipitates were separated on a 10% gel and transferred to Immobilon P. The membrane was immunoblotted for phosphotyrosine and the bands corresponding to pp60^{IRS3} and IRS-1 were analyzed on a Molecular Dynamics phosphorimager. The relative intensity of pp60^{IRS3} and the IRS-1/IRS-2 is displayed in the bar graph.

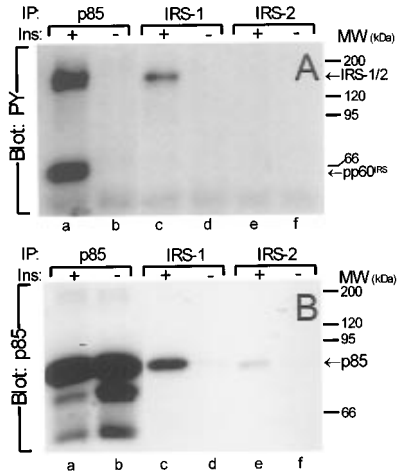

FIGURE 3: IRS-1, IRS-2, and pp60^{IRS} associate independently with p85. Isolated adipocytes (1 mL of packed cells from a 200 g rat) were incubated without (−) or with (+) 80 nM insulin for 5 min. Cell lysates were immunoprecipitated with 5 μL of αp85^{PAN} (lanes a and b), αIRS-1 (lanes c and d), or αIRS-2 (lanes e and f). The immune complexes were separated by SDS−PAGE and transferred to Immobilon P. The membrane was immunoblotted with αPY (panel A), stripped, and reblotted with αp85^{PAN} (panel B).

During insulin stimulation, IRS-1, IRS-2, and pp60^{IRS3} may associate independently with p85 providing three pathways to regulate the PI-3 kinase. Lysates from basal and insulin-stimulated adipocytes were immunoprecipitated with antibodies directed against p85, IRS-1, or IRS-2. Immunoblotting with αPY revealed that αp85 coprecipitated IRS-1 and pp60^{IRS3}. However, αIRS-1 immunoprecipitated IRS-1 and p85, but pp60^{IRS3} was not detected (Figure 3, panels A and B). Tyrosine-phosphorylated IRS-2 was not detected in αIRS-2 immunoprecipitates, whereas a small amount of p85 was immunoprecipitated from insulin-stimulated cells with αIRS-2 (Figure 3, panels A and B). Thus, IRS-1 and pp60^{IRS3} form separate complexes with p85 and appear to be the major tyrosine phosphorylated proteins engaging PI-3 kinase during insulin stimulation of rat adipocytes; however,

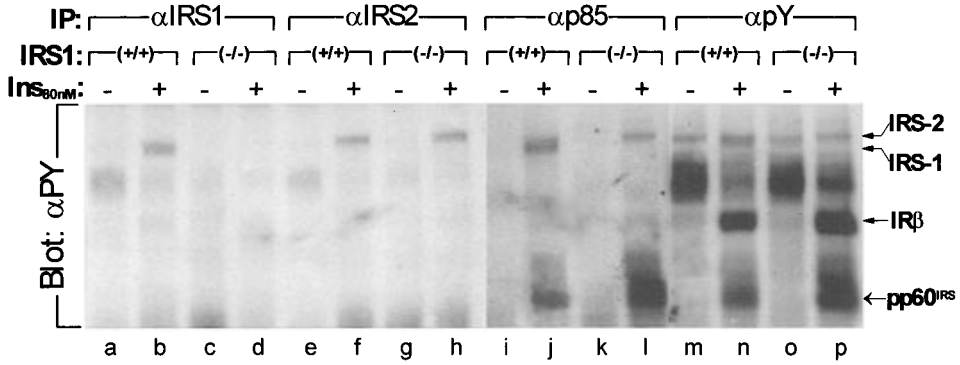

FIGURE 4: pp60^{IRS} associates with p85 in murine adipocytes lacking IRS-1. Adipocytes were isolated from 10 IRS1$^{-/-}$ mice (3 months old) or age-matched control mice IRS1$^{+/+}$ and stimulated without or with 80 nM insulin for 5 min as indicated. Lysates were immunoprecipitated with αIRS1 (lanes a−d), αIRS2 (lanes e−h), αp85 (lanes i−l) or αPY (lanes m−p), separated by SDS−PAGE, transferred to Immobilon P (Millipore), and immunoblotted with αPY.

IRS-2 plays a surprisingly small role in these cells during insulin stimulation.

pp60^{IRS3} Associates with p85 in the Absence of IRS-1. To directly examine the relationship between IRS-1 and pp60^{IRS3}, experiments were conducted with isolated adipocytes from IRS-1-deficient (IRS-1$^{-/-}$) mice and age-matched wild-type mice. As expected, IRS-1 was tyrosine phosphorylated during insulin stimulation of normal murine adipocytes, whereas it was absent from the IRS1$^{-/-}$ mice (Figure 4). Unlike the rat adipocytes described above, the level of IRS-2 tyrosine phosphorylation in wild-type mouse adipocytes was comparable to IRS-1, and this was found to be the same in IRS1$^{-/-}$ adipocytes (Figure 4).

In normal mouse adipocytes stimulated with insulin for 5 min, p85 was predominately associated with IRS-1 and pp60^{IRS3}, as shown above with rat adipocytes. Even though IRS-2 was phosphorylated in the murine cells, it was poorly associated with p85, as revealed by the absence of the upper band in the αPY immunoblot of the αp85 immunoprecipitate (Figure 4, lanes i and j). However, in the absence of IRS-1, both IRS-2 and pp60^{IRS3} associated with p85 during insulin stimulation. Whereas the IRS-2 signal remained weak, the phosphorylation of pp60^{IRS3} was stronger than that found in the wild-type adipocytes (Figure 4). Thus, phosphorylation of IRS-2 and pp60^{IRS3} was independent of IRS-1, and pp60^{IRS3} phosphorylation or association with p85 occurred more strongly without competition from IRS-1. However, without specific antibodies against pp60^{IRS3}, it is not possible to distinguish between these alternatives (Patti et al., 1995b).

pp60^{IRS3} and IRS-1 Use a Common Mechanism To Engage p85. The PI-3 kinase regulatory subunit, p85, contains several interaction modules, including one SH3 domain followed by a bcr-homology region, two SH2 domains, and several proline-rich motifs (Kapeller & Cantley, 1994). Since pp60^{IRS3} associates rapidly and strongly with p85 during insulin stimulation, we hypothesized that both SH2 domains may bind tyrosine phosphorylation sites in pp60^{IRS3} as previously found for IRS-1 (Rordorf-Nikolic et al., 1995; Backer et al., 1992). To examine this possibility, two synthetic phosphopeptides based on established p85 binding sites in IRS-1 were tested for their ability to displace pp60^{IRS3} or IRS-1 from p85 immune complexes. One peptide contained a single phosphorylated YMPM motif (Tyr$_{608}$), and the other contained two phosphorylated YMPM motifs (Tyr$_{608}$ and Tyr$_{628}$). Immuncomplexes were prepared with p85^{NT} to avoid interference by the αp85, which binds the nSH2 domain. Dissociation of pp60^{IRS3} and IRS-1 from αp85^{NT} was more sensitive to the bisphosphopeptide ($ED_{50} \approx 0.05$ nM) than the monophosphopeptide ($ED_{50} > 1$ μM); the nonphosphorylated peptides were unreactive (Figure 5, panels A and B). Moreover, the monophosphopeptide displayed similar sensitivity with αp85^{NT} immunopreciptates from testis (Figure 5C). These results suggest that pp60^{IRS3}, like IRS-1, contains at least two tyrosine phosphorylated sites, probably the YMXM motifs, which interact with each SH2 domain in p85 and are best dissociated with a synthetic peptide containing two phosphorylated YMXM motifs.

pp60^{IRS3} Binds to Insulin Receptor Derived NPXY Peptides. To engage the activated insulin receptor, pp60^{IRS3} may contain IRS-like interaction domains, including a pleckstrin homology (PH) domain or a phosphotyrosine binding (PTB) domain. Specific antibodies against the PH domain or the PTB domain in IRS-1 or IRS-2 were not helpful in this regard, as they failed to react with pp60^{IRS3}. This is not surprising since these antibodies do not cross-react between IRS-1 or IRS-2. Unfortunately, a functional analysis for the PH domain was impossible, as its ligand binding specificity is unknown. In contrast, the PTB domain in IRS-1 and IRS-2 binds to peptides containing phosphorylated NPXY motifs (Yenush et al., 1996). Thus, it is possible to test for the presence of a PTB domain by specific binding of pp60^{IRS3} to immobilized peptides containing phosphorylated NPXY motifs (Wolf et al., 1995).

Lysates from insulin-stimulated rat adipocytes were incubated with immobilized peptides based on the amino acid sequence around Tyr960 in the NPEY motif of the insulin receptor; the association of IRS-1 and pp60^{IRS3} to these immobilized peptides was assessed by immunoblotting with αPY. As expected, no tyrosine phosphorylated protein bound to the nonphosphorylated NPEY peptide. By contrast, a 60 kDa protein which comigrated with pp60^{IRS3} in αp85 immunoprecipitates bound to the phosphorylated NPXY peptide; however, IRS-1 was poorly detected (Figure 6). Substitution of glutamic acid at the Y^{-1} position with alanine reduced the binding of pp60^{IRS3}; however, this mutation enhanced the binding of IRS-1, as previously shown (Wolf et al., 1995). As expected, substitution of asparagine with alanine at the Y^{-3} position inhibited the binding of IRS-1 and pp60^{IRS3} (Figure 6). Thus, at the functional level, pp60^{IRS3} appears to contain a PTB domain or associate with a protein that contains a PTB domain.

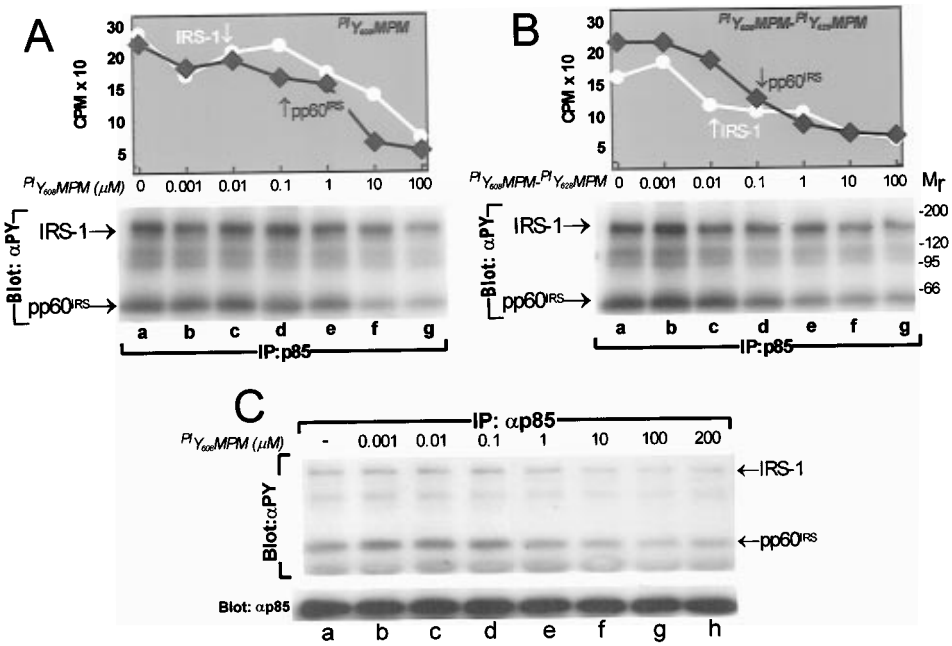

FIGURE 5: pp60^{IRS3} and IRS-1 compete for p85 binding. Isolated adipocytes were stimulated with 80 nM insulin for 2 min (panel A). Then cell lysates were incubated for 16 h at 4 °C with αp85^{NT} and without or with 0.001 μM, 0.01, 0.1, 1.0, 10.0, and 100.0 mM of the following peptides: (1) ^{Pi}YMPM$_{608}$, DDGPY$_{Pi}$MPMSPGV and (2) ^{Pi}YMPM$_{608}$, ^{Pi}YMPM$_{628}$, DDGPY$_{Pi}$PIMPMSP(GA)$_5$GNGDPY$_{Pi}$MPMSPKS [(GA)$_5$ represents a spacer containing five repeating GlyAla dipeptides]. The immune complexes were collected, resolved by SDS−PAGE, and detected by immunoblotting with αPY (lower portion of panels A and B). The amounts of IRS-1 (solid white circles) or pp60^{IRS3} (solid black diamonds) associated with the immuncomplexes were qualified with a phosphorimager. The open circle or open diamond show displacement of IRS-1 or pp60^{IRS3}, respectively, by 10 mM nonphosphorylated ^{Pi}YMPM$_{608}$ peptide (panel A). Testis homogenates prepared from insulin-stimulated rats were incubated with αp85^{NT} and varying concentrations of peptide A were analyzed as described above (panel C). The curves shown are representative of three independent experiments.

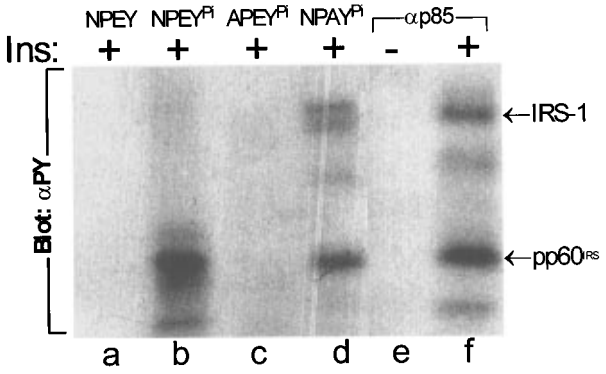

FIGURE 6: Binding of pp60^{IRS3} to phosphorylated NPXY-peptides. Isolated adipocytes from six rats were stimulated with 80 nM insulin for 2 min, and lysates were made as described in the Materials and Methods. Lysates from 1.0 mL of packed adipocytes were incubated with 5 mg of immobilized peptide: (1) NPEY, GGYLASS**NPEY**-LSASD; (2) NPEY^{PI}, GGYLASS**NPEY**(Pi)LSASD; (3) APEY^{PI}, GGYLASS**APEY**(Pi)LSASD; (4) NPAY^{PI}, GGYLASS**NPAY**(Pi)-LSASD. The resin was washed twice with lysis buffer containing 0.05% NP40. Bound proteins were eluted with 100 μL of 2× Laemmli buffer, resolved by SDS−PAGE, and transferred to Immobilon P and detected by immunoblotting with αPY. Equal portions of lysate from stimulated and unstimulated adipocytes were immunoprecipitated with αp85, resolved by SDS−PAGE, and immunoblotted with αPY (lanes e and f). This experiment was conducted twice with identical results.

DISCUSSION

Recent evidence suggests that activation of the PI-3 kinase by the insulin signaling system is necessary and possibly sufficient for insulin-induced GLUT 4 translocation (Morris et al., 1996; Hara et al., 1994; Dudek et al., 1997). Thus, molecules such as IRS-1, IRS-2, and pp60^{IRS3} may be important regulators of glucose uptake. On the basis of work with the IRS proteins, several features emerged which are essential for an efficient insulin receptor substrate, including protein−protein interaction domains that mediate receptor coupling and tyrosine phosphorylation motifs that engage SH2 proteins (Yenush & White, 1997; Sun et al., 1995). On the basis of experiments in this report, pp60^{IRS3} possesses several features which are similar to the IRS proteins. Displacement of p85 from pp60^{IRS3} is most sensitive to peptides containing two YMPM motifs, suggesting that pp60^{IRS3} contains at least two phosphotyrosine residues that bind both SH2 domains of p85. pp60^{IRS3} also binds to recombinant SH2 domains of fyn, SHP2, and Grb-2 (data not shown), suggesting that YXXI/L motifs also occur in this substrate (Sun et al., 1996; Zhang-Sun et al., 1996). Finally, pp60^{IRS3} binds to peptides containing phosphorylated NPXY motifs, suggesting that it contains a phosphotyrosine binding (PTB) domain. Thus, pp60^{IRS3} appears to be a multiphosphorylated signaling protein in the IRS protein family.

Our experience with IRS-1 deletion mutants indicates that the PH domain contributes significantly to insulin-stimulated IRS-1 phosphorylation, whereas the PTB domain alone is insufficient but significantly enhances the coupling (Yenush et al., 1996). The PTB domains in IRS-1 and Shc are structurally similar to pleckstrin homology domains, as both are composed of two antiparallel β-sheets capped by an α-helix (Lemmon et al., 1996). However, physiologically relevant binding partners for the IRS-1 and IRS-2 PH domains are unknown, whereas the PTB binds phosphorylated NPXY motifs (Eck et al., 1996). pp60^{IRS3} binds more strongly than IRS-1 to the phosphorylated NPEY motif in the insulin receptor, strongly suggesting the presence of a PTB domain. By contrast, substitution of alanine for the

glutamic acid at the Y^{+1} position in the NPEY motif reverses this relative affinity making IRS-1 the stronger binder. Thus, the PTB domain alone may be sufficient for sensitive coupling between $pp60^{IRS3}$ and the native insulin receptor, although a PHP domain is expected by analogy to IRS-1 and IRS-2.

Our results suggest that $pp60^{IRS3}$ may be the initial substrate of the activated insulin receptor in adipocytes. The increased affinity between $pp60^{IRS3}$ and the native NPEY motif of the activated insulin receptor may mediate the relatively rapid phosphorylation of $pp60^{IRS3}$ compared to IRS-1. As a result, $pp60^{IRS3}$ associates maximally with p85 after 2−3 min of insulin stimulation, whereas IRS-1 and IRS-2 reach steady state after 5 min. Bisphosphorylated peptides displace $pp60^{IRS3}$ from p85 immune complexes more sensitively than monophosphorylated peptides, suggesting that $pp60^{IRS3}$ contains at least two phosphorylation sites which bind to both SH2 domains in p85. Previous reports show that activation of PI-3 kinase occurs maximally when both SH2 domains in p85 are occupied by a phosphorylated YMXM motif (Rordorf-Nikolic et al., 1995). Thus, in addition to IRS-1, $pp60^{IRS3}$ is likely to be a strong activator of the PI-3 kinase in adipocytes.

IRS1-deficient mice are hyperinsulinemic and small, but not diabetic as the fasting glucose levels are normal (Araki et al., 1995). Insulin-stimulated PI-3 kinase activity and glucose uptake is only partially reduced in murine adipocytes from the $IRS1^{-/-}$ mouse, suggesting that alternative pathway(s) for insulin signaling exist in this tissue (Araki et al., 1995; Patti et al., 1995). IRS-2 appears to compensate for the absence of IRS-1 in murine hepatocytes as the stoichiometry of its tyrosine phosphorylation increases several-fold resulting in nearly normal activation of the PI-3 kinase. By contrast, skeletal muscle lacking IRS-1 is significantly insulin resistant owing to a persistently low level of IRS-2 expression and absence of enhanced tyrosine phosphorylation (Yamauchi et al., 1996). Like skeletal muscle, IRS-2 appears to play a small role in mouse and rat adipocytes, whereas $pp60^{IRS3}$ provides the major alternative pathway to PI-3 kinase in the absence of IRS-1. Although the levels of $pp60^{IRS3}$ cannot be measured without specific antibodies during insulin stimulation of $IRS1^{-/-}$ mice, more tyrosine phosphorylated $pp60^{IRS3}$ is immunoprecipitated with αPY and more associates with p85. Moreover, $pp60^{IRS3}$ clearly dominates over IRS-2 in the adipocytes from the mice. Since the phosphorylation of the insulin receptor is not changed in these cells, we tentatively conclude that $pp60^{IRS3}$ phosphorylation increases in $IRS1^{-/-}$ adipocytes because IRS-1 is not present to compete.

The rapid phosphorylation of $pp60^{IRS3}$ is consistent with an important role in glucose transport regulation. The stimulation of glucose uptake in adipocytes occurs 2−3 min following insulin stimulation, most consistent with the phosphorylation of $pp60^{IRS3}$. Previous studies indicate that $pp60^{IRS3}$ associates with the plasma membrane, placing it physically closer to the insulin receptor kinase. By contrast, IRS-1 is located in the cytosol and low-density membranes (Kelly & Ruderman, 1993) and in GLUT4 vesicles immediately after insulin stimulation where it may also play an important regulatory role (Heller-Harrison et al., 1996). Perhaps $pp60^{IRS3}$ links the insulin signal to structures in the plasma membrane that are required to retain the GLUT4 vesicle, whereas IRS-1 signals to the GLUT4-containing endosome. It is worth noting that $IRS1^{-/-}$ adipocytes display reduced insulin-stimulated glucose uptake, even though the phosphorylation of $pp60^{IRS3}$ is elevated. Thus, IRS-1 is an essential element in the signaling pathway that cannot be entirely replaced with either IRS-2 or $pp60^{IRS3}$. The relative contributions of IRS-1 and $pp60^{IRS3}$, along with IRS-2 and Gab-1 will be resolved when appropriate cell lines expressing various levels of each docking protein are available.

Several insulin-stimulated 60 kDa phosphotyrosine-containing proteins have been described in various cell types, but the adipocyte protein that binds PI-3 kinase was reported first (Keller et al., 1991); it is almost certainly $pp60^{IRS3}$. By contrast, a 62 kDa substrate occurs in various cells that binds to the SH2 domain in $p21^{ras}$-GAP (Hosomi et al., 1994; Ogawa et al., 1994; Kaplan et al., 1990; Ellis et al., 1990; Roth et al., 1992; Milarski et al., 1995). $p62^{rasGAP}$ is a common target of several protein-tyrosine kinases, including v-Abl, v-Src, v-Fps, v-Fms, and activated receptors for IGF-1, EGF, csf-1, as well as the insulin receptor. Recently, a subtrate for bcr-abl that binds to ras-Gap was purified and cloned (Carpino et al., 1997; Yamanashi & Baltimore, 1997). This protein, called $p62^{dok}$, reacts with monoclonal antibodies raised against $p62^{rasGAP}$, suggesting they are identical (Yamanashi & Baltimore, 1997). The $p62^{dok}$ contains a recognizable PH domain at its NH_2-terminus that is distantly similar to the PH domain in IRS-1, IRS-2, and Gab-1. $p62^{dok}$ may contain a PTB domain, but it contains little amino acid sequence similarity to IRS-1; however, two conserved arginine residues known to bind phosphotyrosine in the $IRS1^{PTB}$ domain appear to be correctly positioned (Eck et al., 1996). The COOH-terminus of $p62^{dok}$ contains multiple tyrosine phosphorylation sites in motifs that recognize various SH2 proteins, but none are expected to bind PI-3 kinase.

In summary, $pp60^{IRS3}$ may generally resemble IRS-1 and IRS-2, being composed of a PH domain, a PTB domain, and a short tail of tyrosine phosphorylation sites which engage and activate proteins with SH2 domains, such as the PI-3 kinase. The subcellular distribution of $pp60^{IRS3}$ and IRS-1 is distinctly different, which presumably enhances specific signaling pathways needed in adipocytes during insulin receptor signaling. The recent isolation of a cDNA encoding the 60 kDa adipocyte substrate, presumably $pp60^{IRS3}$, will provide important insight into the complex pathways regulating the insulin signal in adipocytes (Lavan et al., 1997).

ACKNOWLEDGMENT

We would like to thank Drs. Barbara Kahn and Karen Housenecht for helpful advice. We would also like to thank Jonathon Winnay for excellent technical assistance.

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BI9630974