The 60 kDa Insulin Receptor Substrate Functions Like an IRS Protein (pp60IRS3) in Adipose Cells

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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 pp60IRS3 to distinguish it from other tyrosine phosphorylated proteins of similar size. During insulin stimulation, p85 associated with pp60IRS3 more rapidly than with IRS-1 or IRS-2. In mice lacking IRS-1, p85 associated more strongly with pp60IRS3 than with IRS-2, suggesting that pp60IRS3 provides an alternate pathway in these cells. Synthetic peptides containing two phosphorylated YMPM motifs displace pp60IRS3 and IRS-1 from cp85 immune complexes, suggesting that pp60IRS3, like IRS-1, engages both SH2 domains in p85. Moreover, pp60IRS3 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 pp60IRS3 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., 1993; Kanai et al., 1993; Zhang-Sun et al., 1996). Here, we propose the name pp60 IRS3 because our results suggest it contains a PTB domain and binds 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 cp85 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.

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
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: \(\alpha\)IRS-1 was raised against a synthetic peptide containing residues 1221–1234 of rat IRS-1 (Sun et al., 1991); \(\alpha\)IRS-2 was made using a GST-fusion protein containing residues 619–746 of mouse IRS-2 (Sun et al., 1995). Antiphosphotyrosine antibody (\(\alpha\)PY) was prepared with phosphotyramine coupled to keyhole limpet hemocyanin (White & Backer, 1991); 4G10 was 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.

**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 (50 mg/kg). The rats were treated without or with insulin (3.5 mg/kg) and treated without or with 80 nM insulin for 5 min. The effect of 80 nM insulin for 5 min on 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.
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 (αp85NT), alleviating concerns that pp60IRS3 is one of the small p85 homologs (data not shown, also see Figure 5). Moreover, antibodies against p110α immunoprecipitated both pp60IRS3 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 pp60IRS3, 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, pp60IRS3 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 pp60IRS3 was detected by αp85 immunoprecipitation from adipose and testis of insulin-stimulated rats, whereas a small amount of p85 was observed in adipocytes. This interaction was attributed to a weak but stable complex between the insulin receptor and the IRS proteins, as previously discussed (Backer et al., 1993). The pp60IRS3 was detected by αp85 immunoprecipitation from adipose and testis of insulin-stimulated rats, whereas a small amount of p85 was observed in adipocytes. However, αIRS-1 (lanes c and d), or αIRS-2 (lanes e and f). The immunocomplexes were separated by SDS–PAGE and transferred to Immobilon P. The membrane was immunoblotted with αPY (panel A), stripped, and reblotted with αp85PAN (panel B).

During insulin stimulation, IRS-1, IRS-2, and pp60IRS3 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 p85 coprecipitated IRS-1 and pp60IRS3. However, αIRS-1 immunoprecipitated IRS-1 and p85, but pp60IRS3 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 pp60IRS3 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,
IRS-2 plays a surprisingly small role in these cells during insulin stimulation.

**pp60IRS** Associates with p85 in the Absence of IRS-1. To directly examine the relationship between IRS-1 and pp60IRS, 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 pp60IRS, 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 p85 immunoprecipitate (Figure 4, lanes i and j). However, in the absence of IRS-1, both IRS-2 and pp60IRS associated with p85 during insulin stimulation. Whereas the IRS-2 signal remained weak, the phosphorylation of pp60IRS was stronger than that found in the wild-type adipocytes (Figure 4). Thus, phosphorylation of IRS-2 and pp60IRS was independent of IRS-1, and this occurred more strongly without competition from IRS-1. However, without specific antibodies against pp60IRS, it is not possible to distinguish between these alternatives (Patti et al., 1995b).

**pp60IRS 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 pp60IRS associates rapidly and strongly with p85 during insulin stimulation, we hypothesized that both SH2 domains may bind tyrosine phosphorylation sites in pp60IRS as previously found for IRS-1 (Rordorf-Nikolic et al., 1995; Bäcker 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 pp60IRS or IRS-1 from p85 immune complexes. One peptide contained a single phosphorylated YMPM motif (Tyr960), and the other contained two phosphorylated YMPM motifs (Tyr960 and Tyr963). Immunocomplexes were prepared with p85NT to avoid interference by the αp85, which binds the nSH2 domain. Dissociation of pp60IRS and IRS-1 from αp85NT was more sensitive to the bisphosphopeptide (ED50 ≈ 0.05 nM) than the monophosphopeptide (ED50 > 1 μM); the nonphosphorylated peptides were unreactive (Figure 5, panels A and B). Moreover, the monophosphopeptide displayed similar sensitivity with αp85NT immunoprecipitates from testis (Figure 5C). These results suggest that pp60IRS, 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.

**pp60IRS Binds to Insulin Receptor Derived NPXY Peptides.** To engage the activated insulin receptor, pp60IRS 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 pp60IRS. 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 pp60IRS 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 NPXY motif of the insulin receptor; the association of IRS-1 and pp60IRS to these immobilized peptides was assessed by immunoblotting with αPY. As expected, no tyrosine phosphorylated protein bound to the nonphosphorylated NPXY peptide. By contrast, a 60 kDa protein which comigrated with pp60IRS in αp85 immunoprecipitates bound to the phosphorylated NPXY peptide; however, IRS-1 was poorly detected (Figure 6). Substitution of glutamic acid at the Y1 position with alanine reduced the binding of pp60IRS, 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 Y3 position inhibited the binding of IRS-1 and pp60IRS (Figure 6). Thus, at the functional level, pp60IRS appears to contain a PTB domain or associate with a protein that contains a PTB domain.
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 pp60IRS3 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, pp60IRS3 possesses several features which are similar to the IRS proteins. Displacement of p85 from pp60IRS3 is most sensitive to peptides containing two YMPM motifs, suggesting that pp60IRS3 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, pp60IRS3 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

FIGURE 5: pp60IRS3 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 αp85NT and without or with 0.001 µM, 0.01, 0.1, 1.0, 10.0, and 100.0 mM of the following peptides: (1) PyYMMP96, DDGPYPyMPMSPGV and (2) PyYMMP96, PyYMMP25, DDGPYPyPIMPMSPG(VGA)3, GNGDPPyPyPMSPPKS [G(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 pp60IRS3 (solid black diamonds) associated with the immune complexes were quantified with a phosphorimager. The open circle or open diamond show displacement of IRS-1 or pp60IRS3, respectively, by 10 mM nonphosphorylated PyYMMP96 peptide (panel A). Testis homogenates prepared from insulin-stimulated rats were incubated with αp85 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 pp60IRS3 to phosphorylated NPYX-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, GGYLASSNPY-LSASD; (2) NPYFP, GGYLASSNPY(P)-LSASD; (3) APEYFP, GGYLASSAPEY(P)-LSASD; (4) NPAYFP, GGYLASSNPAY(P)-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.

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, pp60IRS3 possesses several features which are similar to the IRS proteins. Displacement of p85 from pp60IRS3 is most sensitive to peptides containing two YMPM motifs, suggesting that pp60IRS3 contains at least two phosphotyrosine residues that bind both SH2 domains of p85. pp60IRS3 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, pp60IRS3 binds to peptides containing phosphorylated NPYX motifs, suggesting that it contains a phosphotyrosine binding (PTB) domain. Thus, pp60IRS3 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 NPYX motifs (Eck et al., 1996). pp60IRS3 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 pp60IRS3 and the native insulin receptor, although a PHP domain is expected by analogy to IRS-1 and IRS-2.

Our results suggest that pp60IRS3 may be the initial substrate of the activated insulin receptor in adipocytes. The increased affinity between pp60IRS3 and the native NPEY motif of the activated insulin receptor may mediate the relatively rapid phosphorylation of pp60IRS3 compared to IRS-1. As a result, pp60IRS3 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 pp60IRS3 from p85 immune complexes more sensitively than monophosphorylated peptides, suggesting that pp60IRS3 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, pp60IRS3 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 pathways(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 pp60IRS3 provides the major alternative pathway to PI-3 kinase in the absence of IRS-1. Although the levels of pp60IRS3 cannot be measured without specific antibodies during insulin stimulation of IRS1−/− mice, more tyrosine phosphorylated pp60IRS3 is immunoprecipitated with tPY and more associates with p85. Moreover, pp60IRS3 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 pp60IRS3 phosphorylation increases in IRS1−/− adipocytes because IRS-1 is not present to compete.

The rapid phosphorylation of pp60IRS3 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 pp60IRS3. Previous studies indicate that pp60IRS3 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 pp60IRS3 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 pp60IRS3 is elevated. Thus, IRS-1 is an essential element in the signaling pathway that cannot be entirely replaced with either IRS-2 or pp60IRS3. The relative contributions of IRS-1 and pp60IRS3, 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 pp60IRS3. By contrast, a 62 kDa substrate occurs in various cells that binds to the SH2 domain in p21ras-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). p62rasGAP is a common target of several protein-tyrosine kinases, including v-Ab1, v-Src, v-Fps, v-Fms, and activated receptors for IGF-1, EGF, csf-1, as well as the insulin receptor. Recently, a substrate for bcr-abl that binds to ras-Gap was purified and cloned (Carpino et al., 1997; Yamanashi & Baltimore, 1997). This protein, called p62dok, reacts with monoclonal antibodies raised against p62rasGAP, suggesting they are identical (Yamauchi & Baltimore, 1997). The p62dok contains a recognizable PH domain at its NH2-terminus that is distant similar to the PH domain in IRS-1, IRS-2, and Gab-1. p62dok 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 IRS1PTB domain appear to be correctly positioned (Eck et al., 1996). The COOH-terminus of p62dok contains multiple tyrosine phosphorylation sites in motifs that recognize various SH2 proteins, but none are expected to bind PI-3 kinase.

In summary, pp60IRS3 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 pp60IRS3 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 pp60IRS3, will provide important insight into the complex pathways regulating the insulin signal in adipocytes (Lavan et al., 1997).

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