The 60-kDa Phosphotyrosine Protein in Insulin-treated Adipocytes Is a New Member of the Insulin Receptor Substrate Family*

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Brian E. Lavant‡, William S. Lane§, and Gustav E. Lienhard

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 and the §Harvard Microchemistry Facility, Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

A 60-kDa protein that undergoes rapid tyrosine phosphorylation in response to insulin and then binds phosphatidylinositol 3-kinase has been previously described in adipocytes and hepatoma cells. We have isolated this protein, referred to as pp60, from rat adipocytes, obtained the sequences of tryptic peptides, and cloned its cDNA. The predicted amino acid sequence of pp60 reveals that it contains an N-terminal pleckstrin homology domain, followed by a phosphotyrosine binding domain, followed by a group of likely tyrosine phosphorylation sites, four of which are in the YXXM motif that binds to the SH2 domains of phosphatidylinositol 3-kinase. The overall architecture of pp60 is thus the same as that of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), and furthermore both the pleckstrin homology and phosphotyrosine binding domains are highly homologous (about 50% identical amino acids) to these domains in both IRS-1 and IRS-2. Thus, pp60 is a new member of the IRS family, which we have designated IRS-3.

The insulin receptor is a tyrosine kinase that is activated upon insulin binding. Signaling from this receptor proceeds primarily by its tyrosine phosphorylation of substrate proteins, which then act as docking proteins for one or more SH2 domains containing proteins. Docking of these proteins in turn activates specific signal transduction pathways. The substrate docking proteins that have been molecularly characterized to date are the closely related IRS-1 and IRS-2, and more recently the pleckstrin homology and phosphotyrosine binding domains are highly homologous (about 50% identical amino acids) to these domains in both IRS-1 and IRS-2. Thus, pp60 is a new member of the IRS family.

EXPERIMENTAL PROCEDURES

Preparation of Affinity Matrix—GST itself and the GST fusion protein with the N-terminal SH2 domain of the 85-kDa subunit of PI 3-kinase (GST-NSH2) were prepared as described (5). These were covalently attached via sulfhydryl groups on the GST (the SH2 domain itself has no sulfhydryl groups) to activated thiopropyl Sepharose 6B beads (Sigma). The beads with coupled GST (1.3 mg/ml) or GST-NSH2 (1.8 mg/ml) were placed in columns, washed with 10 volumes of 5 mM dithiorthiole in 120 mM Tris-HCl, 1 mM EDTA, pH 6.8, to cleave the remaining 2-pyridyl disulfide, and then washed with 30 volumes of 5 mM N-ethylmaleimide in this buffer to block the free sulfhydryl groups. Protein not covalently bound to the beads was released with 25 volumes of 4% SDS, 2 mM N-ethylmaleimide, 100 mM Tris-HCl, 1 mM EDTA, 10% glycerol, pH 6.8. Finally, the columns were washed extensively with 150 mM NaCl, 10 mM sodium phosphate, pH 7.4. The SDS treatment released only about 10% of protein, and subsequently the GST-NSH2 exhibited full activity in binding pp60.

Isolation of pp60 and Sequencing of Peptides—Rat adipocytes were prepared and treated with insulin as described (3). The cells were lysed in hot SDS buffer, the lysate was diluted with a buffer containing nonionic detergent, and particulate matter removed by centrifugation and filtration, exactly as described in Ref. 3, with the exception that the nonionic detergent was nonylethylene glycol dodecyl ether (Thesit™ from Boehringer Mannheim) rather than octylethylene glycol dodecyl ether. The cell extract (350 ml) from the adipocytes of 150 rats was passed at 0.14 ml/min through a 1.5-ml column of immobilized GST and then through a 0.2-ml column of immobilized GST-NSH2. Once the extract was applied, the GST column was disconnected, and a 0.2-ml portion of it was treated exactly as the GST-NSH2 column to serve as the control. The columns were washed with 20 ml of 1% Thesit in 20 mM Tris-HCl, 150 mM NaCl, 1 mM sodium vanadate, pH 7.4, with protease inhibitors (2 μg/ml aprotinin, 2 μl leupeptin, 0.2 mM pepastatin A) and then with 20 ml of 0.1% Thesit in the same buffer. The beads from each column (about 0.2 ml) were transferred to low protein-binding microfuge tubes, and a hole was pierced in the bottom of each using a 26 gauge needle. Adherent liquid was removed by centrifuging briefly with each tube inside a second tube. Bound proteins were then eluted from the beads in an SDS buffer (4% SDS, 1 mM EDTA, 1 mM sodium vanadate, 10% glycerol, 100 mM Tris-HCl, pH 6.8, with the protease inhibitors given above) by the same method. Beads were eluted successively with two 90-μl portions of SDS buffer, followed by two 180-μl portions. The eluates are referred to in order of elution as P (combined 90-μl eluates), P1 and P2 from the GST-NSH2 and similarly, G, G1, and G2 from the GST. To estimate the yield of pp60, samples containing the original extract, the depleted extract, and the eluate fractions were quantitatively immunoblotted for Tyr(P) as described (3). Approximately 90% of the purified pp60 was in fraction P, with most of the remainder in fraction P1.

Eluate fractions P and G were each separated on single lanes of a 5–12% acrylamide gradient gel. The pp60 in the lane with fraction P was detected by copper staining for protein (Bio-Rad); this area along with the corresponding area from the lane with G was excised. After carboxymethylation in a gel, the bands were subjected to tryptic digestion in a gel as described in Ref. 6 without the addition of 0.02% TWEEN. The resulting peptide mixture was separated by microbore high-performance liquid chromatography; HPLC, high performance liquid chromatography; IRS, insulin receptor substrate; PCR, polymerase chain reaction; PH, pleckstrin homology; PTB, phosphotyrosine binding; RACE, rapid amplification of cDNA ends; bp, base pair(s); nt, nucleotide(s).
performance liquid chromatography using a Zorbax C18 1.0 mm by 150-mm reverse-phase column on a Hewlett-Packard 1090 HPLC/1040 diode array detector. Optimum fractions were chosen based on differential UV absorbance at 205, 277, and 292 nm, and the sequences of eight peptides unique to the P fraction were determined by automated Edman degradation on an Applied Biosystems 477A or 477A sequencer. The average initial amino acid yield for the peptides sequenced was 820 ± 310 fmol. Strategies for peak selection, reverse-phase separation, and Edman microsequencing have been previously described (7). Complementary peptide sequence information was obtained on 10% of the digest mixture by collisionally induced dissociation using microcapillary electrospray ionization/tandem mass spectrometry on a Finnigan TSQ7000 triple quadrupole mass spectrometer (8).

**pp60 cDNA**—Total RNA was obtained from rat adipocytes using the Trizol reagent (Life Technologies), and mRNA was subsequently purified from it using the Fast-Track kit (Invitrogen). The adipocytes of 24 rats yielded approximately 4 μg of purified mRNA. An oligo(dT) primed cDNA library of the Marathon Ready® type was prepared for us from this mRNA by Clontech. Tryptic peptide g (see Fig. 1D) served as the basis for the design of a mixed sense oligonucleotide containing deoxyinosine (I) at positions of high degeneracy (5′-TTRYTTCIGGIC-CIVTTATAYGARTT-3′; where Y is T or C and R is A or G). 3′ RACE was performed with the Marathon Ready cDNA using this primer (20 μM) and the AP1 primer (2 μM) of the Marathon Ready kit, according to the manufacturer’s instructions. A major 700-bp product was obtained that was reamplified and then gel purified. After filling the 5′ and 3′ ends with Klenow DNA polymerase, the piece was digested with NotI (a site introduced during cDNA synthesis) and cloned into NotI/EcoRV digested pBluescript II (SK−) (Stratagene). The insert was sequenced (nt 1499–1699, see Fig. 2) and was found to encode tryptic peptide h.

The 5′ end of the cDNA was obtained by 5′ RACE with the Marathon-Ready cDNA and a combination of the AP1 primer and an antisense primer derived from the 3′ RACE product (nt 1565–1589). Two major PCR products of approximately 1600 and 1800 bp were generated in the initial amplification and gel purified as a mixture. Reamplification of the mixture with nested primers (AP2 of the Marathon-Ready kit and an upstream antisense primer (nt 1531–1556)) again generated a mixture of PCR products. Two major PCR products were sequenced from its 3′ end and found to be identical from nt 619 to 1530. Upstream of nt 619 the sequence was a mixture, indicating that the two PCR products diverged at this point. To obtain sequence upstream of nt 619, the mixture of PCR products was subcloned into pBluescript II (SK−) and parts of the inserts from some clones were sequenced. Primers based upon this sequence were then used to sequence directly the 5′ end of the cDNA by generating the mixture of the two PCR products. These gave a single sequence at the most 5′ end (nt 1–618) and a mixture of sequences downstream of nt 618; this indicated the presence of an intervening sequence of two PCR products. This mixture of PCR products was directly sequenced from its 3′ end and found to be identical from nt 619 to 1530. The major Tyr(P) proteins had mobilities corresponding to the N-terminal SH2 domain of the 85-kDa subunit of PI 3-kinase as described under “Experimental Procedures” and “Results and Discussion.” Samples of the fractions eluted with SDS from the GST-NSH2 column (P1 and P2) or from the control GST column (G1 and G2) were separated on a 5–12% polyacrylamide gradient gel, transferred to nitrocellulose, and then immunoblotted for Tyr(P) (lanes 1–4) or stained for protein with colloidal gold (lanes 5–8). Lanes 1–4 contained 0.5% of each SDS elute fraction, and lanes 5–8 contained the remainder. Molecular mass standard proteins were run on lanes 9–11 at several loads (μg of each shown). B, the amino acid sequences of eight tryptic peptides (designated a–h) from pp60 are given. Uppercase and lowercase one-letter abbreviations for the amino acids indicate assignments made with high and low confidence, respectively. x designates a position for which no assignment could be made.

**RESULTS AND DISCUSSION**

**Purification of pp60**—The method for the purification of pp60 was based on our previous finding that pp60 is efficiently adsorbed from extracts of insulin-stimulated adipocytes by the N-terminal SH2 domain of the 85-kDa subunit of PI 3-kinase as a GST fusion protein (3). An extract of insulin-stimulated adipocytes from 150 rats was passed sequentially through a column of immobilized GST alone and then through a column containing the GST-NSH2 fusion protein. After the adsorption step, the columns were separated, each was washed, and adsorbed proteins were eluted with SDS. Fig. 1A (lanes 1–4) shows the eluted Tyr(P) proteins as detected by anti-Tyr(P) immunoblotting. The major Tyr(P) proteins had mobilities corresponding to those expected for pp60 and IRS-1. Smaller amounts of Tyr(P) proteins at approximately 97 and 145 kDa were also present. The 97-kDa protein is most likely the β subunit of the insulin receptor, which is known to bind to the N-terminal SH2 domain of PI 3-kinase (9); the identity of the 145-kDa protein is unknown. There was specific binding to the GST-NSH2 column; no Tyr(P) proteins were present in the elute from the GST column (compare lane 1 with 3). Protein staining with colloidal gold showed that two major proteins were eluted specifically from the GST-NSH2 column (Fig. 1A, lanes 5–8); these migrated with the Tyr(P) forms of pp60 and IRS-1. From quantitative immunoblotting of the adipocyte lysate and the SDS eluate fractions of the column for Tyr(P) (data not shown), we determined that approximately 30% of the Tyr(P) form of pp60 was recovered in the purification. In addition, from this data and that in Fig. 1A, we estimate that approximately 500 ng (8 pmol) of pp60 were isolated from the adipocytes of 150 rats.

To obtain peptides from pp60, the bulk of the SDS eluate from the GST-NSH2 column (about 90%, with the remainder used for the analyses described above) was run in a single lane on a gradient gel, and the gel slice containing pp60 was treated with trypsin. Tryptic peptides were isolated by HPLC, and the sequences of eight peptides were determined (Fig. 1B). A search of the data base using the BLAST program revealed no significant matches with sequences in known proteins.

**cDNA Encoding pp60**—Initially a PCR product encoding the 3′ end of pp60 was generated in a 3′ RACE reaction using a degenerate primer based upon the sequence of peptide g and a Marathon-Ready cDNA library from rat adipocytes. Subsequently, the 5′ end of the pp60 cDNA was obtained by a 5′ A New Member of the IRS Family

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**Fig. 1.** Purification of pp60 from insulin-stimulated rat adipocytes and sequences of tryptic peptides. A, pp60 was purified as described under “Experimental Procedures” and “Results and Discussion.” Samples of the fractions eluted with SDS from the GST-NSH2 column (P1 and P2) or from the control GST column (G1 and G2) were separated on a 5–12% polyacrylamide gradient gel, transferred to nitrocellulose, and then immunoblotted for Tyr(P) (lanes 1–4) or stained for protein with colloidal gold (lanes 5–8). Lanes 1–4 contained 0.5% of each SDS elute fraction, and lanes 5–8 contained the remainder. Molecular mass standard proteins were run on lanes 9–11 at several loads (μg of each shown). B, the amino acid sequences of eight tryptic peptides (designated a–h) from pp60 are given. Uppercase and lowercase one-letter abbreviations for the amino acids indicate assignments made with high and low confidence, respectively. x designates a position for which no assignment could be made.

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**TABLE**

| Sequence     | Description                                                                 |
|--------------|-----------------------------------------------------------------------------|
| a AFGLSSG5YR | Amino acid sequence from peptide a                                           |
| b LSL15      | Amino acid sequence from peptide b                                           |
| c XEALFLGDs  | Amino acid sequence from peptide c                                           |
| d xxxDVPAPYEIPaLAlAA | Amino acid sequence from peptide d                                         |
| e AQQQYVnpxer | Amino acid sequence from peptide e                                          |
| f MGSA5YR    | Amino acid sequence from peptide f                                          |
| g FLPGFLYTEFK| Amino acid sequence from peptide g                                          |
| h WRPWMAQ9pr | Amino acid sequence from peptide h                                          |
form estimated by SDS gel electrophoresis. The explanation for this difference most likely is an aberrantly low mobility on electrophoresis, which is frequently the case for phosphorylated proteins.

Several types of evidence establish that the predicted protein is the 60-kDa protein that undergoes tyrosine phosphorylation in response to insulin. First, as noted above, the isolation of the cloned protein was based on a known binding property of pp60. Second, as described below, the structure of the protein is that expected for a substrate of the insulin receptor; the predicted sequence contains, as expected, several potential PI 3-kinase binding motifs. Third, we have prepared affinity purified rabbit antibodies against the C-terminal peptide (14 amino acids) of the predicted protein and shown that these react with pp60. The tyrosine phosphorylated form of pp60 was isolated from a lysate of insulin-treated adipocytes by adsorption with GST-NSH2 or with antibodies against Tyr(P), as described in Ref. 3. Immunoblotting of each adsorbate with the antibodies against the C terminus detected only a 60-kDa protein. When this experiment was performed with a lysate of basal adipocytes, no protein was detected (data not shown).

Domains and Tyr(P) Motifs in pp60—The amino acid sequence of pp60 was compared with the protein data base using the BLAST P program and also was examined for potential sites of tyrosine phosphorylation. This revealed that pp60 contains in the following order from its N terminus: a PH domain that is highly homologous to the PH domain in IRS-1 and IRS-2, a PTB domain that is highly homologous to the PTB domain in IRS-1 and IRS-2, and, distributed over the C-terminal third of the protein, a number of likely tyrosine phosphorylation sites in motifs that can bind SH2 domain-containing proteins (Fig. 3 and see below). The architecture of pp60 is strikingly similar to that of IRS-1 and IRS-2. Although the latter two proteins are larger (1231 and 1321 amino acids, respectively), each contains an N-terminal PH domain, followed by a PTB domain, followed by a group of tyrosine phosphorylation sites at which a variety of SH2-domain containing proteins dock (2, 11). Thus, pp60 is a new member of the IRS family, and henceforth we refer to it as IRS-3.

The PH domain of IRS-3 consists of 100 amino acids (residues 32–131) and exhibits 50 and 45% identity with the PH domains of IRS-1 and IRS-2, respectively (Fig. 3 and see below). The architecture of pp60 is strikingly similar to that of IRS-1 and IRS-2. Although the latter two proteins are larger (1231 and 1321 amino acids, respectively), each contains an N-terminal PH domain, followed by a PTB domain. Although the PH domains generally show a great deal of variation (12) and suggest that there is a common function for the PH domain in the three IRSs. In this regard, the PH domain of IRS-1 is necessary for its efficient in vivo tyrosine phosphorylation by the insulin receptor, although it does not appear to interact directly with the receptor (13–15).

The PTB domain of IRS-3 consists of 115 amino acids (residues 160–274) and exhibits 48 and 53% identity with the PTB domains of IRS-1 and IRS-2, respectively (Fig. 3B). This high degree of homology is notable, because the sequences of PH domains generally show a great deal of variation (12) and suggests that there is a common function for the PH domain in the three IRSs. In this regard, the PH domain of IRS-1 is necessary for its efficient in vivo tyrosine phosphorylation by the insulin receptor, although it does not appear to interact directly with the receptor (13–15).

The PTB domain in IRS-3 consists of 115 amino acids (residues 160–274) and exhibits 48 and 53% identity with this domain in IRS-1 and IRS-2, respectively (Fig. 3B). This high degree of homology is notable, because the sequences of PH domains generally show a great deal of variation (12) and suggests that there is a common function for the PH domain in the three IRSs. In this regard, the PH domain of IRS-1 is necessary for its efficient in vivo tyrosine phosphorylation by the insulin receptor, although it does not appear to interact directly with the receptor (13–15).

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domain complexed with a 9-residue Tyr(P) peptide similar to the sequence surrounding Tyr960 in the insulin receptor has been determined (17). Remarkably, 14 of the 19 amino acids in IRS-1 that interact with the bound peptide (Fig. 6 of Ref. 17) are identical in IRS-3, including the two arginines whose guanidinium groups contact the phosphate of the Tyr(P) residue; the remaining five differences are conservative substitutions. This suggests that IRS-3 will also be found to bind via its PTB domain to the activated insulin receptor by association with the segment containing Tyr(P)960.

Outside of the PH and PTB domains there are no regions of extended homology between IRS-3 and IRS-1/2. Although IRS-1 and IRS-2 contain a region just downstream of the PTB domain referred to as the SAIN domain, which participates in the interaction with the insulin receptor, and IRS-2 also contains a domain even further downstream (residues 591–733) that also interacts with the receptor (15, 18–20), neither of these are present in IRS-3.

Several of the potential tyrosine phosphorylation sites in IRS-3 lie within motifs that conform to the established recognition specificities of SH2 domains (21, 22). Most notably, there are four YXXM motifs (Tyr343, Tyr352, Tyr362, and Tyr392); this is the motif to which each SH2 domain of the PI 3-kinase 85-kDa subunit binds. Given the strong association of the Tyr(P) form of IRS-3 with both SH2 domains, one or more of these sites is almost certainly phosphorylated in vivo. The occurrence of a linear array of four YXXM motifs suggests that tandem motifs are phosphorylated and then bind simultaneously to the two SH2 domains on the 85-kDa subunit; such a bidentate interaction has been shown to result in very high affinity binding (23). Among the other potential tyrosine phosphorylation sites of IRS-3, there is one (Tyr321) that would be expected to bind to the SH2 domain of Grb2, the adaptor for SOS (the GDP-releasing factor for Ras), and another (Tyr466) that could bind to N-terminal SH2 domain of either the Tyr(P) phosphatase SHP2 or phospholipase Cγ. It remains to be determined whether these or other SH2 domain proteins are associated with the Tyr(P) form of IRS-3. Because the Tyr(P) forms of IRS-1 and IRS-2 function as docking/effector proteins for PI 3-kinase, Grb2, and SHP2 (2), the similarity of IRS-3

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**Fig. 3. Structure of IRS-3 (pp60) and homology with IRS-1/2.** A, the organization of IRS-3, showing the PH and PTB domains and the potential sites of tyrosine phosphorylation. The latter are those tyrosine residues that have one or more acidic residues within the immediate five upstream amino acids (24). B, the PH and PTB domains from rat IRS-1, rat IRS-2, and rat IRS-3 were aligned with the PILEUP program (GCG Wisconsin). Identical amino acid residues at a position are on a black background, and conserved amino acid residues are on a gray background. The percentage values at the end of each sequence are the percentage of identical amino acids in a pairwise comparison of each to the IRS-3 sequence. The PH and PTB domains of IRS-1 and IRS-2 are 69 and 75% identical to each other, respectively (11). The two conserved Arg residues of the IRS-1 PTB domain that contact the Tyr(P) of the bound peptide similar in sequence to that around Tyr960 of the insulin receptor are marked with an asterisk.
with IRS-1/2 extends to at least one and probably several interactions with SH2 domain proteins.

**Implications**—The rapid tyrosine phosphorylation of IRS-3 in response to insulin and the identification of it as a member of the IRS family strongly indicates that it is a substrate for the insulin receptor. However, this remains to be demonstrated. Besides the insulin receptor, a variety of other receptors, including the related receptor for insulin-like growth factor I, signal through tyrosine phosphorylation of IRS-1/2 (1, 2). Thus, IRS-3 may also participate in signal transduction from other receptors. A major challenge now is to elucidate the role that each IRS plays in insulin action.

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