The Insulin-elicted 60-kDa Phosphotyrosine Protein in Rat Adipocytes Is Associated with Phosphatidylinositol 3-Kinase*

Brian E. Lavan and Gustav E. Lienhard†

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755-3844

(Received for publication, October 26, 1992)

Insulin stimulates the tyrosine phosphorylation of a 60-kDa protein (pp60) in rat adipocytes. After insulin treatment of these cells, pp60, as well as the 160-kDa insulin receptor substrate-1 (IRS-1), were found to be associated with the enzyme phosphatidylinositol 3-kinase (PtdIns-3-kinase) in separate complexes. By contrast, pp60 was not detected in insulin-treated mouse 3T3-L1 adipocytes, which contain abundant IRS-1. PtdIns-3-kinase complex. The pp60-PtdIns-3-kinase complex was located in both the soluble and membrane fractions of the rat adipocytes. Fusion proteins containing the isolated src homology 2 domains from the 85-kDa subunit of PtdIns-3-kinase bound to pp60 in lysates of insulin-treated rat adipocytes. This finding indicates that the most likely mode of association of pp60 with PtdIns-3-kinase is through binding of phosphotyrosyl residues in pp60 to these domains. By immunoaffinity chromatography on a monoclonal antibody against phosphotyrosine, pp60 was purified in high percentage yield from insulin-stimulated rat adipocytes, but the low amount of the protein obtained (about 3 ng from the adipocytes of one rat) precluded sequence analysis.

The cellular effects of insulin are mediated through the insulin receptor, a heterotetrameric protein consisting of two α and two β subunits located in the plasma membrane. The α subunits are located entirely extracellularly and constitute the binding site for insulin, while the β subunits are transmembrane. Upon insulin binding, a protein tyrosine kinase intrinsic to the β subunit is activated, leading to phosphorylation of this subunit on tyrosine residues. Initiation of many, if not all, the effects of insulin require activation of the insulin receptor as a tyrosine kinase (1-3). In addition to the insulin receptor, several other phosphotyrosyl polypeptides appear after insulin challenge of various cell types (4).

The cellular effects of insulin are mediated through the insulin receptor, a heterotetrameric protein consisting of two α and two β subunits located in the plasma membrane. The α subunits are located entirely extracellularly and constitute the binding site for insulin, while the β subunits are transmembrane. Upon insulin binding, a protein tyrosine kinase intrinsic to the β subunit is activated, leading to phosphorylation of this subunit on tyrosine residues. Initiation of many, if not all, the effects of insulin require activation of the insulin receptor as a tyrosine kinase (1-3). In addition to the insulin receptor, several other phosphotyrosyl polypeptides appear after insulin challenge of various cell types (4).

Tyrosine phosphorylation of such proteins is likely to be part of the pathway for signal transmission from the insulin receptor to insulin-sensitive effector systems. Identification and characterization of insulin-elicted Tyr(P) proteins is therefore important in understanding the molecular basis of insulin signaling.

In this regard, we and others have recently purified a widely distributed, insulin-elicted Tyr(P) polypeptide of 160-185 kDa, now referred to as IRS-1 (insulin receptor substrate-1) (5, 6). Highly related cDNA clones encoding this protein in rat liver (7), human hepatocellular carcinoma (8), and mouse 3T3-L1 adipocytes, have been isolated. The full role played by IRS-1 in insulin signaling is unknown, but one function appears to be to couple the insulin receptor to activation of the enzyme PtdIns-3-kinase. The Tyr(P) form of IRS-1, generated by phosphorylation by the activated insulin receptor, associates with PtdIns-3-kinase (7, 9, 10), and this association stimulates the activity of the enzyme (10). PtdIns-3-kinase consists of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit which contains two src homology-2 (SH2) domains (11-15). SH2 domains are domains of about 100 amino acids that bind strongly to Tyr(P) in specific peptide sequences in signaling proteins (reviewed in Ref. 16). Two types of evidence indicate that the association of IRS-1 with PtdIns-3-kinase involves binding to the SH2 domain of the latter. First, we have shown that the isolated SH2 domains of PtdIns-3-kinase bind the Tyr(P) form of IRS-1 (9). Second, IRS-1 has nine potential tyrosine phosphorylation sites that lie in the sequence YXXM, a consensus sequence for binding to PtdIns-3-kinase (7, 17).

In the course of investigating the association of IRS-1 with PtdIns-3-kinase (9), we examined whether other insulin-elicted Tyr(P) proteins have the same property. Insulin treatment of rat adipocytes elicits the rapid tyrosine phosphorylation of a protein of approximately 60 kDa (hereafter referred to as pp60), as well as that of IRS-1 and the β subunit of the insulin receptor (18-23). Estimated from the intensity of its signal on Tyr(P) immunoblots, pp60 is a major insulin-elicted Tyr(P) protein, comparable to IRS-1 and the insulin receptor β subunit. To investigate associations of pp60 with PtdIns-3-kinase we have used antibodies to the 85-kDa catalytic subunit of PtdIns-3-kinase and evidence is presented that the association occurs via the SH2 domains of the kinase. In addition, we describe an efficient procedure for the purification of pp60 from insulin-stimulated rat adipocytes.

**EXPERIMENTAL PROCEDURES**

*Isolation of Rat Adipocytes and Preparation of Extracts.—*Isolated adipocytes were prepared from epididymal fat pads of fed Sprague-Dawley rats (150-175 g) by collagenase digestion in Krebs-Ringer bicarbonate buffer supplemented with 30 mm Heps, 1% bovine serum albumin, 3 mm glucose, and 200 mm adenosine, at final pH 7.4, as

---

* This work was supported in part by a postdoctoral fellowship from the Juvenile Diabetes Foundation International (to B. E. L.) and National Institutes of Health Grant DK 42816. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 603-650-1627; Fax: 603-650-1128.

1 The abbreviations used are: Tyr(P), phosphotyrosine; C12E8, octaethylene glycol dodecyl ether; EP475, 1-trans-epoxysuccinyl-L-leucylamido-3-methyl-butanine; IRS-1, insulin receptor substrate-1; PMMA, phospholipid-amidyl fluoride; PtdIns, phosphatidylinositol; SDS, sodium dodecyl sulfate; SH2, src homology 2.

2 S. R. Keller, R. Aebersold, C. W. Garner, and G. E. Lienhard, submitted for publication.

5921
detailed in Honnor et al. (24). Adipocytes were washed in the above buffer, resuspended to a cell density of 1.2 × 10^6 cells/ml, and stimulated with 100 nM insulin for 5 min or left in the basal state. Cells were subsequently washed three times in albumin-free buffer; 100 nM insulin was included in the wash buffer for the insulin-stimulated state, and 1 mg/ml bovine serum albumin (BSA) for the basal state. To prepare the soluble, membrane, and cytoskeleton fractions, adipocytes from treated cultures (25; 10 cm plate) containing 3 × 10^6 cells were homogenized at room temperature in 7.5 ml of Buffer A (50 mM Heps, 150 mM NaCl, 1 mM EGTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10% glycerol, 1.5 mM MgCl₂, 1 mM sodium orthovanadate, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 1 mM PMSF, at pH 7.0) using 10 up-and-down strokes in a glass homogenizer with a Teflon pestle driven at 1250 rpm. The extract was subsequently centrifuged at 116,000 × g, for 1 h at 4 °C. The soluble fraction (infranatant) was retained, and the fat cake discarded. The pellet was resuspended in 7.5 ml of 1% Triton X-100 in Buffer A and recentrifuged as above to yield the solubilized membrane preparation. The Triton X-100-insoluble material was solubilized in 7.5 ml of SDS sample buffer to give the cytoskeleton fraction.

For immunoprecipitation experiments, nondenatured and denatured lysates were prepared from the adipocytes isolated from eight cultures as follows. Nondenaturing detergent solubilization was accomplished by mixing 17 ml of 1% Triton X-100 in Buffer A directly with the cells at room temperature. For denatured extracts, the adipocytes were lysed by vigorous mixing with 1.3 ml of 4% SDS in 100 mM Hepes, 300 mM NaCl, 80 μM leupeptin, 80 μg/ml aprotonin, 8 mM PMSF, 80 μM sodium orthovanadate, 0.5 mM leupeptin, 0.5 mM aprotinin, 0.5 mM leupeptin, 5 μM leupeptin, 5 μM aprotinin, 5 mM pepstatin A, 5 μM pepstatin A, and 5 mM leupeptin, 5 mM leupeptin, and recentrifuged to prepare a solubilized membrane extract. The Triton X-100-insoluble material was solubilized in 7.5 ml of SDS sample buffer to give the cytoskeleton fraction.

Immunoprecipitations and Immunoblotting—Immunoprecipitations of IRS-1, p85, and Tyr(P) proteins from the extracts of rat adipocytes were performed as follows. The extracts were incubated with primary antibody for 30 min (soluble and membrane fractions) or 2 h (nondenatured and denatured lysates) and the immune complexes collected by mixing for 1 h (soluble and membrane fractions) or 2 h (nondenatured and denatured lysates) with 20 μl of protein A-Sepharose. The immunoprecipitates were washed three times with the solubilization buffer. The immunoprecipitates of these proteins from the lysates of 3T3-L1 adipocytes were performed in a similar way. The antibodies used are given in the figure legends. These amounts were sufficient to adsorb at least 50% of the IRS-1, 70% of the p85, and 90% of the Tyr(P) proteins, as assessed by immunoblotting of the initial lysate and the lysate after immunoadsorption (data not shown). Immune complexes were dissociated by adding 60 μl of SDS sample buffer and heating at 65 °C for 10 min. After removal of the beads, the SDS sample was heated at 100 °C for 3 min. The SDS sample buffer consisted of 4% SDS, 20 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 100 mM Tris-HCL, at pH 6.8 with protease inhibitors (10 μM EP475, 10 μM leupeptin, 10 μg/ml aprotonin, 1 μg/ml pepstatin A, 1 mM PMSF) and phosphatase inhibitors (10 μM sodium orthovanadate, 10 mM phenyl phosphate).

Samples were separated by SDS-gel electrophoresis on small 10% acrylamide slab gels and transferred electrophoretically to Immobilon-P (Millipore, Bedford, MA) at 400 mA for 3 h. The transfer buffer consisted of 25 mM Tris, 190 mM glycine, 20% methanol, 0.005% SDS. Proteins were immunoblotted as follows: Tyr(P) proteins, with the affinity-purified rabbit anti-Tyr(P) as detailed in Lavan et al. (9); IRS-1, with the affinity purified rabbit antibodies against the carboxyl-terminal peptide, at 4 μg/ml; p85, with the anti-p85 antiserum at a dilution of 1:500. Tyr(P) immunoblots were blocked with 30% glycerol/7% bovine serum albumin. Tyrosine phosphorylated Tyr(P) antibodies were used as a standard. The amounts of the antibodies used are given in the figure legends. These amounts were sufficient to detect at least 50% of the IRS-1, 70% of the p85, and 90% of the Tyr(P) proteins, as assessed by immunoblotting of the initial lysate and the lysate after immunoadsorption (data not shown). Immune complexes were dissociated by adding 60 μl of SDS sample buffer and heating at 65 °C for 10 min. After removal of the beads, the SDS sample was heated at 100 °C for 3 min. The SDS sample buffer consisted of 4% SDS, 20 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 100 mM Tris-HCL, at pH 6.8 with protease inhibitors (10 μM EP475, 10 μM leupeptin, 10 μg/ml aprotonin, 1 μg/ml pepstatin A, 1 mM PMSF) and phosphatase inhibitors (10 μM sodium orthovanadate, 10 mM phenyl phosphate).

Cell Culture and Preparation of Extracts from 3T3-L1 Adipocytes—3T3-L1 adipocytes were carried as fibroblasts and differentiated into adipocytes as previously described (25). 10-cm plates of adipocytes at 10–12 days post-differentiation were incubated for 2 h in serum-free medium and treated with 300 nM insulin for 3 min or left in the basal state. To prepare soluble, membrane, and cytoskeleton fractions, cells from a plate (about 10^6 cells with 5 mg of total protein) were scraped at room temperature into 2 ml of Buffer B (20 mM Tris-HCL, 140 mM NaCl, 30 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotonin, 1 μg/ml leupeptin, 10 μg/ml PMSF, at pH 7.5) and homogenized in a glass homogenizer with a Teflon pestle by 25 hand-driven up-and-down strokes at room temperature. The homogenate was centrifuged at 105,000 × g for 1 h at 4 °C, and the infranatant removed. After discarding the fat cake, the membrane pellet was resuspended in 2 ml of 1.5% Cl₂E₈ in Buffer B and recentrifuged to prepare a solubilized membrane extract. The Cl₂E₈-insoluble material was solubilized in 0.5 ml of SDS sample buffer and used as the cytoskeleton fraction.

Whole cell lysates were prepared under nondenaturing or denaturing conditions for use in immunoprecipitation experiments as follows. For lysis under nondenaturing conditions, the cells on one 10-cm plate were scraped at room temperature into 2 ml of 1.5% Cl₂E₈ in Buffer B and recentrifuged to prepare a solubilized membrane extract. The Cl₂E₈-insoluble material was solubilized in 0.5 ml of SDS sample buffer and used as the cytoskeleton fraction.
Association of pp60 with Phosphatidylinositol 3-Kinase

RESULTS

Insulin-elicited Tyr(P) Proteins in Rat and 3T3-L1 Adipocytes—The distribution of insulin-elicited Tyr(P) proteins between soluble, membrane, and cytoskeletal fractions was examined in both rat and 3T3-L1 adipocytes by immunoblotting of these fractions from basal and insulin-treated cells with antibodies against Tyr(P) (Fig. 1). Insulin-elicited Tyr(P) proteins of 160 and 95 kDa were observed in both cell types, the former being largely soluble and the latter largely membrane bound. In contrast, a 60-kDa Tyr(P) protein, found in both the soluble and membrane fractions, was observed in rat adipocytes but not in 3T3-L1 adipocytes (compare Fig. 1A, lanes 2 and 4, with Fig. 1B, lanes 2 and 4). Also, two soluble Tyr(P) proteins of about 42 and 40 kDa were detected in insulin-treated 3T3-L1 adipocytes but not in rat adipocytes (compare lane 2 of Fig. 1B with that of Fig. 1A). The identities of the 160-, 95-, 42-, and 40-kDa Tyr(P) polypeptides are IRS-1 (see below and Refs. 5–7), the β subunit of the insulin receptor (9, 18), and most probably, the extracellular signal regulated protein kinases 1 and 2 (28), respectively. The identity of the 60-kDa protein (pp60) which has been found in insulin-treated rat adipocytes in other studies (see introduction), is unknown. Low levels of the insulin receptor β subunit were detected in the cytoskeletal fraction of both rat and 3T3-L1 adipocytes (Fig. 1, A and B, lanes 5 and 6).

Association of Insulin-elicited Tyr(P) Polypeptides with PtdIns-3-kinase in Rat Adipocytes—In order to determine whether PtdIns-3-kinase was associated with any of these Tyr(P) proteins, the approach of immunoadsorption and immunoblotting was adopted. Lysates of basal and insulin-treated cells were immunoadsorbed with antibodies against IRS-1, the 85-kDa subunit of PtdIns-3-kinase (p85), and Tyr(P), and the immunoadsorbates were immunoblotted with all three antibodies. The lysates were prepared under both nondenaturing conditions (solubilization in the nonionic detergent, Triton X-100) and denaturing conditions (solubilization in hot SDS/dithiothreitol, followed by N-ethylmaleimide and an excess of Triton X-100), in order to maintain or disrupt protein-protein associations, respectively. This method allowed us to assess whether a precipitating polypeptide was present in the immunoprecipitate as a result of association with the antigen protein or simply as a consequence of cross-reactivity with the precipitating antibodies. Since protein-protein interactions should be disrupted in extracts prepared under denaturing conditions, the amount of a polypeptide precipitated as a consequence of association should be reduced under these conditions.

pp60, detected by anti-Tyr(P) immunoblotting, was highly enriched in the PtdIns-3-kinase immunoprecipitate from the nondenatured lysate of insulin-treated adipocytes (Fig. 2A, compare lane 2 with 8). Co-immunoprecipitation of the pp60 with PtdIns-3-kinase was completely abolished when the lysate was prepared under denaturing conditions (Fig. 2A, compare lane 18 with 8). In contrast, the ability of anti-p85 to precipitate p85 was found not to differ between the nondenatured and denatured lysates, since similar levels of PtdIns-3-kinase were recovered from each (Fig. 2B, lanes 7 and 8 versus 17 and 18). These observations indicate that pp60 co-immunoprecipitated with PtdIns-3-kinase as a result of association, rather than as a result of cross-reaction of anti-p85 with pp60. It was not possible to assess whether pp60 was also associated with PtdIns-3-kinase under basal conditions because there are no antibodies against the pp60 protein and because it contained little or no Tyr(P) prior to insulin stimulation (Fig. 1A, lanes 1 and 3, and Fig. 2A, lane 9).

As expected (see the introduction) insulin treatment of rat adipocytes promoted the association of PtdIns-3-kinase with IRS-1. Under basal conditions the two were associated to a small extent and the extent of this association was markedly
increased with insulin treatment (Fig. 2A, lanes 7 and 8; Fig. 2B, lanes 5 and 6; and Fig. 2C, lanes 7 and 8). The increased association paralleled the increased tyrosine phosphorylation of IRS-1 (Fig. 2A, lanes 5 and 6). This association was substantially reduced in lysates prepared under denaturing conditions (Fig. 2A, lane 18 versus 8; Fig. 2B, lane 16 versus 6; and Fig. 2C, lane 18 versus 8). The observation that some slight association of the Tyr(P) form of IRS-1 with p85 occurred after SDS denaturation at 100 °C (Fig. 2A, lane 18) may be explained by partial renaturation of the SH2 domains of p85.

If both pp60 and IRS-1 were associated in a ternary complex with PtdIns-3-kinase, then some pp60 would be expected in the anti-IRS-1 immunoprecipitates. The absence of any association paralleled the increased tyrosine phosphorylation of IRS-1 and Fig. 3. Association between pp60 and PtdIns-3-kinase occurred in both the soluble and membrane fractions of 3T3-L1 adipocytes (Fig. 3A, lanes 8 and 18). The association between IRS-1 and PtdIns-3-kinase occurred almost entirely in the soluble fraction (Fig. 3A, lane 8 versus 18; Fig. 3B, lane 6 versus 16; Fig. 3C, lane 8 versus 18). This finding is consistent with PtdIns-3-kinase and IRS-1 being mainly cytosolic proteins in rat adipocytes (Fig. 3B, lanes 1 and 2 versus 11 and 12; Fig. 3C, lanes 1 and 2 versus 11 and 12). Interestingly, insulin caused some translocation of PtdIns-3-kinase to the membrane fraction (Fig. 3B, lane 11 versus 12 and 17 versus 18). The extent of translocation was small (Fig. 3B, compare lane 12 with 2 and lane 18 with 8), but it was also observed in a replicate experiment.

Association of Insulin-elicited Tyr(P) Polypeptides with PtdIns-3-kinase in 3T3-L1 Adipocytes—In order to compare further the rat and 3T3-L1 adipocytes, immunoprecipitation and immunoblotting with antibodies against IRS-1, p85, and Tyr(P) were also carried out with nondenatured and denatured lysates of 3T3-L1 adipocytes (Fig. 4). With the nondenatured lysate, anti-p85 immunoprecipitates contained the Tyr(P) form of IRS-1 but not of the insulin receptor β subunit (Fig. 4A, lane 8). The band in Fig. 4A, lane 6, with an electrophoretic mobility slightly less than that of the insulin receptor β subunit is probably an antibody-derived band, since it was present in the basal immunoprecipitate (Fig. 4A, lane 2).
Association of pp60 with Phosphatidylinositol 3-Kinase

Fig. 3. Association of soluble and membrane-bound pp60 with PtdIns-3-kinase in rat adipocytes. Soluble and membrane fractions were prepared from basal (−) and insulin (+) treated rat adipocytes as described under “Experimental Procedures.” Aliquots (1.5 ml) of the fractions at 1.3 mg/ml (SOLUBLE) or 0.9 mg/ml (MEMBRANE) were immunoprecipitated as detailed in the legend to Fig. 2. Samples of the immunoprecipitates and the original fraction were immunoblotted for Tyr(P) (A), p85 (B), and IRS-1 (C). Lanes with lysate samples contained 1.2% of the total fraction prepared from the adipocytes of one rat. Lanes with immunoprecipitates are derived from 37% of the fraction from one rat. On this particular blot the signals from the Tyr(P) forms of IRS-1 and pp60 in the lysate sample (lane 2) were relatively weak. The autoradiograms presented are representative ones from an experiment performed twice.

Fig. 4. Association of IRS-1 with PtdIns-3-kinase in 3T3-L1 adipocytes. Lysates of basal (−) and insulin (+) treated 3T3-L1 adipocytes were prepared under non-denaturing (C12E8) and denaturing (SDS/C12E8) conditions, as described under “Experimental Procedures.” 1.2-ml aliquots of 1 mg/ml cell lysates were immunoprecipitated, as outlined in the legend to Fig. 2, with the exception that 10 µl of anti-IRS-1 (peptide 3) antiserum was used to immunoprecipitate IRS-1 in place of anti-IRS-1 (COOH-terminal). Samples of the immunoprecipitates and the original lysates were immunoblotted for Tyr(P) (A), p85 (B), and IRS-1 (C). Lanes with lysate contain samples equivalent to 0.2% of the cells from a 10-cm plate. Lanes with immunoprecipitates contain samples derived from 5% of the cells on a 10-cm plate. The autoradiograms presented are representative ones of an experiment performed three times.

7) and the control immunoprecipitates (Fig. 4A, lanes 3 and 4). As was the case with the lysates of rat adipocytes, the association of PtdIns-3-kinase with the Tyr(P) form of IRS-1 was drastically reduced in lysates prepared under denaturing conditions (Fig. 4A, lane 18 versus 8). Association of IRS-1 with p85 occurred to some extent in the basal state, since the anti-IRS-1 immunoprecipitate from basal cells contained some p85 (Fig. 4B, lane 5); the extent of association was significantly increased as the result of the increased tyrosine phosphorylation of IRS-1 in response to insulin (Fig. 4B, lane 6). In the complimentary immunoprecipitation, IRS-1 was barely detectable in the anti-p85 immunoprecipitate from basal cells, while a larger amount was present in this immunoprecipitate after insulin stimulation (Fig. 4C, lanes 7 and
8). These associations were absent in extracts prepared under denaturing conditions (Fig. 4B, lanes 15 and 16 versus 5 and 6; Fig. 4C, lanes 17 and 18 versus 7 and 8). This finding again indicates that co-immunoprecipitation is due to association rather than to cross-reactivity of the antisera.

As was the case in the rat adipocytes, PtdIns-3-kinase was present in the anti-Tyr(P) immunoprecipitate after insulin treatment, but this association was abolished in denatured extracts (Fig. 4B, lane 10 versus 20). There was no detectable insulin-elicted Tyr(P) protein of 85 kDa either in total cell lysates (Fig. 4A, lanes 2 and 12) or in the anti-p85 or the anti-Tyr(P) immunoprecipitates (Fig. 4A, lanes 8, 10, 18, and 20). Thus, insulin did not cause the tyrosine phosphorylation of p85 in 3T3-L1 adipocytes.

It is important to note that in sharp contrast to the results with rat adipocytes, pp60 was not detected in either the anti-p85 or the anti-Tyr(P) immunoprecipitates of 3T3-L1 adipocytes (compare Fig. 4A, lanes 8, 10, and 20, with Fig. 2A, lanes 8, 10, and 20). Since immunoprecipitation followed by immunoblotting is a procedure that concentrates pp60 and therefore markedly enhances its signal (Fig. 2A, compare the pp60 signals in lanes 2 and 12 with those in lanes 8, 10, and 20), the absence of pp60 in these immunoprecipitates is further evidence that the amount of this protein is very low in 3T3-L1 adipocytes relative to rat adipocytes.

Association of pp60 with the SH2 Domains of PtdIns-3-kinase—In order to assess whether the association of pp60 with PtdIns-3-kinase could be mimicked by the SH2 domains of its 85-kDa subunit alone, we employed glutathione S-transferase fusion proteins containing the amino- and carboxyl-terminal SH2 domains of p85. Insulin-stimulated lysates of rat adipocytes were prepared under denaturing conditions, in order to dissociate endogenously associated PtdIns-3-kinase, and were then adsorbed with the glutathione S-transferase fusion proteins bound to glutathione-Sepharose. The original lysate, depleted lysate, and adsorbates were subsequently blotted for Tyr(P) (Fig. 5). pp60 associated with both the PtdIns-3-kinase amino- and carboxyl-terminal SH2 domains, as did IRS-1 and the β subunit of the insulin receptor. (Fig. 5, lanes 6 and 7). No association of these proteins was observed with the glutathione S-transferase alone (Fig. 5, lane 5). These associations were also observed with the SH2 domains of PtdIns-3-kinase present as TyrP fusion proteins in an experiment where the adsorption was carried out with approximately 50 times less fusion protein/assay compared to the amount of the glutathione S-transferase fusion proteins (data not shown; see Ref. 9 for method).

Purification of pp60 from Rat Adipocytes—To purify pp60 from rat adipocytes we used immunoadfinity chromatography on anti-Tyr(P). Insulin-treated rat adipocytes were lysed in hot SDS/dithiothreitol, alkylated with N-ethylmaleimide, and finally diluted in an excess of nonionic detergent. This lysate was passed over a column of an immobilized monoclonal anti-Tyr(P) antibody. The column was washed and adsorbed Tyr(P) proteins eluted with phenyl phosphate. Fig. 6 shows the profile of eluted Tyr(P) proteins, as determined both by anti-Tyr(P) immunoblotting and by colloidal gold staining for protein. The purified material contained four major Tyr(P) bands (Fig. 6, left panel). We identified three of these Tyr(P) proteins as IRS-1, the β subunit of the insulin receptor, and pp60 by comparing their electrophoretic mobilities with those of the Tyr(P) bands in the total cell lysate from insulin-treated adipocytes. The fourth band, which migrates directly underneath pp60, most probably is a constitutive Tyr(P) protein. When the purification was performed on a small scale with lysate from both basal and insulin-treated adipocytes, this protein was also present in the phenyl phosphate eluate from the basal lysate, whereas as expected IRS-1, the β subunit of the insulin receptor, and pp60 were not (data not shown). When the same proteins eluted with phenyl phosphate were visualized with a sensitive colloidal gold stain (Fig. 6, right panel), bands corresponding to IRS-1, the insulin receptor β subunit, pp60, and the constitutive Tyr(P) protein were observed. On the basis of its identical electrophoretic mobility in both blots and also its rather broad appearance, the colloidal gold band corresponding to pp60 is likely to be purified pp60.

The recoveries of Tyr(P) proteins were estimated by quantitative immunoblotting for Tyr(P). Samples of total lysate, depleted lysate from the flow-through of the anti-Tyr(P) immunoaffinity column, and the purified Tyr(P) proteins were each separated at several loads on a single gel and immunoblotted with the combination of polyclonal anti-Tyr(P) and [32P]-labeled protein A. In a preparation that started with the adipocytes from 30 rats, 75% of the pp60 from the total cell lysate was adsorbed by the anti-Tyr(P) column, and subsequently 50% of the adsorbed pp60 was recovered in the Tyr(P) proteins eluted with phenyl phosphate. Thus the overall yield of pp60 was 37%. A similar yield was obtained for the β subunit of the insulin receptor (21%). A rough estimate of the amount of pp60 purified by this procedure can be made by visual comparison of its intensity upon colloidal gold staining with the intensities of various amounts of the protein standards (Fig. 6, right panel). On this basis, 3 ng of pp60 were purified from the adipocytes of one rat by the combination of immunoaffinity chromatography and preparative scale polyacrylamide electrophoresis. Due to the low abundance of pp60, it was not feasible to purify enough to carry out sequence analysis on tryptic peptides, a procedure that typically requires about 100 pmol (6 μg) (29).

**DISCUSSION**

Our results confirm those of earlier studies showing that with rat adipocytes insulin induces the tyrosine phosphorylation of a 60-kDa protein, in addition to the insulin receptor...
and IRS-1 (18–23). A major new finding of this study is that the Tyr(P) form of pp60, like that of IRS-1, is associated with PtdIns-3-kinase. The primary evidence for this is that under non-denaturing conditions, pp60 was immunoprecipitated by an antiserum against the 85-kDa subunit of PtdIns-3-kinase. We have also obtained this result with two monoclonal antibodies against the a form of p85, the epitopes for which are located in the \( \beta \)c-like and SH3 domains, as well as with an antiserum specific for the carboxyl-terminal peptide of the 110-kDa subunit of the enzyme (all kindly provided by Dr. M. Waterfield, Ludwig Institute for Cancer Research, London; data not shown). Consistent with this finding, the isolated SH2 domains of PtdIns-3-kinase, expressed as the glutathione S-transferase fusion proteins, efficiently bound the Tyr(P) form of pp60. This observation also indicates that the association of pp60 with PtdIns-3-kinase most likely occurs through the binding of its phosphotyrosine phosphorylation sites to these domains in the kinase. This mode of association would also account for the absence of a ternary complex of PtdIns-3-kinase, pp60, and IRS-1, since IRS-1 also probably binds to PtdIns-3-kinase via the SH2 domains (9).

Previous studies have shown that insulin treatment of rat adipocytes causes the appearance of PtdIns-3-kinase activity in anti-Tyr(P) immunoprecipitates (30, 31, 42). A priori the basis of this effect could either be tyrosine phosphorylation of PtdIns-3-kinase itself or association of PtdIns-3-kinase with Tyr(P) proteins. Our study strongly supports the latter. In addition to pp60, PtdIns-3-kinase was also found in association with the Tyr(P) form of IRS-1. Moreover, no tyrosine phosphorylation of p85 was detected by anti-Tyr(P) immunoblotting of the lysates or of the anti-Tyr(P) or anti-p85 immunoprecipitates.

We found that all of the PtdIns-3-kinase complex with IRS-1, and most with pp60, was located in the soluble fraction, although in the latter case some complex was observed in the membrane fraction. This agrees with the results of a recent study, in which PtdIns-3-kinase activity immunoprecipitated with anti-Tyr(P) was located in both the soluble and membrane fractions of insulin-treated rat adipocytes (42). In a separate study, however, all of the PtdIns-3-kinase activity immunoprecipitated with anti-Tyr(P) was located in the membrane fraction (31). A possible explanation for these discrepancies may lie in the different buffers used for homogenization of the cells. Ours and that in (42) contained relatively high concentrations of salts (300 mM), whereas that in (31) contained only 25 mM salts.

The functional consequence of the association of pp60 with PtdIns-3-kinase remains to be determined. In the case of IRS-1, it has recently been demonstrated that association of its Tyr(P) form with PtdIns-3-kinase activates the kinase about 3-fold (10). Since both pp60 and IRS-1 probably associate via the SH2 domains of the kinase, a reasonable expectation is that association with pp60 is also activating. The rapid appearance of PtdIns 3,4- and 3,4,5-phosphates seen upon exposure of adipocytes to insulin (32) may thus involve signaling through both pp60 and IRS-1.

The identity of pp60 is currently unknown. There is considerable evidence that this is a unique protein, rather than a proteolytic fragment of IRS-1 or the \( \beta \) subunit of the insulin receptor. First, Mooney and Bordwell (23) have labeled rat adipocytes with \( ^{32}P \), isolated these three Tyr(P) proteins by immunoadsorption with anti-Tyr(P) followed by gel electrophoresis, and generated one-dimensional peptide maps with both V8 proteinase and chymotrypsin. Most of the bands in
the maps of pp60 did not correspond to bands in those of IRS-1 or the β subunit. Second, our finding that pp60, but not the insulin receptor, is associated with PtdIns-3-kinase also indicates that pp60 is not a fragment of the insulin receptor. Also, since pp60 is largely a soluble protein, it would have to be derived from the cytoplasmic domain of the β subunit of the receptor, but the mobility of the entire domain on gel electrophoresis corresponds to only 48 kDa (33). Finally, we have immunoblotted the mixture of Tyr(P) polypeptides purified from rat adipocytes (see Fig. 6) with antibodies against the carboxyl-terminal peptide of the insulin receptor (34), kindly provided by Dr. Robert Smith, Joslin Diabetes Center, Boston, MA) and with antibodies against peptides corresponding to amino acid residues 764–777 and 1222–1235 (carboxyl terminus) in rat IRS-1 (7). In each case the antibodies reacted with the expected polypeptide but did not react with pp60 (data not shown). In combination with the report that antibodies against a peptide corresponding to amino acid residues 489–503 of rat IRS-1 do not react with pp60 (35), the result with our antibodies against IRS-1 peptides also eliminates IRS-1 as the precursor of pp60.

Although we were able to purify pp60 in high percentage yield from rat adipocytes, the actual amount was too low to obtain the sequence of peptides. Unfortunately, rat adipocytes are the most abundant source of this protein known to date. This Tyr(P) protein was not detected in the mouse 3T3-L1 adipocytes, and in another study it was not detected in human adipocytes (36). Also, it has not been observed in the other major insulin-sensitive cell types, liver (6, 37, 38) and muscle (33, 41). The lack of detection of this protein in other cell types may be accounted for by low abundance or alternatively because of limited tyrosine phosphorylation or rapid dephosphorylation. Further characterization of this insulin-elicited Tyr(P) protein will require discovery of a richer source or an approach different from purification. In this regard, we have noted that pp60 is similar in size to that of the tyrosine kinases of the src family (39). However, an antibody against the carboxyl-terminal peptide common to src, yes, and fyn (40), kindly provided by Dr. Sara Courtneidge, EMBL, Heidelberg) did not immunoblot purified pp60 under conditions where a rat brain homogenate gave a strong signal (data not shown). This result indicates that pp60 is not one of these kinases.

Acknowledgments—We are deeply indebted to Dr. Tony Pawson, Samuel Lunenfield Research Institute, Mount Sinai Hospital, Toronto, for the bacterial strains expressing the glutathione S-transferase fusion proteins, and to Drs. Drucker, Smith, Courtneidge and Waterfield, for various antibodies (see text).

REFERENCES

1. Olefsky, J. M. (1990) Diabetes 39, 1099–1106
2. Murakami, M. S., and Rosen, O. M. (1991) J. Biol. Chem. 266, 22653–22660
3. Wilden, P. A., Siddle, K., Haring, E., Backer, J. M., White, M. F., and Kahn, C. R. (1992) J. Biol. Chem. 267, 13719–13727
4. Kasuga, M., Izumi, T., Toh, K., Shibata, T., Momomura, K., Tashiro-Hashimoto, Y., and Kadowaki, T. (1990) Diabetes Care 13, 317–326
5. Keller, S. R., Kitagawa, K., Aebersold, R., Lienhard, G. E., and Garner, C. W. (1991) J. Biol. Chem. 266, 12917–12920
6. Rothenberg, P. L., Lane, N. J., Kadowaki, T., Backer, J., White, M., and Kahn, C. R. (1991) J. Biol. Chem. 266, 8302–8311
7. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) Nature 352, 73–77
8. Nishikawa, M., and Wands, J. R. (1992) Biochem. Biophys. Res. Commun. 183, 280–285
9. Lavan, B. E., Kuknis, M. R., Garner, C. W., Anderson, D., Reodrik, M., (1990) J. Biol. Chem. 265, 11631–11636
10. Backer, J. M., Myers, M. G. Jr., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Fu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., and Druker, B. J. (1993) EMBO J. 11, 3469–3479
11. Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S., and Courtneidge, C. S. (1990) J. Biol. Chem. 265, 19704–19711
12. Escobedo, J. A., Navanesanattasoo, S., Kavanagh, W. M., Milisky, D, Fried, Y. A., and Williams, L. T. (1991) Cell 65, 75–82
13. Skolnik, E. Y., Margolis, B., Mohammad, M., Lowenstein, E., Fischer, R., Drenpa, A., Ullrich, A., and Schlessinger, J. (1991) Cell 65, 83–90
14. Otani, M., Hilles, L., Goutt, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hean, J., Totty, N., Smith, A. J., Morgan, S. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1991) Cell 70, 419–425
15. Hiles, I. D., Otani, M., Volinia, S., Fry, M. J., Goutt, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hean, J. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1992) Cell 70, 419–425
16. Koe, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) Cell 69, 283–292
17. Cantley, L. C., Auger, K. R., Carpenter, C. L., Duckworth, B. C., Graziani, A., Kapeller, R., and Solomon, S. (1991) Cell 64, 281–302
18. Momomura, K., Toh, K., Sato, Y., Taka, A., and Kasuga, M. (1998) Biochem. Biophys. Res. Commun. 155, 1181–1186
19. Esaki, O. (1980) J. Biol. Chem. 254, 16118–16122
20. Mooney, R. A., Bordwell, K. L., Lubowsky, S., and Casellini, E. J. (1989) Endocrinology 124, 422–429
21. Mooney, R. A., Esaki, O., and Kasahara, M. (1990) Biochem. Biophys. Acta 1054, 89–94
22. Ahler, A., Smith, J. A., Randazzo, P. A., Rothenberg, P. L., and Jaret, L. (1991) J. Biol. Chem. 266, 16741–16745
23. Mooney, R. A., and Bordwell, K. L. (1992) Endocrinology 130, 1533–1538
24. Humes, R. L., Dillon, G. S., and Londos, C. (1986) J. Biol. Chem. 260, 17301–17306
25. Frost, S. C., and Lane, D. M. (1986) J. Biol. Chem. 261, 2464–2466
26. Park, J. T., Sharma, B. R., and Shaher, J. A. (1986) Arch. Biochem. Biophys. 248, 175–189
27. McGlaue, C. J., Ellis, C., Roedrik, M., Anderson, D., Moenatai, G., Reich, A. D., Panayotou, G., Ed, P., Berstein, A., Waterfield, M. D., and Pawson, T. (1992) Mol. Cell Biol. 12, 991–997
28. Cob, M. H., Boulton, T. G., and Robbins, D. J. (1991) Cell Regul. 2 965–968
29. Aebersold, R. H., Leavitt, J., Saavedra, R. A., Hood, L. E., and Kent, S. B. (1992) J. Biol. Chem. 267, 8302–8307
30. Childs, A. D., Kowalski-Chauvel, A., Cormont, M., and Van Obbergen, E. (1992) Eur. J. Biochem. 207, 599–606