Detection of a 60 kDa tyrosine-phosphorylated protein in insulin-stimulated hepatoma cells that associates with the SH2 domain of phosphatidylinositol 3-kinase

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Activation of the tyrosine kinase activity of the insulin receptor by autophosphorylation leads to phosphorylation of cellular substrates on tyrosine. Thus far, the best characterized is the insulin receptor substrate (IRS) 1, which has been proposed to serve as a docking protein for other molecules involved in signal transduction. A number of other proteins that become phosphorylated in response to insulin have been identified, some of which are reported to be tissue-specific. A 60 kDa phosphoprotein has been detected in adipocytes after insulin stimulation [Lavan and Lienhard (1993) *J. Biol. Chem.* **268**, 5921–5928]. We have identified a protein of similar molecular mass in rat hepatoma cells transfected with the human insulin receptor. The 60 kDa protein in hepatoma cells is tyrosine-phosphorylated in response to insulin in a dose-dependent

manner, with maximal phosphorylation occurring at 50 nM insulin. Although the dose-response of p60 phosphorylation mirrors that of IRS-1, the time course is slightly slower, with maximal phosphorylation observed 5 min after addition of insulin. Like the adipocyte protein, the 60 kDa protein detected in liver cells binds to the SH2 domain of the p85 regulatory subunit of phosphatidylinositol 3-kinase, but not to other SH2 domains. Binding of p60 to p85 is similar to the interaction between p85 and IRS-1 in that a tyrosine-phosphorylated peptide containing the YVXM motif can inhibit the association. The presence of this 60 kDa tyrosine-phosphorylated protein in adipocytes and hepatoma cells suggests that it represents another important intermediate in the insulin-receptor signal-transduction pathway.

INTRODUCTION

Growth-factor receptors undergo autophosphorylation on tyrosine residues resulting in the generation of binding sites for signalling proteins that contain *src* homology (SH) 2 domains [1–3]. These are conserved protein structural motifs that have been shown to direct intra- and inter-molecular interactions by binding to specific sequences containing tyrosine phosphate [2,4,5]. The binding of SH2-domain-containing proteins to tyrosine-phosphorylated receptor tyrosine kinases may target substrates to the receptor for phosphorylation. For example, the SH2 domains of phospholipase C γ (PLC γ) can bind the activated epidermal-growth-factor [6], nerve-growth-factor [7] and platelet-derived-growth-factor [8] receptors. After binding, PLC γ becomes phosphorylated, in some cases resulting in subsequent activation of the enzyme [9]. Proteins with SH2 domains may also serve as adaptors or activators. For example, occupation of the SH2 domain of the 85 kDa regulatory subunit of phosphatidylinositol 3-kinase (PI-3-kinase) by phosphotyrosine results in increased activity of the 110 kDa catalytic domain [10]. Receptor binding to the SH2 domain of Grb2 induces the recruitment of the guanylyl nucleotide exchange factor, *ras*, to the membrane, where its substrate, p21^{ras}, is localized [11,12].

Although the insulin receptor belongs to the tyrosine kinase family of receptors, proteins containing SH2 domains are more frequently detected by association with receptor substrates than with the receptor itself. Tyrosine phosphorylation of the well-characterized insulin receptor substrate IRS-1 at multiple sites induces the binding of several proteins containing SH2 domains (for review see ref. [13]), including the 85 kDa regulatory subunit of PI-3-kinase [14], Grb2 [15,16], the protein tyrosine phosphatase

SHPTP2 [17,18] and Nck [19]. This phosphorylation-induced association between p85 and IRS-1 is thought to be responsible for increased PI-3-kinase activity [10]. IRS-1–SHPTP2 binding may lead to an increase in the phosphatase activity of this protein, although there is no direct evidence yet to support this possibility [20]. The role of the Grb2–IRS-1 association is less clear. Although IRS-1 can complex with Grb2 in cells [15,16,21], this association does not always lead to *ras* activation [22]. The Shc protein can also be phosphorylated on tyrosine in response to insulin; this substrate serves as a docking site for the SH2 domain of Grb2, and the Shc–Grb2 complex may be necessary for activation of *ras* [15].

The pleiotropic nature of insulin action suggests the existence of other substrates of the receptor that bind SH2 domains. A 60 kDa adipocyte protein, which is tyrosine-phosphorylated in response to insulin, was recently demonstrated to bind the SH2 domains of p85 [23]. In this study we show that a protein with similar properties is also present in liver cells. These results confirm the presence of another IRS that serves as a binding site for SH2 domains.

MATERIALS AND METHODS

Materials

Insulin was from Eli Lilly. ¹²⁵I-labelled Protein A was purchased from Amersham Corp. The polyclonal anti-phosphotyrosine serum used has been described [24]. Anti-PI-3-kinase antibody was from UBI. Anti-[GTPase-activating protein (GAP)] sera was a gift from Dr. Stuart Decker [24]. Glutathione–Sephadex was purchased from Pharmacia. The cloning and purification

Abbreviations used: GAP, GTPase-activating protein; GST, glutathione S-transferase; HTC-IR cells, hepatoma cells; IRS-1, insulin receptor substrate 1; PLC, phospholipase C; PI-3-kinase, phosphatidylinositol 3-kinase; SH, *src* homology.

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of glutathione S-transferase (GST) fusion proteins have been described [25,26].

Cell culture

Rat hepatoma cells transfected with the human insulin receptor (HTC-IR) were generously given by Dr. Chin Sung and Dr. Ira Goldfine (University of California, San Francisco, CA, U.S.A.), and were grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum.

Immunoblots

Cells were seeded in 150 mm dishes. When confluent, they were treated with the indicated concentrations of insulin for 5 min (unless otherwise indicated). After hormone treatment, the cells were washed with cold PBS and lysed in 1 ml of HNTG buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 μ M sodium orthovanadate, 100 mM NaF, 30 mM *p*-nitrophenol phosphate, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin). The lysates were clarified by centrifugation and the supernatants were incubated with glutathione-Sepharose beads containing bound GST fusion proteins or specific antibodies and Protein A-agarose. The following GST fusion proteins were used: the C-terminal SH2 domain of p85 (residues 624–706); the N-terminal SH2 domain of p85 (residues 321–440); a GAP fusion protein containing both the N- and C-terminal SH2 domains (residues 117–444); a PLC γ fusion protein with both the N- and C-terminal SH2 domains (residues 550–756); the SHC SH2 domain (residues 378–471); the full-length Grb2 protein. All of these fusion proteins have been used previously to bind tyrosine-phosphorylated receptors [7,21,22,24–26]. After incubation with the fusion proteins or antibody, the beads were washed four times with HNTG buffer and samples were solubilized in 50 μ l of SDS gel sample buffer. Proteins were separated by electrophoresis on 8 % polyacrylamide gels and transferred to nitrocellulose. Immunoblotting with anti-phosphotyrosine antibodies was performed as described [24].

Peptide-inhibition experiments

Phosphotyrosine-containing peptides were synthesized, purified and characterized as described [27]. To test for inhibition of binding, phosphotyrosine-containing and unphosphorylated peptides were added to the cell lysates at the indicated concentrations before the addition of fusion protein–glutathione-Sepharose beads. The incubations were then carried out as described above.

RESULTS

In order to look for additional proteins phosphorylated on tyrosine in response to insulin, HTC-IR cells were treated with insulin, and the cell lysates were incubated with glutathione-Sepharose beads containing bound fusion proteins of various SH2 domains. The samples were then analysed by anti-phosphotyrosine immunoblotting. Incubation of HTC-IR cell lysates with the C-terminal SH2 domain of the p85 regulatory subunit of PI-3-kinase led to the detection of three tyrosine-phosphorylated proteins in insulin-treated cells (Figure 1). Sepharose beads containing glutathione alone did not precipitate any tyrosine-phosphorylated proteins (results not shown). The 185 kDa protein was demonstrated to be IRS-1 by immunoblotting, and the 95 kDa protein co-migrated with immunoprecipitated insulin receptor β -chain (results not shown). The third protein migrated with an apparent molecular mass of 60 kDa. The identity of this

Figure 1 Insulin-stimulated tyrosine-phosphorylated proteins that bind to the SH2 domain of p85

HTC-IR cells were either untreated (–) or treated (+) with 100 nM insulin for 5 min. The cells were lysed as described in Materials and methods section and the lysates were incubated with glutathione beads containing a bound fusion protein of the C-terminal SH2 domain of p85 and GST. Bound proteins were subjected to anti-phosphotyrosine immunoblotting as described in the Materials and methods section. Molecular sizes (kDa) are indicated on the left.

protein is unknown, although a protein with similar properties was reported in insulin-stimulated adipocytes [23].

To investigate further the tyrosine phosphorylation of this protein, we examined the dose–response to insulin. HTC-IR cells were treated with increasing concentrations of insulin, and p85-binding proteins were isolated as described above. The phosphotyrosine content was assayed by immunoblotting (Figure 2a). Phosphorylation of p60 could be detected after treatment of cells with as little as 0.5 nM insulin. In contrast, receptor phosphorylation was not detected by this assay until 5 nM insulin was present. This probably reflects a lower affinity of the receptor for the fusion protein compared with p60, as tyrosine phosphorylation of the receptor can be detected at lower concentrations of insulin after immunoprecipitation with anti-receptor antibodies [results not shown].

To evaluate further the insulin-dependent phosphorylation of p60 we also examined the time course. HTC-IR cells were treated with 100 nM insulin for various lengths of time, and the p85-binding proteins were analysed by anti-phosphotyrosine immunoblotting (Figures 2b and 2c). Tyrosine phosphorylation of p60 was detected as early as 1 min after stimulation, but did not reach maximal levels until 10 min of insulin treatment. The phosphotyrosine content of p60 remained high throughout 30 min of insulin stimulation. This differed from the time course of receptor and IRS-1 phosphorylation detected with the fusion protein. IRS-1 phosphorylation reached maximal levels as early as 1 min after stimulation, and decreased by 5 min of insulin treatment. Tyrosine phosphorylation of the insulin receptor β -subunit also reached maximal levels at 1 min but remained high over the next 30 min.

We next examined the specificity of the interaction between p60 and p85 (Figure 3). GST fusion proteins containing either the N- or C-terminal SH2 domains of p85 were compared for their ability to bind p60. HTC-IR cells were treated with 100 nM insulin for 5 min and then lysed as described above. The lysates were incubated with glutathione beads containing the two different p85 SH2 domains, and precipitated proteins were analysed by anti-phosphotyrosine immunoblotting. Figure 3 demonstrates that, whereas the C-terminal SH2 domain of p85 was able to bind p60 efficiently, the N-terminal SH2 domain was

Figure 2 Time course and dose-response of p60 phosphorylation

(a) HTC-IR cells were incubated with the indicated concentrations (nM) of insulin for 5 min. The cells were lysed and p85-binding proteins were isolated as described in Figure 1. The bound proteins were analysed by anti-phosphotyrosine immunoblotting. Molecular sizes (kDa) are indicated at the left. (b) HTC-IR cells were incubated with 100 nM insulin for the indicated period of time (min). The cells were lysed and p85-binding proteins were isolated and analysed as described above. Similar results for both time course and insulin dose-response were obtained in five separate experiments. (c) Autoradiograms from (b) were quantified by densitometry (BioImage; Millipore). ●, p60; □, insulin receptor β ; △, IRS-1.

Figure 3 Specificity of the p60-p85 interaction

HTC-IR cells were either untreated (—) or treated (+) with 100 nM insulin for 5 min, and then lysed as described. The cell lysates were incubated with glutathione beads containing bound fusion proteins of either the C-terminal (C-term.) or N-terminal (N-term.) SH2 domain of p85 and GST. The bound proteins were analysed by SDS/PAGE, followed by anti-phosphotyrosine immunoblotting.

Figure 4 Binding of different 60 kDa phosphoproteins to p85 and GAP

HTC-IR cells were either untreated (—) or treated (+) with 100 nM insulin for 2 or 10 min, as indicated, and then lysed as described in the Materials and methods section. The cell lysates were incubated with anti-p85 or anti-GAP antibodies. Bound proteins were analysed by SDS/PAGE, followed by anti-phosphotyrosine immunoblotting.

Figure 5 Inhibition of the p85-p60 interaction by a tyrosine-phosphorylated peptide

HTC-IR cells were either untreated (—) or treated (+) with 100 nM insulin for 5 min, and then lysed as described above. The cell lysates were incubated with glutathione beads containing the C-terminal p85 SH2 domain fusion protein in the presence of the indicated concentration (nM) of the tyrosine-phosphorylated peptide, YVPM. Bound proteins were analysed by anti-phosphotyrosine immunoblotting.

incapable of binding. In contrast, both N- and C-terminal SH2 domains of p85 were capable of binding efficiently to phosphorylated IRS-1. Fusion proteins containing the SH2 domains of ras GAP, Src, Shc, Grb2 and PLC were all unable to bind p60.

Recently a 62 kDa tyrosine-phosphorylated protein was detected in insulin-treated cells that co-precipitated with ras GAP [28,29]. To explore the relationship of this phosphoprotein to the p85-associated phosphoprotein described here, cells were treated with or without insulin, and lysates were immunoprecipitated with anti-p85 or anti-GAP sera, followed by SDS/PAGE and blotting with anti-phosphotyrosine antibodies (Figure 4). As described above for the SH2 fusion protein, both p60 and IRS-1 were detected in anti-p85 immunoprecipitates. Both proteins were tyrosine-phosphorylated in response to insulin, although phosphorylation of p60 was slower than that detected for IRS-1 (Figure 2(b)). More detailed time-course studies revealed that the differences in the kinetics of phosphorylation of IRS-1 and p60 observed with anti-p85 precipitation were virtually identical with those found with p85 SH2 fusion proteins (results not shown). Interestingly, an insulin-sensitive tyrosine-phosphorylated protein was also detected in anti-GAP immuno-

precipitates. This protein had a higher level of basal phosphorylation than that observed for the p85-associated species, and migrated slightly faster on SDS/PAGE. This protein is likely to be the 62 kDa GAP-associated protein that has been detected complexed to other SH2 domain proteins [30,31].

The SH2 domains of p85 have also been shown to bind other proteins including IRS-1 [15], insulin-like-growth-factor-I receptor [32] and platelet-derived-growth-factor receptor [33]. Studies with phosphorylated peptides indicate that the preferred binding site for p85 contains a phosphorylated tyrosine within a YMXM or YVXM motif [18,32,34,35]. We examined whether a phosphopeptide containing the sequence YVPM could inhibit the interaction between p85 and p60. Lysates from insulin-stimulated HTC-IR cells were incubated with p85 beads in the presence of increasing concentrations of phosphopeptide (Figure 5). Concentrations as low as 5 nM significantly decreased binding, and 25 nM peptide completely inhibited the interaction. Non-phosphorylated peptide had no effect (results not shown). These results suggest that the interaction between the p85 SH2 domain and p60 is similar to that observed for binding of IRS-1 of platelet-derived-growth-factor receptor.

DISCUSSION

Autophosphorylation of the insulin receptor activates the intrinsic tyrosine kinase and leads to phosphorylation of intracellular substrates. In many cases these substrates are present at low concentrations and their phosphorylation cannot be detected by anti-phosphotyrosine immunoblotting of whole-cell lysates. Although immunoprecipitating with anti-phosphotyrosine antibodies before immunoblotting allows increased detection of particular substrates, not all tyrosine-phosphorylated proteins are precipitated. The identification of SH2 domains as phosphotyrosine-binding moieties has provided a means of finding new tyrosine kinase substrates. For example, a novel 38 kDa protein that is phosphorylated in response to nerve growth factor was identified by its ability to bind to the SH2 domains of PLC γ [36]. Similarly, in this study we demonstrate the insulin-dependent phosphorylation of a 60 kDa protein and its association with the SH2 domain of the p85 regulatory subunit of PI-3-kinase.

Phosphorylation of p60 is not detectable by immunoblotting whole-cell lysates, but can be seen if the proteins are first isolated by virtue of their binding to the SH2 domain of p85. The specificity of this interaction is demonstrated by the fact that SH2 domains from other proteins, including GAP, Shc, Grb2 and PLC γ , are unable to bind p60. Antibodies to p85 co-immunoprecipitate phosphorylated p60, suggesting that this interaction occurs *in vivo*, although we have not conclusively demonstrated that these proteins are identical. A protein of similar molecular mass and biological properties was also identified in adipocytes [23]. The adipocyte 60 kDa protein was found by anti-p85 immunoprecipitation performed under non-denaturing conditions, and was shown to bind p85 through its SH2 domains. The presence of this p85-binding protein in adipocytes and liver cells suggests that it may be an important component of the insulin-receptor signal-transduction pathway. Interestingly, this p85-associated 60 kDa phosphoprotein is apparently different from that detected in anti-GAP immunoprecipitates [30,31]. This latter protein is also phosphorylated on tyrosine in response to insulin, and may represent the p62^{rec}-associated protein [30].

Insulin treatment stimulates the tyrosine phosphorylation of IRS-1 at multiple sites. This provides binding sites for other molecules that may be involved in propagating the signal from

the receptor. The binding of p85 to IRS-1 has been demonstrated in several cell types, and activation of PI-3-kinase by IRS-1-derived phosphopeptides indicates that the interaction between IRS-1 and p85 *in vivo* leads to activation of the enzyme. As both IRS-1 and p60 appear to bind p85 through its SH2 domains, p60 may also be capable of activating PI-3-kinase, suggesting functional redundancy between these two molecules. One possible explanation may lie in their cellular localization. Perhaps p60 and IRS-1 differentially target PI-3-kinase for distinct functions. Lavan and Lienhard [23] showed that IRS-1-associated p85 was predominantly detected in a cytoplasmic fraction, whereas the complex between adipocyte p60 and p85 was detected in both cytoplasmic and membrane fractions. Moreover, the proposed function of PI-3-kinase in membrane vesicular transport [37] raises the possibility that these p85-binding proteins differentially target certain membrane fractions, perhaps including those containing transporter molecules or other receptors [38]. In this regard, a recent report [39] has shown that inhibition of PI-3-kinase activity can block insulin-stimulated glucose transport in adipocytes. Alternatively, the different time course of phosphorylation of these two proteins may reflect different functions, including the possibility that p60 is responsible for initiating a second wave of PI-3-kinase activation. The answers to these questions will require the generation of specific probes for p60.

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