Insulin and insulin-like growth factor 1 stimulate the phosphorylation on tyrosine of a 160 kDa cytosolic protein in 3T3-L1 adipocytes

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Insulin and IGF-1 (insulin-like growth factor 1) rapidly stimulate the phosphorylation on tyrosine of a 160 kDa cytosolic protein (pp160) in intact 3T3-L1 adipocytes. Half-maximal phosphorylation of pp160 is attained with either 4 nM-insulin or 20 nM-IGF-1. A semi-quantitative immunoblotting procedure using anti-phosphotyrosine antibody revealed that the insulin-stimulated 3T3-L1 adipocyte possesses approx. 3×10^5 and 0.6×10^5 phosphotyrosyl sites, respectively, in pp160 and insulin receptor β -subunit. Removal of insulin from stimulated cells results in the rapid (within 15 min) loss of phosphate groups from tyrosyl residues in both pp160 and receptor β -subunit. Whereas pp160 remains maximally phosphorylated on tyrosine for up to 60 min in the presence of 100 nM-insulin, IGF-1 at the same concentration induces only a transient response that is maximally 50 % of that observed with insulin. pp160 is not phosphorylated on tyrosine in response to platelet-derived growth factor or epidermal growth factor. Although pp160 appears to be a soluble cytoplasmic protein, in the presence of 1 mM-ZnCl₂ it becomes membrane-associated. In view of its apparent cytoplasmic localization and its inability to bind to either wheat-germ agglutinin or concanavalin A, pp160 does not appear to be a typical glycoprotein growth-factor receptor. Our results suggest that pp160 may be a physiologically important cellular substrate of the insulin-receptor tyrosine kinase in the intact 3T3-L1 adipocyte.

INTRODUCTION

Several hormone and growth-factor receptors, including those for insulin and IGF-1, are ligand-stimulated tyrosine kinases [1–8]. Insulin and IGF-1 receptors are heterotetrameric ($\alpha_2\beta_2$) cell-surface glycoproteins, consisting of α -subunits, which bind hormone, and β -subunits, which possess tyrosine kinase catalytic domains [1–6,9–13]. Autophosphorylation of the β -subunit of the insulin receptor, which occurs in response to physiological hormone concentrations both in cell-free receptor preparations and in intact cells [1–6,14,15], markedly enhances tyrosine kinase activity toward exogenous model protein substrates (substrate phosphorylation, refs. [15–20]). Substantial evidence from several laboratories demonstrates that insulin-receptor autophosphorylation is a prerequisite for many of the acute and long-term effects of insulin [21–25]. The precise role of hormone-stimulated substrate phosphorylation in insulin and IGF-1 action remains to be established.

White *et al.* [26] showed the rapid insulin-stimulated phosphorylation on tyrosine of a 185 kDa protein in intact Fao hepatoma cells. Subsequently, several laboratories found that insulin and IGF-1 stimulated the phosphorylation on tyrosine of similar, if not identical, high-molecular-mass proteins in various intact cell systems [25,27–30]. Recently, Häring *et al.* [31] reported a 46 kDa membrane protein in intact rat adipocytes that becomes phosphorylated on tyrosine in response to insulin. Finally, Bernier *et al.* [32] have demonstrated the insulin-stimulated tyrosine phosphorylation of a cyto-

solic 15 kDa protein in intact phenylarsine oxide-treated 3T3-L1 adipocytes. By virtue of the kinetics and concentration-dependence of their respective hormone-stimulated tyrosine phosphorylations, each of these proteins has been implicated as a possible cellular substrate of either the insulin- or IGF-1-receptor tyrosine kinase.

In this paper we report the rapid insulin- and IGF-1-stimulated phosphorylation on tyrosine of a 160 kDa soluble cytoplasmic protein (pp160) in intact 3T3-L1 adipocytes. Using a semi-quantitative immunoblotting technique with anti-phosphotyrosine antibodies, we demonstrate the extent, time course, ligand-concentration dependence and reversibility of hormone-stimulated pp160 tyrosine phosphorylation. In addition, we show that pp160 becomes membrane-associated *in vitro* in the presence of 1 mM-ZnCl₂.

EXPERIMENTAL

Cells

3T3-L1 preadipocytes were cultured and differentiated in 10 cm-diam. culture dishes as previously described [33]. Briefly, 3T3-L1 preadipocytes were grown to confluence, and adipose conversion was induced with isobutylmethylxanthine, dexamethasone and insulin. Finally differentiated 3T3-L1 adipocytes were used for experiments 7–11 days after the induction of adipocyte conversion. Insulin was removed from the medium 2 days before experiments.

Abbreviations used: pp160, phosphorylated 160 kDa protein; IGF-1, insulin-like growth factor 1; IGF-2, insulin-like growth factor 2; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PAGE, polyacrylamide-gel electrophoresis; BSA, bovine serum albumin.

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Hormone additions and cell lysis

Cell monolayers (10 cm culture dishes, containing 1.2×10^7 cells) were washed once and then incubated for 12–16 h at 37 °C in Dulbecco's modified Eagle's medium devoid of serum. Specified hormones were added directly to the medium for the indicated periods of time. The medium was then removed and the cell monolayer was immediately frozen by placing the culture dish on a mixture of solid CO₂ and acetone. After rapid thawing to 4 °C, cells were scraped with a rubber policeman into two 500 μ l portions of Buffer A, containing 20 mM-Hepes, pH 7.4, proteinase inhibitor cocktail [15] including 0.3 mM-phenylmethanesulphonyl fluoride (Sigma), 1 mM-Na₃VO₄, 1 μ M-CaCl₂ and 100 μ M-ZnCl₂. Buffer A was designed to minimize phosphotyrosyl-protein dephosphorylation during subsequent sample processing. Zinc and Na₃VO₄ have previously been shown to inhibit phosphotyrosyl phosphatases effectively [34,35]. Cells were lysed by ten passages through a ball-bearing stainless-steel homogenizer [36], and nuclei and cellular debris were removed by centrifugation at 4 °C for 20 s in a Beckman Microfuge. This discarded fraction contained insignificant amounts of tyrosine-phosphorylated pp160 or insulin-receptor β -subunit (results not shown). The post-nuclear supernatant was immediately collected for analysis or further fractionated by centrifugation in a total volume of 200 μ l for 5–15 min at 210000 *g* at 4 °C in a Beckman Airfuge. The supernatant and crude membrane pellet were individually analysed (see below).

Preparation and purification of sheep anti-phosphotyrosine antibody

Phosphotyrosine was coupled to BSA by using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (Sigma). Phosphotyrosine and [¹⁴C]phosphotyrosine were prepared from tyrosine (Sigma) and [¹⁴C]tyrosine (NEN; 40–60 mCi/mmol) as previously described [37]. A solution containing BSA (18 mg/ml), 20 mM-phosphotyrosine and trace amounts of [¹⁴C]phosphotyrosine was adjusted to pH 4.5 with 1 mM-HCl before addition of the carbodi-imide (solid) to 100 mM (final volume 10 ml). After overnight incubation at room temperature, the solution was exhaustively dialysed against PBS (138 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, 0.9 mM-CaCl₂, 0.5 mM-MgCl₂, pH 7.4).

Sheep were immunized by multiple subcutaneous injections of 2 mg of phosphotyrosyl-BSA in complete Freund's adjuvant, and boosted at 3-week intervals with phosphotyrosyl-BSA in incomplete Freund's adjuvant. Sera obtained by plasmaphoresis were analysed for phosphotyrosine binding by equilibrium dialysis against [¹⁴C]phosphotyrosine as previously described [38]. Anti-phosphotyrosine antibodies were purified by sequential precipitation in 40%-satd. (NH₄)₂SO₄ and affinity purification with phosphotyrosine immobilized to Affigel 10 [prepared according to the manufacturer's (Bio-Rad) specifications]. For affinity purification, precipitated antibody was resuspended and dialysed against 10 mM-imidazole buffer, pH 7.4, containing 150 mM-KCl (IBS). A 5-fold excess of phosphotyrosine in phosphotyrosyl-Affigel (4–8 μ mol of phosphotyrosine/ml of packed resin) was added over estimated phosphotyrosine-binding sites. After overnight incubation at 4 °C, the gel was collected in a column and washed with IBS until the

protein concentration of the eluate was < 0.14 mg/ml. Specific anti-phosphotyrosine antibodies were eluted with 10 mM-phosphotyrosine in IBS, followed by 10 mM-phosphotyrosine in IBS containing 4 M-urea. Both fractions were re-precipitated in 40%-satd. (NH₄)₂SO₄ and dialysed exhaustively against IBS. There was no detectable difference in phosphotyrosine binding between these two fractions.

Immunoblotting with anti-phosphotyrosine antibody

Cell fractions were subjected to SDS/PAGE in 7%-acrylamide gels (Laemmli [39]), and proteins were then electrophoretically transferred to 0.45 μ m-pore-size nitrocellulose filters (Sartorius) in standard Towbin [40] buffer at 300 mA for 4 h at room temperature in a Bio-Rad Transblot apparatus. Under these conditions < 10% of the Coomassie-Blue-stainable protein remains in the gel, whereas < 1% of the transferred pp160 and insulin receptor β -subunit pass through the nitrocellulose filter. The filters were incubated in Tween/NaCl buffer (0.02% Tween-20, 20 mM-imidazole, pH 7.5, and 150 mM-NaCl; Kirkegaard and Perry, Gaithersburg, MD, U.S.A.) containing 1% BSA (Sigma, radioimmunoassay grade) for 2 h at room temperature. Affinity-purified sheep anti-phosphotyrosine antibodies were added at 1 μ g/ml in Tween/NaCl buffer containing 0.1% BSA for 12–16 h at 4 °C. The filters were then washed three times with Tween/NaCl buffer and incubated for 4 h at 4 °C with ¹²⁵I-labelled Protein G (24 mCi/mg; Amersham) at 0.1 μ Ci/ml in Tween/NaCl buffer containing 0.1% BSA. The filters were extensively washed, incubated with 1% (v/v) India ink solution in Tween/NaCl buffer to stain proteins, and were washed, dried, and subjected to autoradiography at –70 °C with a Kodak intensifying screen. The average exposure time was 12 h. For quantification, appropriate bands were excised from the filters and counted for radioactivity with a Packard gamma-radiation counter. Each data point in Figs. 4 and 5 represents the difference between stimulated and unstimulated cells; a background value was obtained for pp160, pp116 (a 116 kDa cellular protein that is phosphorylated on tyrosine in a hormone-insensitive manner) and the β -subunit by running unstimulated cell extracts in duplicate adjacent lanes and counting the radioactivity of the region of the gel co-migrating with the protein of interest. The background values for pp160 and the β -subunit were consistently 40% of the maximum hormone-stimulated values. All experiments were performed in triplicate, with virtually identical results. In all cases the intensity of protein staining on the nitrocellulose filters was uniform in all lanes, indicating a uniform yield from each culture dish.

The sensitivity of the blotting procedure was established by dot-blotting different concentrations of *Escherichia coli* glutamine synthetase (EC 6.3.1.2) containing known amounts of phosphotyrosine [41] (Fig. 1). Briefly, glutamine synthetase was purified from *E. coli* by Zn²⁺-, acetone- and (NH₄)₂SO₄-precipitation steps [42,43], adenylated to completion in the presence of adenylyltransferase (EC 3.1.4.15), reduced and carboxymethylated, and quantitatively hydrolysed with micrococcal nuclease [41]. Analysis of phosphotyrosine released after controlled base hydrolysis established that each 50 kDa monomer contained one phosphotyrosine residue. As little as 40 fmol of phosphotyrosine in glutamine synthetase could be detected by the immuno-

Fig. 1. Quantitative immunoblotting using anti-phosphotyrosine antibodies

Portions (2 μ l) of glutamine synthetase containing known amounts of covalently bound phosphotyrosine were applied to a 0.45 μ m nitrocellulose filter and immunoblotted by using sheep anti-phosphotyrosine antibodies and 125 I-Protein G as described in the Experimental section. After autoradiography, the radioactive 'dots' were excised and counted for radioactivity with a Packard gamma counter.

blot procedure, and the assay remained linear up to 400 fmol (Fig. 1). This is in close agreement with the 10 fmol detection limit determined by Morla & Wang [44], using an anti-phosphotyrosine antibody directed against tyrosine-phosphorylated recombinant v-abl (oncogene product of Abelson murine leukaemia virus). Additional studies indicated that the immunoblot assay was linear up to 1.2 pmol of phosphotyrosine in glutamine synthetase and that 10 μ g of BSA did not interfere with the assay when applied to the nitrocellulose filter with the enzyme. Importantly for determination of the phosphotyrosine content of transferred proteins, the immunoblot signal in pp160, pp116 and the β -subunit (see above) varied in a directly linear manner with the amount of cellular extract loaded on the gel (10–200 μ g of protein). This confirms the linear relationship between the phosphotyrosine content in these proteins and the resultant immunoblot signal.

Materials

Insulin was a gift from Dr. Ronald Chance (Eli Lilly, Indianapolis, IN, U.S.A.), and IGF-1 was a gift from Dr. Goesta Enberg (Karolinska Institute, Stockholm, Sweden). Insulin-like growth factor 2 (IGF-2) was a gift from Dr. James Perdue (American Red Cross, Rockville, MD, U.S.A.), and platelet-derived growth factor

Fig. 2. Growth-factor-stimulated tyrosine phosphorylation in intact 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes (10 cm-diam. culture dishes) were serum-starved overnight and incubated for 5 min at 37 °C in cell-culture medium (Dulbecco's modified Eagle's medium) minus serum (C, control) and containing 100 nM-insulin (INS), -IGF-1, -IGF-2 or -EGF, or 1 μ M-PDGF. BSA (1 mg/ml), which was included in the PDGF incubation, did not alter the pattern of protein tyrosine phosphorylation. Cells were then extracted, and the post-nuclear supernatants (150 μ g of protein per lane) were processed for immunoblotting with anti-phosphotyrosine antibodies as described in the Experimental section. Molecular masses (kDa) were assigned by comparing the relative migration in the gel with the following pre-stained standards: myosin, phosphorylase *b*, BSA, ovalbumin, α -chymotrypsinogen, β -lactoglobulin and lysozyme (BRL, Gaithersburg, MD, U.S.A.)

(PDGF) was obtained from Dr. Charles Scher (University of Pennsylvania, Philadelphia, PA, U.S.A.). Phosphoarginine, phosphoserine, phosphothreonine and phosphotyrosine were purchased from Sigma, and phosphohistidine was obtained from Dr. William Bridger (University of Alberta, Edmonton, Canada). Rabbit polyclonal anti-(rat insulin receptor) antibody was a gift from Dr. José Caro (East Carolina Medical School).

RESULTS

Fully differentiated 3T3-L1 adipocytes possess multiple cell-surface peptide-hormone receptors, including those for insulin [15], IGF-1 [15], IGF-2 [15] and EGF [45], some of which have demonstrable hormone-stimulated tyrosine kinase activity. Cells stimulated individually with each of these peptide growth factors (insulin, IGF-1, IGF-2, EGF and PDGF) were homogenized and their phosphotyrosyl-proteins were analysed by SDS/PAGE, followed by immunoblotting with anti-phosphotyrosine antibodies as described in the Experimental section (Fig. 2). Several results were consistently obtained. (i) Unstimulated cells exhibited one major (116 kDa) and three minor phosphotyrosine-

Fig. 3. Specificity of immunoblotting with anti-phosphotyrosine antibodies

Identical post-nuclear supernatants (150 μ g of protein per lane) from insulin-stimulated cells (100 nM for 5 min at 37 °C) were analysed in parallel by SDS/PAGE followed by immunoblotting with anti-phosphotyrosine antibodies as described in the Experimental section. The following phosphoamino acids were included during the incubation with anti-phosphotyrosine antibodies after transfer to nitrocellulose: none (lane 1), 200 μ M-phosphotyrosine (lane 2), 5 mM-phosphoserine (lane 3), 5 mM-phosphothreonine (lane 4), 5 mM-phosphoarginine (lane 5), 1 mM-phosphohistidine (lane 6) and 5 mM- P_i (lane 7). ' β ' indicates the position of the β -subunit.

containing polypeptides (62 kDa, 80 kDa and 130 kDa). (ii) Stimulation of intact cells with insulin results in the phosphorylation on tyrosine of a major 160 kDa protein (pp160), a minor 35 kDa protein (pp35), and a 95 kDa protein, identified as the β -subunit of the insulin receptor by virtue of its ability to adsorb to wheat-germ agglutinin-Sepharose and concanavalin A-Sepharose, its characteristic electrophoretic mobility in two-dimensional non-reducing/reducing SDS/PAGE [15], and its specific immunoprecipitation by anti-(insulin receptor) antibodies. The phosphotyrosine content of pp160 does not change in a consistent pattern with either the addition (Figs. 2, 4 and 5) or withdrawal (Fig. 6) of insulin or IGF-1. In each of four separate experiments the phosphotyrosine content of pp160 varied by no more than $\pm 20\%$ (see the legends to Figs. 4, 5 and 6). (iii) A similar, if not identical, 160 kDa protein is phosphorylated on tyrosine in response to IGF-1 and IGF-2. (iv) A unique set of proteins consistently become phosphorylated on tyrosine in response to each hormone; for example, five phosphotyrosyl-proteins can be detected in PDGF-stimulated cells (35, 59, 71, 80 and 185 kDa). The 185 kDa protein is apparently the PDGF receptor, and the other proteins have molecular masses similar to those of proteins previously identified in other PDGF-stimulated cells [46]. In addition, the 62 kDa phosphotyrosyl-protein observed in control cells is absent from PDGF-treated cells. The 170 kDa EGF-stimulated phosphotyrosyl-protein (Fig 2) is most likely the EGF receptor [45]; the significance of the 71 kDa EGF-stimulated protein remains to be established. Finally, it appears that IGF-2 stimulates the tyrosine phosphorylation of at least two proteins (62 and 80 kDa) in

Fig. 4. Kinetics of phosphorylation on tyrosine of pp160 and insulin-receptor β -subunit

Differentiated 3T3-L1 adipocytes were serum-starved overnight, incubated with either 100 nM-insulin (a) or 100 nM-IGF-1 (b) for various periods of time, and then analysed by SDS/PAGE followed by immunoblotting as described in the Experimental section and Fig. 2 legend. Bands representing pp160 ($\circ$, $\square$) and the β -subunit ($\triangle$, $\circ$) were localized by autoradiography, cut from the nitrocellulose filter, and counted for radioactivity with a gamma counter. For each data point, background counts in bands co-migrating with pp160 and the β -subunit (in duplicate lanes containing extracts from unstimulated cells) were subtracted, and the resultant value was converted into pmol of phosphotyrosine by comparison with a standard curve for tyrosine-phosphorylated glutamine synthetase (see the Experimental section). Virtually identical kinetic results were obtained in three separate experiments. The raw values (c.p.m.) representing phosphotyrosine in pp160 remained fairly constant during the time course (938 ± 84 c.p.m., mean $\pm$ S.E.M.).

addition to pp160 (Fig. 2). In two separate experiments 100 nM-IGF-2 stimulated the tyrosine phosphorylation of pp160 to the same extent as the same concentration of IGF-1 (results not shown).

Phosphotyrosine in pp160 and the insulin-receptor β -subunit is detected only when insulin is added to the intact cell before homogenization, but not when insulin is added at 4 °C during or after cell homogenization (results not shown). Thus insulin-stimulated phosphorylation of pp160 on tyrosine occurs in intact 3T3-L1 adipocytes before cell homogenization, and is not an artifact which occurs during or after disruption of the cells.

The immunoblot procedure with anti-phosphotyrosine antibody is highly specific. Phosphotyrosine (200 μ M) is

Fig. 5. Dependence on insulin and IGF-1 concentrations of phosphorylation (on tyrosine) of pp160 and insulin-receptor β -subunit

Differentiated 3T3-L1 adipocytes were serum-starved and incubated with various concentrations of insulin (a) or IGF-1 (b) for 1 min at 37 °C. Cellular proteins were analysed for their phosphotyrosine content as in Fig. 4: pp160 (○, □), β -subunit (△). The results shown are representative of those obtained in three identical experiments. The raw values (c.p.m.) representing phosphotyrosine in pp116 remained fairly constant during the experiment (677 ± 46 c.p.m., mean $\pm$ S.E.M.).

the only phosphoamino acid which, when co-incubated with anti-phosphotyrosine antibodies, completely abolishes the immunoblot signal from pp160, the insulin-receptor β -subunit and the hormone-insensitive 116 kDa protein (Fig. 3, lane 2). Furthermore, 5 mM-phosphothreonine, -phosphoserine, -phosphoarginine, and 1 mM-phosphohistidine do not interfere with immunoblotting (Fig. 3, lanes 3–6). P_i (5 mM) causes a minor decrement in the immunoblot signal (Fig. 3, lane 7).

Insulin- and IGF-1-activated phosphorylation of pp160 is extremely rapid, the response being maximal within 20–40 s after hormone addition (Fig. 4). Moreover, pp160 remains maximally phosphorylated for up to 60 min in the presence of insulin (results not shown), whereas the response to IGF-1 is relatively transient (Fig. 4b). The β -subunit of the insulin receptor becomes rapidly autophosphorylated ($t_{1/2} = 10$ s) in 3T3-L1 adipocytes after stimulation by insulin (ref. [15], Fig. 4a). The immunoblot data were quantified by comparing the amount of radioactivity associated with either pp160 or

Fig. 6. Kinetics of dephosphorylation of pp160 and insulin-receptor β -subunit after withdrawal of insulin

Differentiated 3T3-L1 adipocytes were serum-starved overnight, incubated with 20 nM-insulin for 5 min at 37 °C, washed three times in warmed culture medium (Dulbecco's modified Eagle's), and then incubated for the indicated periods of time at 37 °C in fresh medium pre-equilibrated for 10 min in the CO_2 incubator. At the end of each incubation, the extents of phosphorylation of pp160 and insulin-receptor β -subunit were analysed as in Fig. 4. The raw values (c.p.m.) representing phosphotyrosine in pp116 were relatively unaffected by the removal of insulin (874, 942, 1116, 776 and 847 c.p.m. at 0, 1, 5, 10 and 15 min respectively).

the β -subunit to that associated with dot-blot (on the same nitrocellulose filter) of known amounts of glutamine synthetase [41], a model protein containing one phosphotyrosine residue per 50 kDa monomer (see the Experimental section, reference [41], and Fig. 1). It can be calculated that a saturating concentration of insulin stimulates the incorporation of phosphate groups into tyrosine residues to the extent of approx. 3×10^5 sites/cell for pp160 and 0.6×10^5 sites/cell for the β -subunit of the insulin receptor (Fig. 4a). These values should be viewed as first approximations, since the calculations used to generate them incorporate several assumptions: (i) that the anti-phosphotyrosine antibodies bind phosphotyrosyl residues of denatured pp160, insulin-receptor β -subunit and glutamine synthetase adsorbed to nitrocellulose with similar affinities; (ii) that pp160 and the insulin-receptor β -subunit transfer from the gel to the nitrocellulose filter quantitatively; and (iii) that phosphotyrosyl-protein phosphatase activity is completely quenched in Buffer A at 4 °C during the 5–10 min required to generate the samples.

Saturating concentrations of IGF-1 stimulate phosphorylation of pp160 to only 50% of the extent observed with insulin (Fig. 4b). A small amount of a 95 kDa phosphotyrosyl-protein is detected in cells stimulated with IGF-1 (Figs. 2 and 4b) and IGF-2 (Fig. 2). In these cases, however, the extent of phosphorylation

Fig. 7. pp160 is a soluble cytoplasmic protein that can become membrane-associated

Serum-starved differentiated 3T3-L1 adipocytes were incubated for 5 min at 37 °C with 100 nM-insulin. The cells were homogenized in Buffer A containing either 0.1 mM- (a) or 1.0 mM (b) Zn^{2+} , after which nuclei and cellular debris were removed by low-speed centrifugation. Membranes were then pelleted at 210000 g for 5 min as described in the Experimental section. The postnuclear supernatant (lane 1), cytosol (lane 2) and membranes (lane 3) from 5×10^6 cells were subjected to immunoblotting with anti-phosphotyrosine antibody and analysed as in Fig. 4.

of the 95 kDa protein was insufficient for analysis. Previous work [15] with 3T3-L1 adipocytes showed that IGF-1 and IGF-2 do not activate phosphorylation of the β -subunit of the insulin receptor, as determined by two-dimensional non-reducing/reducing gel analysis.

The concentration-dependence of hormone-activated pp160 and β -subunit phosphorylation is illustrated in Fig. 5. Half-maximal phosphorylation of pp160 occurs in the presence of 4 nM-insulin or 20 nM-IGF-1. β -Subunit phosphorylation is half-maximal in the presence of 12 nM-insulin, in agreement with previous reports [32]. Preliminary studies indicate that IGF-2 also stimulates phosphorylation of pp160 on tyrosine, with kinetics, concentration-dependence and a maximal response similar to that of IGF-1 (results not shown).

The effects of insulin on pp160 and receptor β -subunit phosphorylation are rapidly reversed, i.e. within 10–15 min, after removal of a saturating concentration of insulin (Fig. 6). As in the previous time course and dose-response experiments (Figs. 4 and 5), the 116 kDa phosphoprotein provides an internal control, since its extent of phosphorylation on tyrosine remains relatively constant after either the addition (Fig. 2) or the removal (Fig. 6) of insulin.

As shown in Fig 7(a), pp160, phosphorylated on tyrosine, fulfils the criteria for a soluble cytoplasmic protein under conditions in which 3T3-L1 membranes are sedimented in the presence of a low concentration (0.1 mM) of or no (results not shown) Zn^{2+} . These centrifugation conditions are sufficient to sediment quantitatively integral membrane proteins, such as the insulin receptor. The apparent cytosolic location of pp160 is maintained whether the cell homogenate is derived from insulin-, IGF-1- or IGF-2-stimulated cells, even when the cells are harvested within 10 s of hormone

addition. These findings strongly suggest that phosphorylated pp160 is a soluble cytoplasmic protein. It is therefore noteworthy that, in the presence of 1 mM- Zn^{2+} , pp160 becomes entirely membrane-associated (Fig. 7b). Moreover, the soluble form of pp160, previously separated from membranes, does not sediment in the presence of 1 mM- Zn^{2+} , indicating that soluble pp160 is not merely precipitated by 1 mM- Zn^{2+} . The addition of 0.1–1.0 mM- Mg^{2+} or $-\text{Ca}^{2+}$ results in a progressive loss of phosphotyrosine in pp160 (apparently as a result of enhanced phosphatase activity), without any evident increase in membrane association (results not shown).

DISCUSSION

We have identified a 160 kDa cytoplasmic protein in intact 3T3-L1 adipocytes that becomes rapidly phosphorylated on tyrosine in response to physiological concentrations of insulin and IGF-1 (Figs. 4 and 5). By a semi-quantitative immunoblotting procedure using anti-phosphotyrosine antibodies (Fig. 1), it was determined that approx. 3×10^5 molecules of phosphate become covalently bound to tyrosyl residues of pp160, and 0.6×10^6 molecules to tyrosyl residues of the insulin-receptor β -subunit, per cell in response to maximal stimulation by insulin (Fig. 4a). Under the steady-state conditions employed in these studies, there are approx. 10^5 high-affinity insulin-binding sites per 3T3-L1 adipocyte [45,47]; thus there is approximate agreement between the number of insulin receptors (i.e. insulin-binding sites) per cell and the number of phosphotyrosine residues in the β -subunit per cell as determined by immunoblotting. These findings represent the first reported semi-quantitative measurement of the extent of insulin-stimulated protein phosphorylation in an intact-cell system.

Our results complement the previous studies by Bernier *et al.* [32], which demonstrated insulin-stimulated tyrosine phosphorylation of a 15 kDa (pp15) protein in phenylarsine oxide-treated 3T3-L1 adipocytes. Indeed, pp15 can be observed on immunoblots (with anti-phosphotyrosine antibodies) of extracts from 3T3-L1 adipocytes treated with both insulin and phenylarsine oxide (D. H. Madoff & M. D. Lane, unpublished work). In fact, the kinetics and insulin-concentration dependence of pp15 tyrosine phosphorylation detected in this manner were comparable with those previously reported [32]. Conversely, Bernier *et al.* demonstrated insulin-independent phosphorylation of at least one high-molecular-mass polypeptide in 3T3-L1 adipocytes treated with insulin, but not with phenylarsine oxide (Fig. 1B in reference [32]). Although this polypeptide was not further characterized, it may prove to be pp160.

The present work suggests that pp160 is a cellular substrate of the insulin-receptor tyrosine kinase in the intact 3T3-L1 adipocyte. pp160 is phosphorylated on tyrosine at a rate that closely parallels the rate of autophosphorylation of the insulin receptor's β -subunit (Fig. 4a), a process known to activate the receptor's tyrosine kinase activity toward exogenous substrates [15–20]. The phosphorylation of pp160 is equally, if not more sensitive, to insulin ($\text{ED}_{50} = 4$ nM) than is β -subunit phosphorylation ($\text{ED}_{50} = 12$ nM) (Fig. 5a), suggesting that there may be rapid amplification of the insulin-stimulated signal (i.e. tyrosine kinase activity) between β -subunit autophosphorylation and the phos-

Table 1. Hormone-stimulated tyrosine phosphorylation and biological responses in 3T3-L1 adipocytes

The hormone concentration is that required for half-maximal or maximal biological activity in intact 3T3-L1 adipocytes.

Hormone	Biological activity	Hormone concn. (nM)		Reference
		Half-maximal	Maximal	
Insulin	Cell surface binding	6	20	D. H. Madoff & M. D. Lane (unpublished work)
	β -Subunit autophosphorylation	8	50	[15]
		12	100	The present paper
	pp160 tyrosine phosphorylation	4	20	The present paper
	2-Deoxyglucose uptake	5	30	[15]
	Glucose oxidation	0.3	10	[56]
	S6 kinase activation	1	1000	[57]
IGF-1	pp160 tyrosine phosphorylation	20	50	The present paper
	2-Deoxyglucose uptake	3	50	[15]

phorylation of pp160. Definitive evidence that pp160, or any other putative substrate of the receptor kinase [26,27,31,32], is directly phosphorylated by the insulin receptor has not yet been demonstrated.

When insulin is withdrawn from maximally stimulated cells, the phosphotyrosine content of pp160 and of the insulin-receptor β -subunit decays at similar rates (Fig. 6). The turnover rate of phosphate groups on tyrosyl residues of the β -subunit on withdrawal of insulin approximates to that previously reported by this laboratory [15]. This phenomenon probably reflects phosphotyrosyl-protein phosphatase activity in the absence of insulin-stimulated kinase activity.

IGF-1 stimulates the phosphorylation of tyrosyl residues on pp160 to an extent 50% of that obtained with insulin (Fig. 4). Studies are needed to determine whether the 160 kDa protein that becomes phosphorylated on tyrosine in response to insulin is the same 160 kDa protein that undergoes phosphorylation in response to IGF-1. It is of interest that the kinetics of pp160 phosphorylation in response to IGF-1 differ markedly from those observed in response to insulin. Incubation of 3T3-L1 adipocytes with IGF-1 results in peak stimulation after 45–60 s, returning to 40% of maximum after 5 min (Fig. 4b), despite the continued presence of the growth factor. The results with IGF-2 are similar (D. H. Madoff & M. D. Lane, unpublished work). A similar kinetic pattern was observed by Izumi *et al.* [27] for IGF-1-stimulated tyrosine phosphorylation of a 185 kDa protein in NRK cells, and by Häring *et al.* [31] for insulin-stimulated tyrosine phosphorylation of a membrane-associated 46 kDa protein in rat adipocytes. The latter investigators did not detect tyrosine-phosphorylated protein(s) of higher molecular mass, such as pp160, in this phylogenetically related adipocyte. The kinetic results have at least two plausible explanations. At early times after hormone addition, substrate phosphorylation may predominate over dephosphorylation; however, at later times phosphatase activity may increase (perhaps as a result of hormonal stimulation), allowing dephosphorylation to dominate. There is indeed precedent for ligand-stimulated phosphatase activation in the phosphoinositol pathway [48]. Alternatively, rapid ligand-dependent receptor desensitization could occur, thereby decreasing kinase activity and lowering the

steady-state amount of phosphotyrosine in pp160. In fact, serine and/or threonine phosphorylation of the insulin receptor β -subunit *in situ* has been associated with a decrease in insulin-stimulated tyrosine kinase activity [49–51] and is a potential mechanism for the latter hypothesis. However, work in our laboratory has failed to detect any change in [32 P]phosphoserine amounts within the β -subunit as a consequence of stimulation by insulin [15].

pp160 does not appear to represent any of the characterized peptide hormone receptors, since it is a soluble cytoplasmic protein in the phosphorylated state (Fig. 7a) and does not bind to either wheat-germ-agglutinin-Sepharose or concanavalin A-Sepharose (D. H. Madoff & M. D. Lane, unpublished work), and thus does not appear to contain complex or high-mannose oligosaccharide chains. This phosphorylated protein is immunoprecipitated with anti-phosphotyrosine antibodies and is specifically released from the immune complex with phosphotyrosine. pp160 is, however, not immunoprecipitated with anti-(insulin receptor) antibodies under conditions which quantitatively immunoprecipitate the insulin receptor. Moreover, pp160 does not appear to exist in a disulphide-linked oligomeric structure with itself or other proteins, since it migrates on the diagonal in two-dimensional non-reducing/reducing SDS/PAGE (results not shown; see [15]).

There are several similarities between pp160 and the 185 kDa protein (pp185) identified by White *et al.* [26] and further characterized by Izumi *et al.* [27]. Both proteins are soluble cytoplasmic constituents (Fig. 7a); ref. [52]) and are rapidly phosphorylated on tyrosine in response to physiological concentrations of both insulin in IGF-1. pp185 has been found in NRK, MDCK and Fao hepatoma cells [26,27,52] and KB cells [53], whereas we have demonstrated pp160 in 3T3-L1 adipocytes as well as in differentiated BC3H-1 myocytes (D. H. Madoff & M. D. Lane, unpublished work). In addition, Gibbs *et al.* [28] have observed a similar 180 kDa protein in 3T3-L1 adipocytes, and Beguinot & Smith [29] have detected a 175 kDa protein in L6 myocytes. Although it has not been possible to compare directly the relative migration of pp160 and pp185 in SDS/PAGE, the data in Fig. 2 strongly suggest that the molecular-mass assignment of pp160 is accurate; whereas pp185 in KB

cells migrates slower than the putative EGF receptor [53], pp160 in 3T3-L1 adipocytes migrates more rapidly than do either the EGF or PDGF receptors (Fig. 2). Whether these proteins subserve similar biochemical roles in insulin and IGF-1 action, and what these roles may be, remain to be determined.

Under appropriate experimental conditions the soluble form of pp160 becomes membrane-bound (Fig. 7b), thus raising the interesting possibility that this protein cycles between the plasma membrane and the cytosol as a result of hormonal stimulation in intact cells. The association of pp160 with cellular membranes is promoted by 1 mM- Zn^{2+} . It is possible that dephospho-pp160 is plasma-membrane-associated, and on undergoing phosphorylation dissociates from the membrane. This could facilitate translocation of phosphotyrosyl-pp160 to the cytoplasmic compartment, where it may serve as an intermediary in signal transmission. Activated pp160 could then recycle back to the plasma membrane either before or following dephosphorylation. In support of the hypothesis is the fact that crude membranes from insulin-stimulated 3T3-L1 adipocytes can carry out the phosphorylation on tyrosine of a 160 kDa membrane-associated protein (D. H. Madoff & M. D. Lane, unpublished work). With this broken-cell system, approx. 0.6×10^5 molecules of phosphate are incorporated per cell into tyrosine residues in both the 160 kDa protein and the β -subunit. This is in fair agreement with the results obtained for intact 3T3-L1 adipocytes (Fig. 4a). Although the bivalent-cation-dependent association of pp160 with cellular membranes remains to be further investigated, it is possible that this property is a general feature of cellular substrates of receptor tyrosine kinases. Indeed, the 35 kDa substrate of the EGF receptor becomes reversibly membrane-bound in a Ca^{2+} -dependent fashion [54,55].

The results summarized in Table 1 demonstrate that the hormone concentrations required for half-maximal and maximal pp160 tyrosine phosphorylation are similar to those required for the activation of several well-characterized biological functions in 3T3-L1 adipocytes. These data, in conjunction with the time course for pp160 tyrosine phosphorylation (Fig. 4), are consistent with the view that pp160 is a physiologically important substrate of the insulin and perhaps IGF-1 tyrosine kinases. Work is needed to purify pp160 in order to test our hypotheses and directly define its role in hormone action.

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