Insulin-induced tyrosine dephosphorylation of paxillin and focal adhesion kinase requires active phosphotyrosine phosphatase 1D

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Insulin stimulation of fibroblasts rapidly induces the tyrosine dephosphorylation of proteins of 68 kDa and 125 kDa, in addition to the tyrosine phosphorylation of the insulin receptor β -chain, insulin receptor substrates 1 and 2, and Shc. Using specific antibodies, the 68 kDa and 125 kDa proteins were identified as paxillin and focal adhesion kinase (pp125^{FAK}) respectively. We have examined whether dephosphorylation of paxillin and pp125^{FAK} requires interaction of the cells with the extracellular matrix. For this, cells were grown on poly(L-lysine) plates, and the tyrosine phosphorylation of pp125^{FAK} and paxillin was increased by addition of lysophosphatidic acid. Under these conditions, insulin still induced the complete dephosphorylation of pp125^{FAK} and paxillin, indicating that this process can occur

independently of the interaction of integrins with extracellular matrix proteins. We also studied whether dephosphorylation of pp125^{FAK} and paxillin results from the action of a phosphotyrosine phosphatase. It was found that phenylarsine oxide, a phosphotyrosine phosphatase inhibitor, prevented the insulin-induced dephosphorylation of pp125^{FAK} and paxillin. Furthermore, this insulin-induced dephosphorylation was also impaired in cells expressing a dominant-negative mutant of phosphotyrosine phosphatase 1D (PTP 1D). Thus we have identified paxillin as a target for dephosphorylation by insulin. In addition, we have obtained evidence that the insulin-mediated dephosphorylation of paxillin and pp125^{FAK} requires active PTP 1D.

INTRODUCTION

The insulin receptor is a member of the receptor tyrosine kinase family of cell surface receptors [1,2]. Among the cellular effects mediated by insulin are the stimulation of glucose uptake, and of glycogen, lipid and DNA synthesis [3,4]. Binding of insulin to its receptor results in autophosphorylation of the insulin receptor β -chain, of its substrates insulin receptor substrate (IRS) 1 and 2, and of the Shc proteins [5–8]. Tyrosine phosphorylation of IRS1, IRS2 and Shc facilitates the binding of SH2-domain-containing proteins [9].

In the case of Shc, tyrosine phosphorylation mediates the formation of the complex between growth-factor-receptor-bound protein 2 (Grb2) and mammalian son of sevenless (mSOS), the latter being a guanine nucleotide exchange factor for p21^{ras} [10–14]. Several lines of evidence suggest that the formation of a Shc–Grb2–mSOS complex reflects a major step in insulin-induced p21^{ras}–GTP formation [15–17]. The formation of p21^{ras}–GTP is involved in the stimulation of the extracellular-signal-regulated kinase (ERK) pathway leading to gene expression. p21^{ras}–GTP is not involved in metabolic signalling, as expression of dominant-negative p21^{ras(Asn-17)} does not interfere with insulin-stimulated glucose uptake or glycogen synthesis [18,19].

Tyrosine-phosphorylated IRS1 associates with the 85 kDa subunit of phosphatidylinositol 3'-kinase (PI 3-kinase), Grb2, dynamin, Nck, phosphotyrosine phosphatase 1D (PTP 1D; also known as Syp, PTP 2C and SH-PTP2) and integrins [20–26]. The

binding of PI 3-kinase to tyrosine-phosphorylated IRS1 reflects the main step in insulin-induced PI 3-kinase activation [27,28]. Activated PI 3-kinase contributes to the activation of the 70/85 kDa ribosomal S6 kinase [29–31]. Inhibition of PI 3-kinase by the fungal metabolite wortmannin is paralleled by inhibition of insulin-induced glucose uptake, glycogen synthesis and lipogenesis, implicating PI 3-kinase in metabolic signalling [18,32–35]. PTP 1D probably acts as a positive regulator by dephosphorylating phosphotyrosines that negatively regulate signalling mediators such as the Src family of kinases [36,37]. In support of a positive role in signalling, it was found that insulin-induced activation of the p21^{ras}/ERK pathway requires the action of a phosphotyrosine phosphatase (PTPase) [38], probably PTP 1D [39–41].

In addition to stimulating the tyrosine phosphorylation of Shc proteins and IRS1, insulin mediates the tyrosine dephosphorylation of focal adhesion kinase (pp125^{FAK}) [40,42]. pp125^{FAK} is a cytosolic tyrosine kinase that is predominantly localized in focal adhesions, where it is bound to the cytosolic part of the β -integrin subunit and to several cytoskeletal proteins, such as tensin and paxillin. Clustering of integrins, as a result of adherence of the cells to extracellular matrix proteins, leads to the activation and tyrosine phosphorylation of pp125^{FAK} [43]. Tyrosine phosphorylation of pp125^{FAK} is implicated in the formation of focal adhesions and the assembly of stress fibres [43].

In the present study, the mechanism of insulin-mediated

Abbreviations used: pp125^{FAK}, focal adhesion kinase; PAO, phenylarsine oxide; IRS1, insulin receptor substrate 1; PTPase, phosphotyrosine phosphatase; PTP 1D, phosphotyrosine phosphatase 1D; Grb2, growth-factor-receptor-bound protein 2; mSOS, mammalian son of sevenless; ERK, extracellular-signal-regulated kinase; PI 3-kinase, phosphatidylinositol 3'-kinase; LPA, lysophosphatidic acid; DMEM, Dulbecco's modified Eagle's medium.

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pp125^{FAK} dephosphorylation was investigated. Evidence is provided that insulin-induced pp125^{FAK} dephosphorylation involves the action of PTP 1D rather than an altered interaction of the cells with the extracellular matrix. In addition, we observed the tyrosine dephosphorylation of a 68 kDa protein. Using specific antibodies, this protein was identified as the pp125^{FAK} substrate paxillin.

MATERIALS AND METHODS

Materials

Phenylarsine oxide (PAO) and poly(L-lysine) were purchased from Sigma (St. Louis, MO, U.S.A.); bovine insulin, hygromycin B and BSA were from Boehringer Mannheim (Mannheim, Germany); lysophosphatidic acid (1-acyl-*sn*-glycerol-3-phosphate; LPA) was kind donated by Dr. W. Moolenaar (Netherlands Cancer Institute, Amsterdam, The Netherlands); Protein G- and Protein A-Sepharose beads were from Pharmacia Biotech (Uppsala, Sweden); enhanced chemiluminescence detection reagents were from Amersham International (Amersham, Bucks., U.K.); lipofectamine, tissue culture media and fetal calf serum were from Life Technologies (Gaithersburg, MD, U.S.A.). Stock solutions of PAO were prepared in DMSO and diluted before use in PBS.

Cell lines

The NIH3T3-derived cell line A14 overexpressing human insulin receptors has been described previously [44]. A14 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 9% (v/v) fetal calf serum. Cell lines overexpressing wild-type PTP 1D were constructed by stable transfection of A14 cells in a 60 mm dish with 1 μ g of a pRK5 plasmid containing PTP 1D cDNA under the control of the cytomegalovirus promoter [45], and 0.1 μ g of pHyg, which contains the hygromycin B phosphotransferase gene, using 10 μ g of lipofectamine. Cell lines expressing dominant-negative PTP 1D were constructed by transfection with 1 μ g of a pRK5 plasmid containing mutant PTP 1D cDNA, in which the triplet encoding Cys-463 (TGC) was changed to that encoding alanine (GCC) [46], and 0.1 μ g of pHyg. Transfected cells were cultured in DMEM containing 9% fetal calf serum and 200 μ g/ml hygromycin B. Colonies were isolated 2–3 weeks after transfection, and identified by immunoblotting of total cell lysates with a polyclonal antibody against PTP 1D. In addition, positive colonies containing mutant PTP 1D were analysed for an impaired insulin-induced phosphorylation of ERK, as described previously [15]. In some experiments, cells were plated for 8 h on dishes coated with 0.1 mg/ml poly(L-lysine). Before each experiment, the cells were serum-arrested for at least 18 h on DMEM containing 0.5% BSA.

Antibodies

Monoclonal anti-pp125^{FAK} antibody 2A7, polyclonal anti-pp125^{FAK} antiserum and polyclonal anti-(PTP 1D) antiserum were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Monoclonal anti-phosphotyrosine antibody PY20 conjugated to horseradish peroxidase, and polyclonal anti-ERK1 antiserum, recognizing both the 42 kDa and 44 kDa isoforms of mitogen-activated protein kinase, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal anti-paxillin antibody was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Polyclonal affinity-purified anti-phosphotyrosine antibodies were described previously [15].

Immunoprecipitations and Western blotting

Serum-arrested cultures grown in 90 mm dishes were incubated with insulin as described in the Results section, rinsed twice with ice-cold PBS containing 1 mM sodium orthovanadate, and lysed in buffer as described before [15]. Lysates were cleared by centrifugation and incubated for 4 h with monoclonal anti-pp125^{FAK} antibody 2A7 (1.5 μ g), monoclonal anti-paxillin antibody (2.0 μ g), polyclonal anti-(PTP 1D) antiserum (5.0 μ g) or polyclonal affinity-purified anti-phosphotyrosine antibodies. pp125^{FAK} and paxillin immune complexes were collected on Protein G-Sepharose beads, whereas PTP 1D and anti-phosphotyrosine immune complexes were collected on Protein A-Sepharose beads. Collected immune complexes were washed four times with lysis buffer [15] and twice with 10 mM Tris/HCl (pH 7.5), 1 mM EDTA, after which the complexes were dissolved in SDS/PAGE sample buffer (10% glycerol, 5% β -mercaptoethanol, 3% SDS, 0.1 M Tris/phosphate, pH 6.8, and 0.01% Bromphenol Blue), and resolved on SDS/PAGE. After SDS/PAGE, proteins were transferred to Immobilon membranes (Millipore, Bedford, MA, U.S.A.) by Western blotting. Filters were incubated with anti-phosphotyrosine antibody PY20 conjugated to horseradish peroxidase, or with polyclonal anti-pp125^{FAK} antiserum followed by incubation with goat anti-(rabbit IgG) conjugated to horseradish peroxidase. Bound horseradish peroxidase was visualized by enhanced chemiluminescence as described before [15].

RESULTS

Insulin-induced tyrosine dephosphorylation of pp125^{FAK} and paxillin

When quiescent A14 cells were incubated with insulin (15 min), anti-phosphotyrosine immunoblots revealed the tyrosine dephosphorylation of a 125 kDa protein (pp125) and of a 68 kDa protein (pp68). In addition, tyrosine phosphorylation of IRS1 and the insulin receptor β -chain was stimulated (Figure 1).

Recently, other groups have reported the tyrosine dephosphorylation of pp125^{FAK} in response to insulin [40,42]. To verify whether our pp125 was identical to pp125^{FAK}, lysates from quiescent and insulin-stimulated A14 cells were immunoprecipitated with a monoclonal antibody directed against

Figure 1 Insulin-induced tyrosine dephosphorylation of pp125 and pp68

Quiescent A14 cells were kept quiescent or incubated with insulin (1 μ M; 10 min). Total cell lysates (30 μ g of protein/lane) were analysed on a Western blot and probed with monoclonal anti-phosphotyrosine antibody PY-20 conjugated to horseradish peroxidase. The arrows indicate the positions of pp125, pp68, IRS1 and the insulin receptor (IR) β -chain. IRS1 and the IR β -chain were identified by reprobing the filter with specific antibodies directed against these proteins; —, quiescent cells; +, insulin-stimulated cells. The Western blot shown is a representative of three experiments.

Figure 2 Identification of pp125 as pp125^{FAK}

Lysates of A14 cells were immunoprecipitated with the monoclonal anti-pp125^{FAK} antibody 2A7 (αFAK). The immunoprecipitates were analysed by Western blotting using monoclonal anti-phosphotyrosine antibody PY-20 conjugated to horseradish peroxidase (αPY). Aliquots (25%) of the pp125^{FAK} immunoprecipitates were analysed with polyclonal anti-pp125^{FAK} antiserum, in order to verify the application of equal amounts of immunoprecipitated pp125^{FAK}. The arrow indicates the position of pp125^{FAK}. —, Quiescent cells; +, insulin-stimulated (2 min; 1 μM) cells. The immunoblot shown is a representative of three experiments.

Figure 3 Identification of pp68 as paxillin

Lysates of quiescent (lanes 1, 3, 5 and 7) or insulin-stimulated (10 min; 1 μM) (lanes 2, 4, 6 and 8) A14 cells were immunoprecipitated (i.p.) with monoclonal anti-paxillin antibody (lanes 1–4), polyclonal affinity-purified anti-phosphotyrosine antibody (P-Tyr; lanes 5 and 6) or monoclonal anti-pp125^{FAK} antibody 2A7 (FAK; lanes 7 and 8). The immunoprecipitates were analysed by Western blotting with polyclonal affinity-purified anti-phosphotyrosine antibody (lanes 1 and 2) or with monoclonal anti-paxillin antibody (lanes 3–8). The immunoblots shown are representative of two independent observations.

pp125^{FAK}. Subsequent analysis of Western blots containing pp125^{FAK} immunoprecipitates with anti-phosphotyrosine antibodies confirmed the tyrosine dephosphorylation of pp125^{FAK} in response to insulin (Figure 2). Aliquots of the pp125^{FAK} immunoprecipitates were analysed on Western blots with polyclonal antiserum against pp125^{FAK} to verify that equal amounts of pp125^{FAK} had been applied (Figure 2).

For the identification of pp68, anti-paxillin antibodies were tested for their ability to react with pp68 on Western blots, based on reports that the tyrosine phosphorylation state of pp125^{FAK} is closely correlated with that of the 68 kDa protein paxillin [47–50]. In total cell lysates, paxillin was detected as a diffuse band, probably due to phosphorylation of the protein. Tyrosine-phosphorylated pp68 was found to co-migrate with the upper part of the broad paxillin band. To positively identify pp68 as paxillin, lysates from quiescent and insulin-incubated A14 cells were immunoprecipitated with monoclonal antibodies against paxillin. Analysis of the paxillin immunoprecipitates from quiescent cell lysates with polyclonal anti-phosphotyrosine antibodies revealed a discrete band, migrating at a position corresponding to the upper part of the broad paxillin band observed in anti-paxillin immunoprecipitates (Figure 3). In lysates from insulin-stimulated cells, tyrosine phosphorylation of paxillin was decreased (Figure 3). An aliquot of the paxillin immunoprecipitates was used to verify the loading of equal amounts of

Figure 4 Insulin-induced dephosphorylation of paxillin and pp125^{FAK} occurs independently of the interaction of integrins with extracellular matrix proteins

Shown is an anti-phosphotyrosine Western blot of total cell lysates (30 μg of protein/lane) of A14 cells grown on poly(L-lysine) plates. Cells were kept quiescent (lane 1), or incubated with 1 μM LPA (lanes 2 and 3; 2 min and 12 min respectively) and/or 1 μM insulin (lane 4, 2 min with LPA followed by 10 min with insulin; lane 5, 10 min with insulin). Arrows indicate the positions of IRS1, pp125^{FAK} (pp125), the insulin receptor (IR) β-chain and paxillin (pp68), as verified by reprobing the stripped filter with specific antibodies directed against these proteins. The immunoblot shown is representative of two independent observations.

paxillin (Figure 3). Similar results showing insulin-induced dephosphorylation of paxillin were obtained when anti-phosphotyrosine immunoprecipitates were stained with anti-paxillin antibodies (Figure 3).

To investigate the presence of a protein complex between pp125^{FAK} and paxillin in quiescent cells, lysates from quiescent and insulin-treated A14 cells were immunoprecipitated with monoclonal anti-pp125^{FAK} antibodies. As shown in Figure 3, paxillin was co-precipitated with pp125^{FAK} in quiescent cells. In insulin-stimulated cells, however, this complex was partially disrupted (Figure 3).

Insulin-induced dephosphorylation of pp125^{FAK} and paxillin occurs independently of the interaction of integrins with extracellular matrix proteins

The degree of tyrosine phosphorylation of pp125^{FAK} and paxillin is increased upon interaction of integrins with extracellular matrix proteins [51–53]. Because A14 cells round up after insulin stimulation (D. M. Ouwens, H. M. M. Mikkers and J. A. Maassen, unpublished work), the interaction of integrins on the cell surface with extracellular matrix proteins is likely to change. This may result in an altered tyrosine phosphorylation state of pp125^{FAK} and paxillin. To examine whether the insulin-mediated tyrosine dephosphorylation of pp125^{FAK} and paxillin requires interaction of the cells with the extracellular matrix, A14 cells were grown on poly(L-lysine) dishes. The cells were grown on the plates for 8 h in order to allow *de novo* synthesis of a functional insulin receptor, which is digested by trypsinization of cultured cells. Figure 4 shows that some tyrosine phosphorylation on pp125^{FAK} was observed in total cell lysates from quiescent cells. Tyrosine phosphorylation on pp125^{FAK} and paxillin was induced by treatment with LPA, a phospholipid which does not act via integrins but exerts its effects through a G-protein-coupled receptor [54,55]. In addition, an unidentified 60 kDa protein was tyrosine-phosphorylated in response to LPA treatment (Figure 4). When LPA treatment was followed by incubation with insulin, complete dephosphorylation of pp125^{FAK} and paxillin was observed. pp60 was also dephosphorylated. These findings indicate that the insulin-induced tyrosine dephosphorylation of pp125^{FAK} and paxillin can occur independently of the interaction of integrins with extracellular matrix proteins.

Figure 5 Inhibition of insulin-induced pp125^{FAK} dephosphorylation by PAO

Shown is an anti-phosphotyrosine Western blot of total cell lysates (30 µg of protein/lane) of A14 cells. The cells were incubated with PAO (5 min; 25 µM) prior to insulin stimulation (5 min; 100 nM). Arrows indicate the positions of IRS1, pp125^{FAK} (pp125), the insulin receptor (IR) β-chain and paxillin (pp68), as verified by reprobing the stripped filter with specific antibodies directed against these proteins. Lanes: —, cells incubated with PAO alone; +, cells incubated with PAO plus insulin. The immunoblot shown is a representative of three experiments.

Insulin-induced dephosphorylation of pp125^{FAK} and paxillin requires active PTP 1D

Evidence is emerging that some insulin-induced cellular responses require the action of a PTPase [38–40]. To study whether activation of a PTPase by insulin may be involved in the dephosphorylation of pp125^{FAK} and paxillin, cells were incubated with the PTPase inhibitor PAO, prior to insulin stimulation. PAO treatment alone slightly increased tyrosine phosphorylation on pp125^{FAK} and paxillin. Incubation of the cells with PAO prevented the insulin-induced tyrosine dephosphorylation of

pp125^{FAK} and paxillin, suggesting a role for a PTPase in pp125^{FAK} and paxillin dephosphorylation (Figure 5).

Regarding pp125^{FAK} dephosphorylation, PTP 1D is a putative candidate to be involved. Previous studies have shown that PTP 1D is activated by insulin treatment, and have implicated this PTPase in early post-receptor effects of the insulin receptor, upstream of the p21^{ras}/ERK pathway [39–41]. In addition, it has been reported that PAO inhibits the activity of PTP 1D *in vitro* [39].

To study the role of PTP 1D in the insulin-induced dephosphorylation of paxillin and pp125^{FAK} in detail, we generated A14 cell lines stably expressing wild-type PTP 1D or a mutant PTP 1D in which Cys-463 was substituted by alanine. Cys-463 is located in the highly conserved HCSAGXXR(S/T)G motif in the PTPase domain [45,56,57]. Both Cys-463 and Arg-469 are implicated in phosphate binding and are crucial for catalytic activity [58]. As a control, A14 cells overexpressing wild-type PTP 1D were constructed. Expression of wild-type or mutant PTP 1D was assessed by immunoblotting of total cell lysates with anti-(PTP 1D) antibodies (Figure 6a). The mutant PTP 1D protein acted as a dominant-negative mutant with respect to activation of the p21^{ras}/ERK pathway, as insulin-induced phosphorylation of ERK was impaired (Figure 6b) [46].

The expression of wild-type or dominant-negative PTP 1D affected the basal tyrosine phosphorylation states of pp125^{FAK} and paxillin. An anti-phosphotyrosine Western blot revealed that overexpression of wild-type PTP 1D slightly reduced the basal phosphotyrosine content on pp125^{FAK} and paxillin, whereas an increase was seen in cells expressing dominant-negative PTP 1D. Insulin stimulation of cells overexpressing wild-type PTP 1D induced a dose-dependent dephosphorylation of pp125^{FAK} and

Figure 6 Insulin-induced dephosphorylation of pp125^{FAK} requires active PTP 1D

(a) Anti-(PTP 1D) Western blot of total cell lysates of parental A14 cells (lane 1), A14 cells overexpressing wild-type PTP 1D (lane 2) and A14 cells expressing dominant-negative PTP 1D (lane 3). The immunoblot shown is representative of three experiments. (b) Anti-ERK Western blot of total cell lysates of A14 cells, A14 cells overexpressing wild-type (WT) PTP 1D and A14 cells expressing dominant-negative (C463A) PTP 1D. Cells were kept quiescent (—) or were stimulated with insulin (5 min; 100 nM) (+). The immunoblot shown is representative of two experiments. (c, d) Anti-phosphotyrosine immunoblots of total cell lysates (30 µg of protein/lane) of A14 cells overexpressing wild-type PTP 1D (c), and A14 cells expressing dominant-negative PTP 1D (d). Cells were kept quiescent (lane 1), or incubated for 15 min with increasing concentrations of insulin (lanes 2–6; 0.1 nM, 1 nM, 10 nM, 100 nM and 1 µM insulin respectively). The arrows indicate the positions of IRS1, pp125^{FAK} (pp125), the insulin receptor (IR) β-chain, paxillin (pp68) and Shc, as verified by reprobing the stripped filter with specific antibodies directed against these proteins. The immunoblots shown are representative of two experiments.

Figure 7 Co-precipitation of pp125^{FAK} and PTP 1D

Total cell lysates of quiescent and insulin-stimulated A14 cells were immunoprecipitated with polyclonal anti-(PTP 1D) antiserum. Shown is an anti-pp125^{FAK} Western blot of the immunoprecipitates; —, quiescent cells; +, insulin-stimulated (10 min; 1 μ M) cells. The immunoblot shown is a representative of three experiments.

paxillin (Figure 6c). In cells harbouring dominant-negative PTP 1D, insulin only slightly attenuated the tyrosine phosphorylation states of paxillin and pp125^{FAK} (Figure 6d). The identities of pp125^{FAK} and paxillin were confirmed by immunoprecipitation of the proteins followed by anti-phosphotyrosine Western blotting (results not shown). Similar results were obtained with two different clonal cell lines. These results implicate a requirement for active PTP 1D in the insulin-induced dephosphorylation of pp125^{FAK} and paxillin.

Regarding the inhibitory effect of dominant-negative PTP 1D on insulin-induced pp125^{FAK} dephosphorylation, we examined whether PTP 1D is found in a complex with pp125^{FAK}. For this, PTP 1D immunoprecipitates were analysed for co-precipitation of pp125^{FAK} by Western blotting with polyclonal anti-pp125^{FAK} antiserum. In quiescent A14 cells, a complex between pp125^{FAK} and PTP 1D was observed (Figure 7). This complex was disrupted after insulin stimulation of the cells, suggesting that the complex is mediated by interactions between phosphotyrosine and SH2 domain-containing proteins.

DISCUSSION

Insulin stimulation of cells induces a variety of responses such as glucose uptake, glycogen formation, the stimulation of DNA, lipid and protein synthesis, and a reorganization of the actin cytoskeleton [3,4,59]. The signal transduction pathways of the insulin receptor involve several proteins which become tyrosine phosphorylated in response to insulin. These proteins include the insulin receptor β -chain, IRS1, IRS2 and Shc [5–8]. In addition to these events, insulin induces the tyrosine dephosphorylation of pp125^{FAK} [40–42].

In the present paper, we describe the tyrosine dephosphorylation of a 68 kDa protein (pp68). The tyrosine phosphorylation state of pp68 closely correlated with the tyrosine phosphorylation state of pp125^{FAK}; both proteins were tyrosine phosphorylated by LPA, and dephosphorylated by insulin with similar kinetics. A close correlation between the tyrosine phosphorylation states of pp68 and pp125^{FAK} has been described previously [47–50]. In those studies, pp68 was identified as paxillin, a protein found in a complex with pp125^{FAK} and c-Src [43,49]. Using specific antibodies, we demonstrated that the 68 kDa protein which was dephosphorylated after insulin stimulation was indeed paxillin. Tyrosine-phosphorylated paxillin and pp125^{FAK} were found in a protein complex which was disrupted after insulin stimulation.

With respect to the insulin-induced dephosphorylation of paxillin and pp125^{FAK}, we examined the possible signalling

pathways contributing to these events. As A14 cells round up after insulin stimulation, we examined whether an altered interaction of integrins with extracellular matrix proteins contributes to insulin-mediated paxillin and pp125^{FAK} dephosphorylation. For this, cells were grown on poly(L-lysine) plates, which abolishes the tyrosine phosphorylation of pp125^{FAK} via integrins. When cells are attached to poly(L-lysine) plates for 40 min, the phosphotyrosine on pp125^{FAK} is absent. In our study, we had to culture the cells on poly(L-lysine) for 8 h to allow *de novo* synthesis of functional insulin receptors, which are digested by trypsinization. After that period of time, some tyrosine phosphorylation of paxillin and pp125^{FAK} was observed, although the phosphotyrosine content on paxillin and pp125^{FAK} was much lower than in cells grown on 'regular' dishes. Under these conditions, phosphorylation of paxillin and pp125^{FAK} was induced by LPA, which acts via a G-protein-coupled receptor. When cells were subsequently incubated with insulin, complete dephosphorylation of paxillin and pp125^{FAK} occurred. We conclude from these results that insulin-mediated paxillin and pp125^{FAK} dephosphorylation does not depend on the interaction of integrins with extracellular matrix proteins.

We next examined the involvement of a PTPase in insulin-mediated pp125^{FAK} and paxillin dephosphorylation. Using PAO, a general PTPase inhibitor, complete inhibition of the insulin-induced dephosphorylation of paxillin and pp125^{FAK} was observed. Recently, evidence was obtained that activation of the p21^{ras}/ERK pathway by insulin requires active PTP 1D [39–41], a PTPase that is sensitive to inhibition by PAO [39]. To study a possible involvement of PTP 1D in insulin-induced paxillin and pp125^{FAK} dephosphorylation, we examined cell lines expressing wild-type or dominant-negative PTP 1D for these events. In cells overexpressing wild-type PTP 1D, the basal tyrosine phosphorylation states of pp125^{FAK} and paxillin were attenuated. In cells expressing mutant PTP 1D, the basal tyrosine phosphorylation states of paxillin and pp125^{FAK} were elevated, and insulin-induced tyrosine dephosphorylation of paxillin and pp125^{FAK} was impaired. An elevation of the basal tyrosine phosphorylation of pp125^{FAK} in CHO cells transiently transfected with the dominant-negative PTP 1D mutant has been reported previously [40]. In that study insulin still induced complete pp125^{FAK} dephosphorylation, which is in contrast to our data using cell lines stably expressing mutant PTP 1D. Possible factors causing this difference may be the level of expression of the mutant PTP 1D protein and the cell lines used (CHO-derived versus NIH3T3-derived).

In view of the known requirement for active PTP 1D in the activation of the p21^{ras}/ERK pathway, the possibility arises that the observed inhibition of insulin-mediated paxillin and pp125^{FAK} dephosphorylation may result from impaired activation of this pathway [39–41]. However, inhibition of the p21^{ras}/ERK pathway by dominant-negative p21^{ras(Asn-17)} [59] had no effect on insulin-mediated paxillin and pp125^{FAK} dephosphorylation. In addition, activation of the p21^{ras}/ERK pathway by EGF had no effect on the tyrosine phosphorylation state of paxillin and pp125^{FAK} (D. M. Ouwers and J. A. Maassen, unpublished work). These observations suggest that dephosphorylation of paxillin and pp125^{FAK} is mediated by a signalling pathway involving PTP 1D that is distinct from the p21^{ras}/ERK pathway (which is also regulated by PTP 1D).

Both pp125^{FAK} and paxillin are implicated in the assembly of focal adhesions and the formation of stress fibres [43,60,61]. Changes in the tyrosine phosphorylation state of these proteins may affect the organization of the cytoskeleton. Recently it was found that insulin stimulates a major reorganization of the actin network, involving regional aggregation of polymerized actin

below the cell surface [62]. It is expected that the insulin-induced dephosphorylation of pp125^{FAK} and paxillin contributes to this response, and our data also indicate that PTP 1D is involved in this process of cytoskeletal reorganization (D. M. Ouwers and J. A. Maassen). To what extent these processes contribute to insulin-mediated responses, such as GLUT4 translocation, remains to be clarified.

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