RESEARCH COMMUNICATION

Insulin and insulin-like growth factor-1 stimulate dephosphorylation of paxillin in parallel with focal adhesion kinase

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Paxillin and focal adhesion kinase (pp125^{FAK}) co-localize in focal adhesions; recently insulin has been shown to stimulate the dephosphorylation of pp125^{FAK}; however, its effect on paxillin is unknown. We show that insulin and IGF-1 can stimulate the dephosphorylation of paxillin in CHOT (overexpress human insulin receptors) and CHOΔCT69 (overexpress insulin receptors lacking C-terminal 69 amino acids) cells. Furthermore, the insulin-receptor C-terminus is not needed for either insulin or IGF-1 to stimulate paxillin or pp125^{FAK} dephosphorylation in the CHO (Chinese-hamster ovary) cell lines used.

INTRODUCTION

Stimulation of the insulin receptor by insulin results in kinase activation, receptor autophosphorylation and the phosphorylation of immediate signalling proteins such as insulin receptor substrate-1 (IRS-1) and Shc [1]. Subsequently a number of signal transduction pathways such as those involving Grb-2/ras/mitogen-activated protein kinase, phosphatidylinositol 3-kinase and the tyrosine phosphatase, Syp, are rapidly activated [1]. Apart from the well-known biological effects of insulin on glucose and lipid metabolism and cell growth, insulin has recently been shown, *in vitro*, to stimulate cytoskeletal changes in cells, including membrane ruffling and actin rearrangements [2,3], effects which probably involve the signalling pathways listed above. These changes play an important role in many eukaryotic cellular functions, including motility, chemotaxis, cell division and endocytosis.

Associated with the cytoskeleton are focal adhesions, dynamic regions of the cell which are sites where actin bundles terminate at the plasma membrane, and where signals from the extracellular matrix may be transduced via integrins [4]. A key enzyme targeted to focal adhesions is focal adhesion kinase (pp125^{FAK}), which appears to play a central role in integrin-mediated signal transduction and a regulatory function in cytoskeletal rearrangements and interactions [5]. Among the proteins known to associate with pp125^{FAK} is the structural protein paxillin, which binds to pp125^{FAK} at a C-terminal sequence [6]. Paxillin has also been implicated in many protein–protein interactions, including binding to vinculin, C-terminal src kinase, p47$^{gag\text{-}crk}$ and pp60$^{c\text{-}src}$ [4]. In addition to extracellular matrix [7], a number of extracellular stimuli such as growth factors [8] and neuropeptides [9,10] induce both pp125^{FAK} and paxillin tyrosine phosphorylation, suggesting that pp125^{FAK} and/or paxillin may be involved in cytoskeletal processes with tyrosine phosphorylation modulating their function or activity.

In contrast with other agents tested to date, insulin stimulates the dephosphorylation of pp125^{FAK} [11,12], however, its effect on paxillin is unknown. We now report the ability of both insulin and insulin-like growth factor-1 (IGF-1) to stimulate the dephosphorylation of paxillin in Chinese-hamster ovary (CHO) cells overexpressing the human insulin receptor (CHOT). In addition, we show that, in CHO cells, the insulin receptor C-terminus is not required for insulin or IGF-1 to stimulate the dephosphorylation of either paxillin or pp125^{FAK}.

EXPERIMENTAL

Materials

The following were generously given: CHOT cells overexpressing human insulin receptors (Dr. L. Ellis, Institute of Biosciences, Texas A & M University, Houston, TX, U.S.A.), CHOΔCT69 cells overexpressing mutant insulin receptors lacking the last 69 amino acids from the β-subunit C-terminus (Dr. J. Tavaré, Department of Biochemistry, University of Bristol, Bristol, U.K.), recombinant IGF-1 (Dr. V. Russo, Royal Childrens Hospital, Melbourne, Vic., Australia), monoclonal antibody 47-9 against the human insulin receptor (Professor K. Siddle, Department of Clinical Biochemistry, University of Cambridge, Cambridge, U.K.), and antibodies 2A7 (monoclonal) and BC3 (polyclonal) against pp125^{FAK} (Dr. J. T. Parsons, Department of Microbiology, University of Virginia, Charlottesville, VA, U.S.A.). The monoclonal antibody against paxillin was purchased from Transduction Laboratories, Lexington, KY, U.S.A. The polyclonal antibody against a peptide corresponding to the amino acids 1223–1242 mapping at the C-terminus of IRS-1 (clone C-20) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A. Anti-phosphotyrosine antibody (clone 4G10) conjugated to horseradish peroxidase (HRP) was purchased from UBI, Lake Placid, NY, U.S.A. Secondary antibodies conjugated to HRP were purchased from Dako Corporation, Carpinteria, CA, U.S.A.

Methods

Cell culture

CHO cell lines were maintained in α-modified Eagle's medium containing 10 % (v/v) fetal-calf serum (FCS).

pp125^{FAK} and paxillin measurements

CHO cell lines plated in 10 cm dishes were used at 60–80 % confluency. Cells were stimulated with insulin or IGF-1 (1.72–

Abbreviations used: IRS-1, insulin receptor substrate-1; pp125^{FAK}, focal adhesion kinase; IGF-1, insulin-like growth factor-1; CHO, Chinese-hamster ovary; HRP, horseradish peroxidase; FCS, fetal-calf serum.

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172 nM) for 10 min at 37 °C in Earle's Balanced Salt Solution (EBSS)/Hepes/0.1 % (w/v) BSA. In some experiments, cells were pre-incubated for 30 min with 1 nM antibody 47-9 before insulin or IGF-1 stimulation. Cells were lysed [150 mM NaCl/50 mM Tris (pH 7.5)/1 % (v/v)/Triton X-100/0.25 % (w/v) deoxycholic acid/1 mM Na_3VO_4/2 mM EGTA] and pp125^{FAK} immunoprecipitated with 2 μg of monoclonal antibody 2A7 overnight at 4 °C. For paxillin immunoprecipitation, cells were lysed and scraped off before immunoprecipitation with 2 μg of anti-paxillin antibody overnight at 4 °C [13]. For IRS-1 immunoprecipitation, cells were serum-starved [0.5 % (v/v) FCS] overnight and lysed before immunoprecipitation with 1 μg anti-IRS-1 antibody overnight at 4 °C. Prior to immunoprecipitation, the volume of each cell lysate was adjusted for cell number. Immunoprecipitates were separated by reducing SDS/PAGE and proteins transferred [25 mM Tris (pH 8.3)/192 mM glycine/20 % (v/v) methanol] to poly(vinylidene difluoride) membranes via Western blotting. To detect phosphorylation of proteins on tyrosine, membranes were probed with HRP-conjugated 4G10 (1:188). To detect protein levels, membranes were probed with polyclonal antibody BC3 (1:500) followed by HRP-conjugated anti-rabbit antibody (1:2000) for pp125^{FAK}, or a monoclonal antibody to paxillin (1:10000), followed by HRP-conjugated anti-mouse antibody (1:5000). Membranes were stripped [62.5 mM Tris/HCl (pH 6.7)/2 % (w/v) SDS/100 mM 2-mercaptoethanol] for 30 min at 50 °C, before re-probing with the desired antibodies. The bands were revealed by enhanced chemiluminescence ('ECL') and quantified by scanning densitometry.

RESULTS AND DISCUSSION

Insulin stimulates the dephosphorylation of the focal-adhesion protein paxillin

Paxillin and pp125^{FAK} co-localize with integrins in focal adhesions. Recently insulin, in contrast with other growth factors, has been shown to stimulate the dephosphorylation of pp125^{FAK} [11, 12], as shown in Figure 1. The structural focal-adhesion protein paxillin is closely linked with pp125^{FAK} via an association site in the C-terminus of pp125^{FAK} [6], and has been proposed to be a substrate for pp125^{FAK} *in vitro* [14]. Figure 1 shows that insulin (172 nM) stimulation of CHOT cells, which overexpress the human insulin receptor (7.7×10^5 receptors/cell) [15], led to a significant reduction in the level of paxillin tyrosine phosphorylation when compared with basal paxillin phosphorylation (35.1 ± 7.4 % of non-stimulated cells). In CHO cells, which express only endogenous hamster insulin receptors (0.03×10^5 receptors/cell) [15,16], paxillin phosphorylation was unaltered in response to insulin (112.2 ± 21.0 %; Figure 1). The protein levels of paxillin were not altered in response to insulin stimulation of either CHOT or CHO cells (106.0 ± 18.0 % and 120.4 ± 8.4 % of non-stimulated cells respectively; $n = 6$).

The mechanism by which insulin stimulates the dephosphorylation of paxillin or pp125^{FAK} is currently unclear; however, it has been postulated to involve the insulin receptor C-terminus, since insulin stimulation of rat 1 fibroblasts overexpressing either mutant ΔCT43 insulin receptors (lacking the last 43 amino acids of the receptor C-terminus) or YF2 insulin receptors (C-terminal tyrosines, Y1328 and Y1334, replaced with phenylalanine) do not mediate dephosphorylation of pp125^{FAK} [11]. In contrast, our results with CHO cells overexpressing the insulin receptor lacking the last 69 amino acids (CHOΔCT69; 6.7×10^5 receptors/cell) [15] show that insulin still mediates dephosphorylation of pp125^{FAK} in these cells (19.3 ± 10.5 % of non-stimulated cells; Figure 1). A similar effect on paxillin phosphorylation was also observed (34.9 ± 13.9 % of non-stimulated cells; Figure 1). These

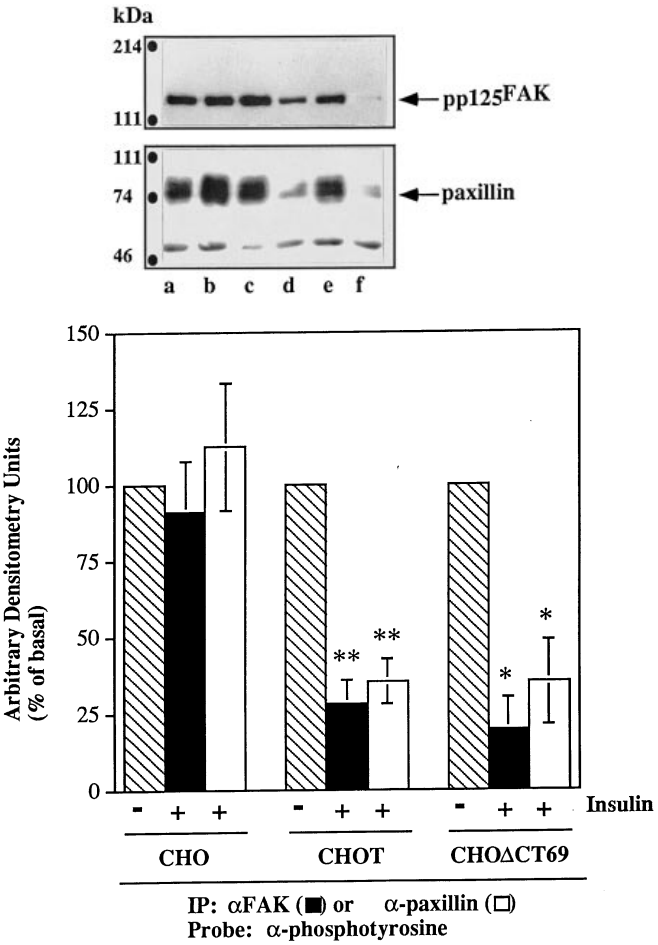

Figure 1 Insulin-stimulated dephosphorylation of pp125^{FAK} and paxillin in CHO, CHOT and CHOΔCT69 cells

Cells were treated in the absence or presence of 172 nM insulin for 10 min at 37 °C. Following immunoprecipitation with the relevant antibody, separation by reducing SDS/7.5%-PAGE and Western transfer, tyrosine phosphorylation of pp125^{FAK} (■) or paxillin (□) was detected using HRP-conjugated 4G10 antibody. Upper panel: the immunoblots are representative of at least three separate experiments. CHO (a and b), CHOT (c and d) and CHOΔCT69 (e and f) cells in the absence (a, c and e) or presence (b, d and f) of insulin. Lower panel: phosphotyrosine levels are expressed as a percentage of the basal activity in each individual CHO cell line and are means ± S.E.M. for at least three separate experiments. The basal phosphotyrosine levels of paxillin were comparable between the cell lines. Basal pp125^{FAK} tyrosine phosphorylation was also similar between the cell lines, although CHOT cells had a higher (but not significant) phosphotyrosine content (results not shown).** $P < 0.01$ and * $P < 0.05$ when compared with basal.

results suggest that the C-terminus of the insulin receptor may not mediate pp125^{FAK} or paxillin dephosphorylation, at least in CHO cells.

IGF-1 stimulates the dephosphorylation of paxillin and pp125^{FAK}

Because the IGF-1 receptor and insulin receptor differ considerably in their C-terminal regions [17], we next examined the effect of IGF-1 on the two focal-adhesion proteins. To ensure that there was no cross-reactivity between the two receptor types [16], we constructed a concentration–response curve from 1.72 to 172 nM insulin or IGF-1. All concentrations of IGF-1 stimulated the dephosphorylation of pp125^{FAK} in both CHOT (Figure 2A) and CHOΔCT69 (results not shown) cells, and was comparable with the effect of insulin over the same concentration range

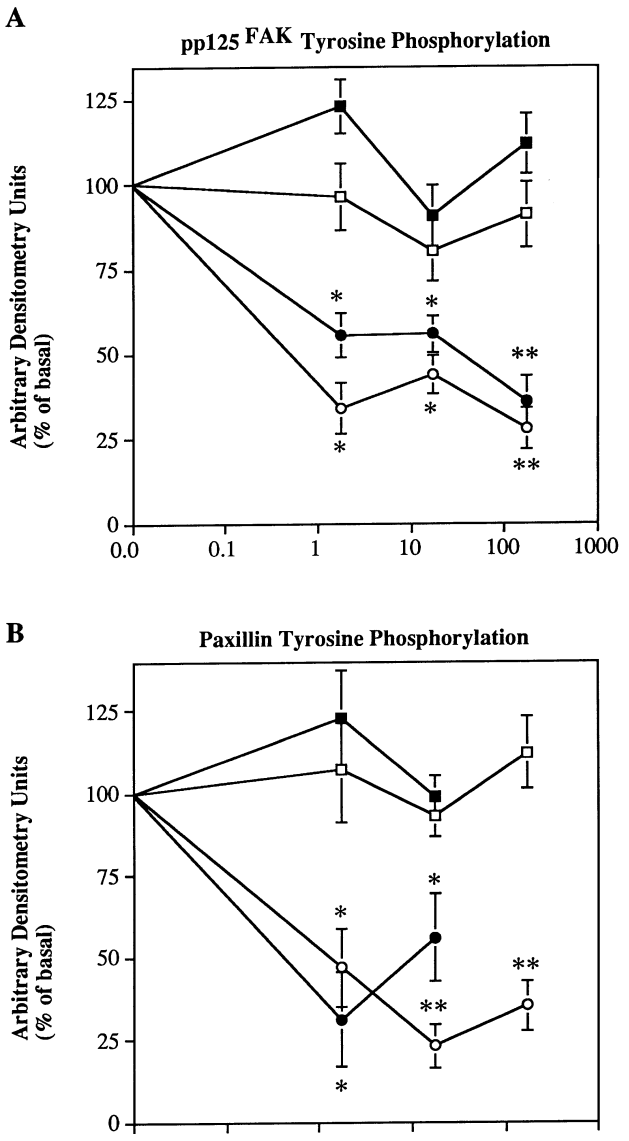

Figure 2 Concentration response for insulin- and IGF-1 stimulated dephosphorylation of pp125^{FAK} (A) and paxillin (B) in CHO (■, □) and CHOT (●, ○) cells

Cells were treated in the presence of 1.72, 17.2 and 172 nM IGF-1(■, ●) or insulin (□, ○) for 10 min at 37 °C. Basal, IGF-1- and insulin-stimulated pp125^{FAK} and paxillin immunoprecipitates were treated as detailed in Figure 1. Phosphotyrosine levels of both pp125^{FAK} and paxillin are expressed as a percentage of the basal activity and are means ± S.E.M. for at least three separate experiments. $^{**}P < 0.01$ and $^{*}P < 0.05$ when compared with basal.

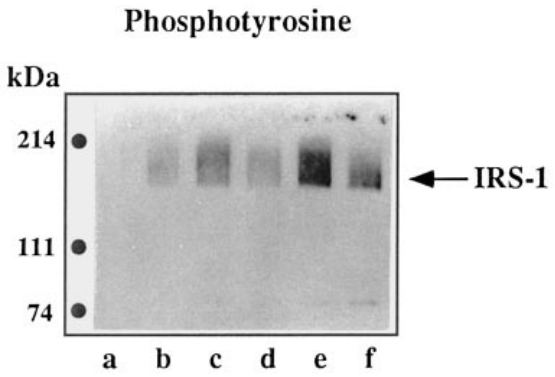

Figure 3 IGF-1 stimulates IRS-1 phosphorylation in CHO (a, b and c) and CHOT (d, e and f) cells

Cells were incubated in either the absence (lanes a and d) or presence of 172 nM insulin (lanes b and e) or IGF-1 (lanes c and f) for 10 min at 37 °C. Basal, insulin- and IGF-1 stimulated IRS-1 immunoprecipitates were prepared as detailed under 'Methods', separated by reducing SDS/7.5%-PAGE and proteins transferred to poly(vinylidene difluoride) membranes via Western blotting. Tyrosine phosphorylation was detected using HRP-conjugated 4G10 antibody. The immunoblot is representative of four separate experiments.

(Figure 2A). This result is in contrast with the lack of IGF-1 effect on pp125^{FAK} phosphorylation in rat 1 fibroblasts over-expressing the wild-type insulin receptor [11]. Interestingly, IGF-1 stimulation of CHO cells did not reduce pp125^{FAK} phosphorylation (Figure 2A), even though CHO cells have the same IGF-1 receptor levels as CHOT cells (3.8×10^4 receptors/cell) [16].

Emphasizing the co-ordinated nature of phosphorylation of paxillin and pp125^{FAK}, IGF-1 also stimulated the dephosphorylation of paxillin in both CHOT (Figure 2B) and CHOΔCT69 (results not shown) cells in a similar manner to insulin (Figure 2B). IGF-1 stimulation of CHO cells did not affect paxillin phosphorylation (Figure 2B). As all CHO cell lines express similar levels of IGF-1 receptors (3.8×10^4 receptors/cell) [16], these results suggest that IGF-1 may not be acting via the IGF-1 receptor. In cells that express high levels of both insulin and IGF-1 receptors (such as CHOT and CHOΔCT69) a proportion of the receptors can assemble as hybrid structures with one chain derived from the insulin receptor and the other chain from the IGF-1 receptor [18]. Perhaps IGF-1 induced dephosphorylation of paxillin and pp125^{FAK} occurs via hybrid receptors. To test this hypothesis we attempted to block hybrid receptors (and insulin receptors) with antibody 47-9, shown previously to inhibit insulin binding [19,20] and block the biological actions of insulin [19]. However, preliminary data showed that 47-9 is only partially able to reverse the effect of insulin on pp125^{FAK} dephosphorylation, suggesting that it is not possible to block the receptors effectively with this antibody (results not shown). We also noted that, in all CHO cell lines, there was stimulation of IRS-1 phosphorylation by insulin and IGF-1 (Figure 3 and [15]), although the effect of insulin was much less in CHO cells (Figure 3, lane b) than in CHOT cells (Figure 3, lane e). Thus differential activation of IRS-1 does not appear to explain the lack of effect of IGF-1 on pp125^{FAK} and paxillin dephosphorylation in CHO cells.

In conclusion, we show in CHOT and CHOΔCT69 cells that insulin stimulates the dephosphorylation of both pp125^{FAK} and an associated structural protein, paxillin, in a co-ordinate manner that is apparently not dependent on the presence of the extreme C-terminus of the insulin receptor. In addition, IGF-1 also stimulates pp125^{FAK} and paxillin dephosphorylation, but only in CHO cells expressing high levels of the insulin receptor. This effect of IGF-1 may be mediated via hybrid receptors where the dephosphorylation signal arises following activation of the insulin-receptor cytoplasmic domain within these hybrids.

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