β 1-Integrins induce phosphorylation of Akt on serine 473 independently of focal adhesion kinase and Src family kinases

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Adhesion by means of β 1-integrins induces the phosphorylation of Akt, an event strictly dependent on the activity of the phosphatidylinositol 3-kinase (PI3K). Binding of the p85 regulatory subunit of PI3K to phosphorylated tyrosine 397 in focal adhesion kinase (FAK) is considered to be the mechanism of cell adhesion-induced activation of class Ia PI3K. Here we show that PI3K-dependent phosphorylation of Akt in response to ligation of β 1-integrins occurs efficiently in the absence of FAK tyrosine phosphorylation. Akt S473 phosphorylation was strongly promoted both in cells expressing the integrin subunit splice variant β 1B, which is unable to activate FAK, and in FAK knockout cells. In addition, we found this phosphorylation to be independent of the Src family kinases Src, Fyn and Yes. These results indicate that a major pathway for adhesion-dependent activation of PI3K/Akt is triggered by the membrane proximal part of the β 1 subunit in a FAK and Src-independent manner.

Keywords: integrin; PI3K; Akt; FAK; Src

EMBO reports (2004) 5, 901–905. doi:10.1038/sj.embor.7400234

INTRODUCTION

Integrins are heterodimeric transmembrane receptors for extracellular matrix (ECM) proteins. Besides mechanically linking the ECM to the cytoskeleton, integrins transduce both outside-in and inside-out signals through multiple pathways. Upon ligand binding, integrins cluster in the cell membrane and undergo a major conformational change in their extracellular domains. These rearrangements lead to the formation of complexes of integrin-associated cytoskeletal and signalling proteins.

Among the signalling events triggered by several integrins, phosphorylation of focal adhesion kinase (FAK) is believed to be central. A number of important signalling intermediates, including Src family kinases, phosphatidylinositol 3-kinase (PI3K), Akt, MAP

kinases and Rho GTPases, can be activated in a FAK-dependent manner (Sieg *et al*, 1999) and participate in the regulation of cell migration, proliferation and apoptosis. However, signalling protein complexes are formed at focal contacts in response to adhesion also in the absence of FAK (Ilic *et al*, 1995). The early signalling events preceding FAK activation, which are triggered by integrins and result in formation of focal contacts, still remain obscure.

Members of the largest subfamily of integrins share β 1 as the common β -subunit. The cytoplasmic domains of β 1A and β 1D splice variants (Armulik, 2002) each contain two conserved NPXY motifs. These motifs are utilized for several interactions, and their involvement in the inside-out activation of integrins (Pfaff *et al*, 1998; Calderwood & Ginsberg, 2003; Tadokoro *et al*, 2003) as well as in the regulation of FAK activation (Wennerberg *et al*, 2000) has been demonstrated. Integrins containing the β 1B splice form, which lacks the carboxy-terminal half of β 1A including the NPXY motifs, are in an inactive conformation and cannot bind the normal β 1-integrin ligands. The β 1B variant has no apparent physiologic function and probably arises through a splicing error (Armulik, 2002). Such integrins can, however, mediate cell adhesion to anti-integrin IgG and the bacterial protein invasins without inducing FAK activation (Armulik *et al*, 2000; Gustavsson *et al*, 2002). Therefore, cells expressing β 1B integrins are useful tools for studying integrin signalling in the absence of FAK activation.

In this study, we have used the cell line GD25 transfected with β 1B but lacking endogenous β 1 expression, and other cell lines lacking specific genes, to identify a novel integrin signalling pathway. We show that PI3K-dependent phosphorylation of Akt is induced by adhesion by means of β 1-integrins independently of FAK or Src family kinases.

RESULTS

The cell lines GD25- β 1A and GD25- β 1B were generated by transfection of the GD25- β 1-deficient cells (Fassler *et al*, 1995; Wennerberg *et al*, 1996; Armulik *et al*, 2000). Both β 1 splice forms were associated with α 3, α 5 and α 6 in these cells (Wennerberg *et al*, 1996; Retta *et al*, 1998) and were expressed at similar levels (Fig 1B). Cell-adhesion induced tyrosine phosphorylation of FAK in the GD25- β 1A but not in GD25- β 1B cells, which was most likely due to differences in the β 1 subunit cytodomain (Fig 1A,C).

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Received 11 May 2004; revised 23 July 2004; accepted 27 July 2004; published online 13 August 2004

Fig 1 | Integrin β1B does not induce FAK activation. (A) Alignment of the C-terminal parts of the β1A and β1B cytoplasmic tails. (B) Expression levels of the respective β1 splice variants in transfected GD25 cells determined by FACS. (C) FAK phosphorylation in the GD25-β1A and GD25-β1B cells. Weaker FAK protein signal in lanes with stronger phosphorylation (lane 3) is due to the incomplete removal of the PY-20 antibody, which is a commonly occurring phenomenon.

β1B integrins induce phosphorylation of Akt

From earlier reports it is evident that binding of the p85 regulatory subunit of PI3K to pY397 of FAK is the mechanism of PI3K activation by β1-integrins (Chen *et al*, 1996), which is required for Akt phosphorylation. Utilizing the established cell lines we set out to further analyse the integrin-induced signalling pathway(s) leading to phosphorylation of Akt. After plating the cells onto glutathione *S*-transferase (GST)-invasin (data not shown) or anti-β1 IgG (Fig 2A), or incubating with GST-invasin or IgG in suspension (Fig 2B), the signal for Akt pS473 was detected in the GD25-β1A cells. Surprisingly, the GD25-β1B cells also induced Akt S473 phosphorylation and the overall induction seemed

Fig 2 | Akt S473 phosphorylation in GD25-β1A and GD25-β1B cells. (A) The time course of Akt S473 phosphorylation in GD25-β1A and GD25-β1B cells adhering to the anti-β1 IgG. (B) Induction of Akt S473 phosphorylation by soluble GST-invasin and anti-β1 IgG in suspended cells. Cell lysates in SDS sample buffer were analysed by western blotting.

stronger compared with GD25-β1A (Fig 2). Comparable results were obtained with two β1B-expressing clones (not shown).

Similar PP2A activity in GD25-β1A and GD25-β1B cells

The serine/threonine phosphatase protein phosphatase 2A (PP2A) is one of the players in the regulation of Akt by integrins (Ivaska *et al*, 2002; Yellaturu *et al*, 2002; Pankov *et al*, 2003). The PP2A assay revealed no difference in the activity of this enzyme regardless of the cell line or whether the cells were suspended or adherent (Fig 3). Whether the stronger and faster induction of Akt phosphorylation in the β1B cells is due to a higher enzymatic activity of PI3K, lower activity of other phosphatases (e.g. PTEN or SHIP), slower turnover of focal complexes in the absence of FAK activity or more favourable conditions for recruitment of relevant signalling proteins to the membrane-associated complexes remains to be clarified.

Effect of kinase inhibitors on Akt phosphorylation

In an attempt to understand which signalling intermediates are involved in the induction of Akt S473 phosphorylation by β1-integrins, the effect of inhibitors for selected kinases was tested (Fig 4). The PI3K inhibitors wortmannin (Fig 4) and LY294002 (not shown) effectively blocked Akt phosphorylation, demonstrating that the signal from both β1 variants to Akt is mediated by PI3K. Src family kinases have been shown to act both upstream and downstream of FAK, and in addition c-Src has been reported to associate directly with and activate PI3K and Akt in response to stimulation of growth factor receptors (Chen *et al*, 2001; Jiang & Qiu, 2003; Frame, 2004). Two widely used inhibitors of Src family tyrosine kinases had different effects on this phosphorylation event: PP2 was strongly inhibitory, whereas SU6656 did not affect pS473 level (Fig 4). The c-Abl tyrosine kinase is activated rapidly during integrin-mediated cell adhesion (Lewis & Schwartz, 1998) and is involved in regulating actin reorganization (Woodring *et al*, 2002). However, the c-Abl inhibitor STI571, which also affects PDGF

Fig 3 | PP2A activity in GD25-β1A and GD25-β1B cells. The PP2A assay was carried out as described in Methods. The relative activity of PP2A was measured and is presented as arbitrary units. Similar results were obtained in three independent experiments. Ad + calcy, adherent cells pretreated with the PP2A inhibitor calcyculin; Ad + C, control antibody; MG + phos, colour reagent (malachite green) mixed with the phosphopeptide; MG, colour reagent alone.

receptor and c-Kit receptor tyrosine kinases (Buchdunger *et al*, 2000), had no detectable effect on Akt S473 phosphorylation (Fig 4).

Akt phosphorylation in FAK, Src, Fyn and Yes null cells

To confirm that FAK activity is not needed for β1-integrin-induced Akt S473 phosphorylation, we used the FAK^{-/-} mouse fibroblasts. In these cells, the β1A-mediated phosphorylation of Akt was strongly induced upon cell adhesion to both GST-invasin and the natural integrin ligand fibronectin (Fig 5). A possible involvement of Src family members was analysed in single, double or triple knockout cells of the Src, Fyn and Yes kinases. Surprisingly, in all cases, prominent phosphorylation of Akt occurred in response to β1-mediated adhesion (Fig 5A and data not shown). This suggests that the mechanisms of the induction of Akt phosphorylation by growth factor receptors and integrins differ with respect to the involvement of Src family kinases. Notably, even in cells lacking Src, Fyn and Yes, PP2 reduced Akt S473 phosphorylation to the background level (Fig 5A and data not shown), indicating that PP2 has an inhibitory effect on kinase(s) outside the Src family.

DISCUSSION

Akt, a downstream effector of PI3K, binds phosphorylated lipids at the membrane by means of its PH domain and mediates anti-apoptotic signals. The final critical steps in the activation of Akt, phosphorylation of T308 in the catalytic domain and S473 in the C-terminal regulatory region, are accomplished by two PI3K-dependent kinases, PDK1 (Alessi *et al*, 1997) and PDK2 (suggested to be ILK by several studies; Persad *et al*, 2001; Troussard *et al*, 2003), respectively. It has been demonstrated that PI3K is essential for the activation of Akt (Datta *et al*, 1996). Therefore, the phosphorylation state of Akt is commonly used as a read-out of PI3K activity.

Fig 4 | Effect of kinase inhibitors on integrin-induced Akt phosphorylation. The GD25-β1A (A) and GD25-β1B (B) cells were pretreated with the indicated inhibitors for 15 min, seeded onto dishes coated with anti-β1 IgG or GST-invasin for 1 h, lysed in SDS sample buffer and subjected to SDS-PAGE and western blotting.

FAK activation has been extensively documented as one of the central events in integrin signalling, and activation of PI3K by FAK has been demonstrated (Chen *et al*, 1996; Shen & Guan, 2001). According to the general view, upon binding the ligand integrins induce autophosphorylation of FAK on Y397. Binding of the SH2 domains of Src family kinase(s) and the p85 subunit of PI3K to FAK pY397 results in the activation of these kinases (Cobb *et al*, 1994; Schaller *et al*, 1994; Chen *et al*, 1996). The Y397-bound Src subsequently phosphorylates FAK to create additional binding sites for other signalling proteins and to increase the catalytic activity of FAK (Schlaepfer *et al*, 1994; Calalb *et al*, 1996; Salazar & Rozengurt, 2001). The involvement of Src family kinases in the phosphorylation of Y397 of FAK has also been suggested (Owen *et al*, 1999). Although FAK can thus participate in and promote several protein interactions, it is not required for assembly of the major components of focal contacts. Instead, a main function of FAK is linked to the turnover of focal contacts (Ilic *et al*, 1995; Wennerberg *et al*, 2000; Parsons, 2003), which is also reflected by its relatively late activation during cell adhesion. Our study shows that stimulation of PI3K occurs in the absence of active FAK as an early step in the integrin-dependent activation of Akt. As the generation of PtdIns-3,4-P and PtdIns-3,4,5-P by PI3K is a prerequisite for the membrane recruitment of a number of proteins involved in signalling cascades, it seems likely that activation of PI3Ks also has a key role in the initial formation of other integrin-associated signalling complexes.

Thus, PI3K can apparently be activated by integrins both downstream of FAK (Chen *et al*, 1996; Akagi *et al*, 2000; Shen & Guan, 2001; Reif *et al*, 2003) and independently of FAK. The possibility that FAK activation would be dependent on PI3K activity is unlikely because phosphorylation of Y397 of FAK was previously found not to be inhibited by wortmannin (Armulik *et al*, 2004). This suggests that PI3K and FAK can be activated by parallel pathways serving as two key events in integrin-mediated signalling.

Fig 5 | Phosphorylation of Akt in cells lacking FAK, Src, Yes and Fyn. The indicated cells were treated as described in the legend to Fig 4. Note that similar levels of Akt pS473 were induced by GST-invasin (A) and fibronectin (B), and that different inhibitors of Src family kinases affected pS473 levels differently.

It is noteworthy that the phosphorylation of Akt is inhibited by PP2, which is widely used as a selective inhibitor of Src family tyrosine kinases, even in cells that lack all three Src family tyrosine kinases expressed in fibroblastic cells (Fig 5). It has been reported that apart from the Src family members, PP2 also inhibits other protein kinases (Bain *et al*, 2003), and our data further exemplify the need to interpret the results obtained with this inhibitor with caution.

Src and PI3K have been shown to act in a closely coordinated manner in the regulation of actin filament remodelling (Fincham *et al*, 2000). The two proteins can bind directly to each other and stimulate each other's enzymatic activity (Gentili *et al*, 2002). Our results suggest that Src family members Src, Fyn and Yes are not required for integrin-mediated activation of the PI3K/Akt pathway in the absence of growth factors. We, therefore, conclude that there is a signalling pathway leading to activation of PI3K by integrins independently of both FAK and Src family kinases. As described in our recent report (Armulik *et al*, 2004), a K756L point mutation in the membrane proximal half of the $\beta 1$ subunit strongly inhibits the integrin-induced S473 phosphorylation of Akt. Together with the above data, our results show that the membrane proximal half of $\beta 1$ is necessary and sufficient to promote this pathway.

METHODS

Cells. The integrin $\beta 1$ knockout mouse fibroblast clone GD25 (Fassler *et al*, 1995) and the stably transfected clones GD25- $\beta 1A$ and GD25- $\beta 1B$ expressing the $\beta 1A$ and $\beta 1B$ isoforms, respectively, of the mouse integrin $\beta 1$ subunit have been described (Wennerberg *et al*, 1996; Armulik *et al*, 2000). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin-streptomycin, fungizone, L-glutamine (2 mM) and puromycin (20 μ g/ml). The FAK^{-/-} cells (clone Du 4) were provided by Dr D. Ilic (San Francisco, CA, USA), SYF cells (Klinghoffer *et al*, 1999) were from the ATCC (CRL-2459), and Src, Fyn, or Yes and Fyn null cells were a gift from Dr K. Wary (Houston, TX, USA).

Antibodies and reagents. The antibodies used were as follows: anti-integrin $\beta 1$ mAb (Ha2/5, BD PharMingen, USA) and pAb (Johansson & Höök, 1984), anti-FAK-pY397 (Biosource, USA), anti-chicken FAK (clone 77) and anti-phosphotyrosine (PY20; BD Transduction Labs, USA), anti-Akt pS473 (CST, USA) and anti-Akt 1/2 (H-136, Santa Cruz, USA). The secondary antibodies were the horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG (Amersham Pharmacia Biotech, Sweden). The Src family tyrosine kinase inhibitors PP2 and SU6656 were from Calbiochem, and wortmannin, soybean trypsin inhibitor and PP2A inhibitor calcyclin were from Sigma-Aldrich (Sweden). The Abl kinase inhibitor STI571 was from Novartis (Switzerland).

Fluorescence-activated cell sorting. Expression levels of the $\beta 1$ -integrin subunit on the GD25- $\beta 1A$ and GD25- $\beta 1B$ cells were determined by fluorescence-activated cell sorting (FACS) analysis as described (Armulik *et al*, 2000).

PP2A assay. The PP2A assay kit was purchased from Upstate Biotechnology (USA). The cells were allowed to attach to GST-invasin or anti- $\beta 1$ IgG-coated dishes for 30 min and otherwise treated as described below (Immunoprecipitation and western blotting section) with the exception of the use of specific inhibitors. The assay was performed according to the manufacturer's instructions.

Immunoprecipitation and western blotting. Tissue culture dishes were coated with 10 μ g anti- $\beta 1$ IgG, 10 μ g GST-invasin or 1% bovine serum albumin (BSA), blocked with 1% BSA and washed with PBS. Cells, serum-starved for 48 h, were detached with trypsin-EDTA, washed once in DMEM containing soybean trypsin inhibitor (1 mg/ml) and left to rest in DMEM at 22 °C for 1 h and for an additional 15 min in the presence of the indicated enzyme inhibitors. Cells were allowed to attach to coated culture dishes for given times at 37 °C.

The cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (20 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 30 mM Na₄P₂O₇, 2 mM EDTA, 1% NP-40, 0.1% SDS, 1% (w/v) Na-deoxycholate, 2 mM PMSF, 2 mM NEM, 1 μ g/ml pepstatin A, 1 μ g/ml leupeptin and 0.2 mM Na₃VO₄) or directly in reducing SDS sample buffer, and the lysates were aspirated through 27G injection needle to shear the DNA. For immunoprecipitation, the RIPA lysates were precleared with plain Sepharose 6B-CL for 1 h followed by incubations with 3 μ g of anti-FAK monoclonal antibody for 2 h and 1.5 μ g of the rabbit-anti-mouse IgG for 30 min. The immune complexes were recovered by 30 min incubation with Protein A-Sepharose, washed twice in lysis buffer and boiled in reducing SDS sample buffer.

The immunoprecipitated proteins and the total cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany). The membranes were blocked in appropriate blocking buffers and incubated with HRP-conjugated anti-phosphotyrosine, or with anti-Akt pS473, followed by peroxidase-conjugated anti-rabbit antibodies. Reactive bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). For loading control, the membranes were stripped and re-probed with antibodies detecting Akt or FAK.

ACKNOWLEDGEMENTS

The work was supported by grants from the Swedish Research Council (no. 7147), Swedish Cancer foundation, King Gustaf V:s 80-års Fond

and Uppsala University. We thank Dr K. Wary and Dr D. Ilić for the gifts of cell lines.

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