The endoproteolytic processing of αvβ5 integrin is involved in cytoskeleton remodelling and cell migration

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Abstract We previously showed that the post-translational cleavage of αv subunit is essential for integrin-dependent signalling and cell adhesion. Here, we report that blocking αv subunit cleavage by expression of α1-PDX, a convertase inhibitor, modified the capacity of cells to change shape, via a remodelling of the actin cytoskeleton upon cell attachment. These changes are associated with cell scattering and with a dramatic increase in cell migration to vitronectin. The αv subunit cleavage is thus essential for integrin function and has a considerable impact on integrin-dependent events, especially those leading to cell migration.

Key words: Proprotein convertase; Endoproteolytic cleavage; αvβ5 integrin; α1-PDX; Actin cytoskeleton; Colon carcinoma

1. Introduction

Cell migration is essential for many important biological events, including embryonic development, inflammatory responses, wound healing and tumour metastasis. Motility requires adhesive interactions of cells with the extracellular matrix (ECM). These interactions are partly mediated by integrins, a family of cell surface adhesion receptors [1,2]. Integrins are heterodimeric proteins produced by the non-covalent association of α and β subunits. Although 18 α and eight β subunits have been described, only 24 different combinations have been identified to date. In addition to their structural role as bridges between the ECM proteins and the actin cytoskeleton, integrins also play a critical role in signal transduction events (for a review see [3–5]).

Some integrin α chains undergo a post-translational cleavage in their extracellular domain by furin and PC5A [6,7]. These proprotein convertases belong to the subtilisin/kexin-like family [8–10]. The potential clinical and pharmacological role of the convertases fostered the development of several inhibitors. The most promising protein-based specific inhibitor of proprotein convertases is the α1-antitrypsin Portland or α1-PDX, a selective inhibitor of furin and, to a lesser extent, of PC5B [11]. α1-PDX blocks the convertase-dependent processing of various precursors, including integrins [12–15].

The role of proteolytic cleavage in integrin function is unclear. Arguably, this post-translational processing of α chain is not required for ligand binding [13,16,17], but is essential for α6β1 integrin activation by phorbol esters [18]. Moreover, it has recently been reported that expression of the uncleaved form of α6 integrin progressively increased relative to the cleaved form during lens cell differentiation, suggesting that the uncleaved form of α6 integrin may have a unique role in the embryonic lens [19].

In a previous report, we showed that inhibition of pro-αv cleavage by over-expression of α1-PDX impaired integrin αvβ5-mediated signal transduction leading to a reduced attachment to vitronectin [13]. In the present study, we further investigated the functional importance of the cleavage defect for cell migration. We report that blocking the αv subunit cleavage by expression of the convertase inhibitor α1-PDX had important repercussions on cytoskeleton remodelling, leading to a dramatic increase in cell migration to vitronectin when cells are stimulated by phorbol 12-myristate 13-acetate (PMA). Thus, our results show that the cleavage of αv subunit is essential for the function of αvβ5 integrin and has a marked impact on integrin-dependent events, especially those leading to cell migration.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Cergy-Pontoise, France) and foetal bovine serum from Bio-Whittaker (Fontenay-sous-Bois, France). Phosphate-buffered saline (PBS) was from Oxoid (Basingstoke, UK). G418, PMA and TRITC-conjugated phalloidin were obtained from Sigma (St-Quentin Fallavier, France). Laminin-1 and vitronectin were prepared according to Timpl et al. [20] and Yatogho et al. [21], respectively. Mouse monoclonal anti-vinculin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and FITC-conjugated goat anti-mouse antibodies were from Jackson (Baltimore, MD, USA).

2.2. Cell scattering

The control (empty vector; PDX0) and stably α1-PDX-transfected (PDX39P) cells, originating from the HT29-D4 adenocarcinoma cell line, were previously described [13]. Cells were grown in DMEM supplemented with 10% foetal calf serum and 400 μg/ml G418.

For cell scattering assay, cells were seeded at low density and allowed to grow for 48 h to obtain small colonies. Medium was changed to DMEM containing 0.1% bovine serum albumin (DMEM/BSA) and cells were treated or not with 100 nM PMA for 4 h at 37°C. Images were taken under an inverted phase-contrast microscope.

2.3. Cell migration assays

Haptotaxis assays were performed using modified Boyden chambers

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; PMA, phorbol 12-myristate 13-acetate

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as described elsewhere [22]. Briefly, the undersurface of the membrane was precoated with purified ECM proteins in PBS (10 μg/ml vitronectin or 30 μg/ml laminin-1) for 1 h at 37°C. The lower reservoir was filled with the appropriate protein in DMEM/BSA and the membrane was placed in the chamber. Cells obtained as a single cell suspension in DMEM/BSA were treated or not with 100 nM PMA prior to being added into the upper reservoir (100 000 cells/cm^2). Following incubation for 5 h at 37°C, non-migratory cells on the upper surface of the membrane were wiped with a cotton swab and migrated cells on the lower surface were stained with 0.125% Coomassie blue. Haptotaxis was determined by counting cells in 10 microscopic fields per well and expressed as the average number of cells per microscopic field (magnification: ×320).

2.4. Cell spreading assay

Cells in single cell suspension in DMEM/BSA were seeded at low density (10 000 cells/cm^2) in substratum-coated well plates as already described [22] and allowed to spread for 2 h at 37°C. Measures of cell spreading were performed on all cells from five randomly chosen microscopic fields (50–70 cells per well). Image acquisition and cell area quantification were performed using a Leica DM IRBE microscope and the Metaview software (Princeton Instruments, Paris, France).

2.5. Fluorescence staining

Serum-starved cells in DMEM/BSA were plated onto vitronectin-coated glass coverslips for 24 h with or without 100 nM PMA. Cells were fixed in 3.7% paraformaldehyde, permeabilised with 70% methanol and ice-cold acetone for 1 min, washed in PBS and blocked for 10 min with blocking buffer (PBS, 1% BSA). Primary antibodies were diluted in blocking buffer and applied for 3 h in a moist chamber at room temperature. After three washes with PBS, coverslips were incubated for 45 min in blocking buffer containing FITC-conjugated secondary antibodies. Actin was visualised by incubation with TRITC-conjugated phalloidin. After washing, samples were permanently mounted with Mowiol. Control staining was performed without either primary or secondary antibodies. Images were captured and

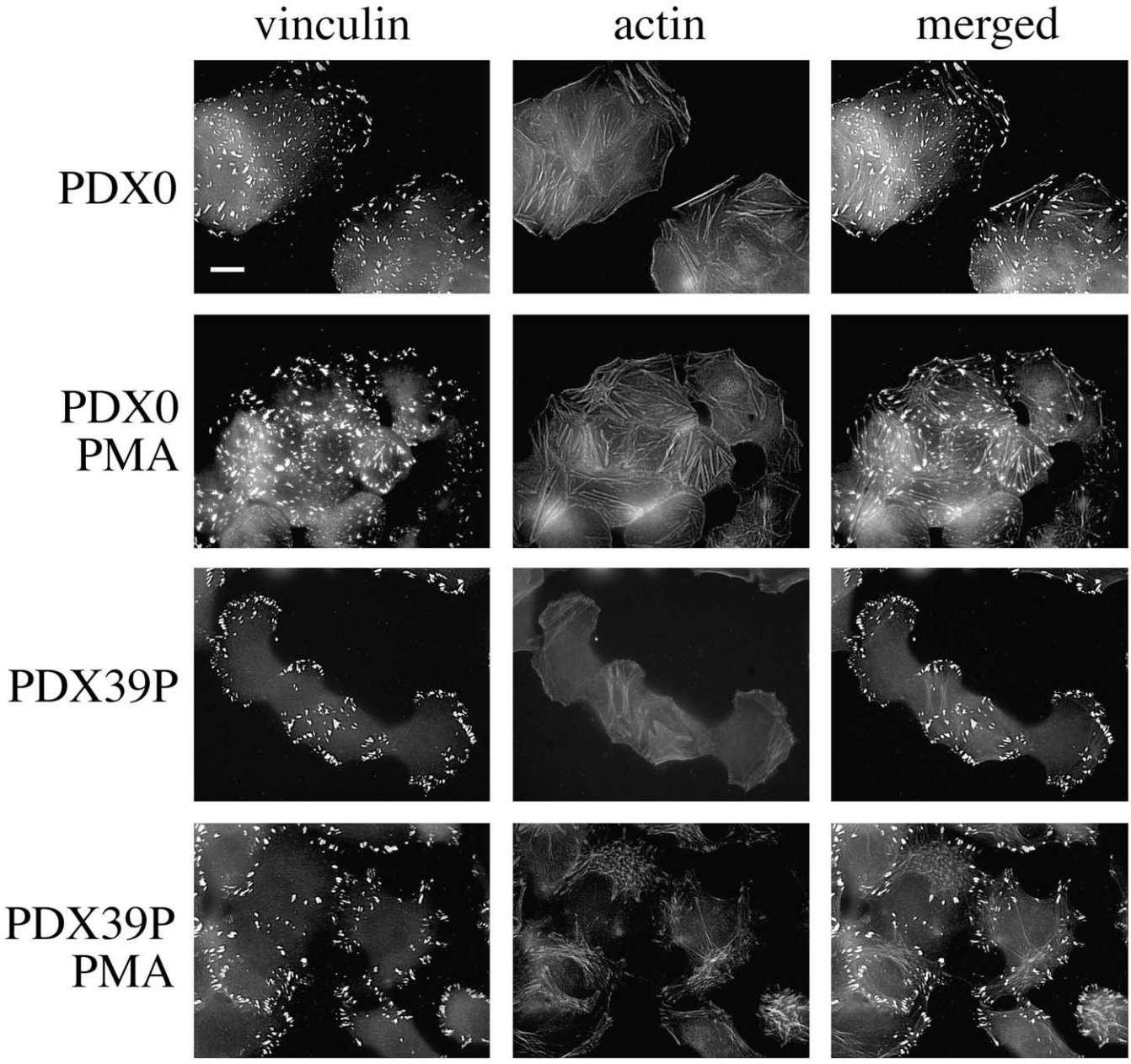

Fig. 1. Effect of α1-PDX expression on the actin cytoskeleton organisation. PDX0 and PDX39P cells were allowed to adhere to a vitronectin-coated surface (10 μg/ml) for 24 h in the presence or absence of 100 nM PMA. After permeabilisation, cells were co-stained for vinculin with an anti-vinculin antibody and FITC-coupled secondary antibody and for actin with TRITC-phalloidin. Scale bar: 10 μm.

analysed using a Leica DM IRBE microscope with cool Snap FX camera and the Metaview software.

3. Results

3.1. α1-PDX expression alters the cell morphology

We previously reported that cells expressing high levels of the convertase inhibitor α1-PDX showed a reduced attachment to vitronectin [13]. Focal adhesions are specialised sites of cell attachment to the ECM where integrin receptors link the ECM to the actin cytoskeleton [1]. To determine whether α1-PDX expression alters the cell distribution of focal adhesions, serum-starved cells were allowed to spread on vitronectin before double staining of actin fibres and vinculin, a component of focal adhesion plaques.

As illustrated in Fig. 1, in mock-transfected cells (PDX0), vinculin was concentrated in dots distributed all over the ventral surface of cells and localised at the end of actin stress fibres. When PDX0 cells were induced to migrate by treatment with the phorbol ester PMA, vinculin labelling was partially redistributed in larger dots along the cell edge in lamellipodia-like extensions. In PDX39P cells, expressing high levels of α1-PDX inhibitor, vinculin immunostaining was already localised at the periphery of untreated cells and the labelling distribution did not change with PMA stimulation. We only noted an increase in the size of stained dots associated with lamellipodia-like extensions, consistent with an enhanced integrin clustering. The same observations were done when labelling of focal adhesions was carried out using anti-focal adhesion kinase (FAK) or anti-phosphotyrosine antibodies (not shown). In addition, it is worth pointing out that the actin cytoskeleton reorganisation in response to PMA treatment was clearly different in the two cell lines (Fig. 1). In the case of PDX0, we only observed a thickening of stress fibres, while PDX39P displayed a complex network of actin filaments.

Cell spreading reflects the capacity of cells to change shape, via a remodelling of the actin cytoskeleton, upon attachment to ECM. We previously reported that PMA-induced migration of HT29-D4 cells is associated with an increased spreading upon phorbol ester stimulation [22]. To determine whether differences in cytoskeleton organisation revealed by fluorescence staining were accompanied by a modification in cell spreading, PDX cells treated or not with PMA were plated on ECM protein substratum. After a 2-h incubation at 37°C, the cell area was determined by computer image analysis. In the absence of treatment, no significant difference could be observed in cell area between both cells, whatever the adhesion substratum (Fig. 2). However, upon PMA stimulation, the cell surface of PDX39P cells plated on vitronectin was significantly higher than that of control cells. In contrast, both cells spread with the same efficiency on laminin (Fig. 2) or type I collagen (not shown), two cleavage-independent substrates [13].

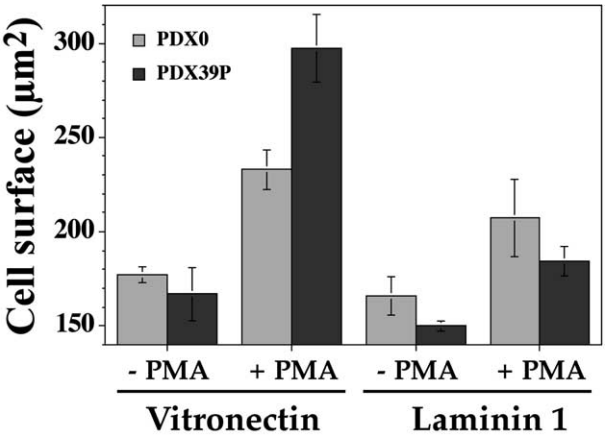

Fig. 2. Effect of α1-PDX expression on cell spreading. Cells were seeded on substratum-coated surface (10 μg/ml vitronectin or 30 μg/ml laminin-1) in the presence or absence of 100 nM PMA. After 2 h at 37°C, cells were fixed with 2.5% glutaraldehyde and their area was measured by computer image analysis. Results (±S.D.) are from a representative experiment of four performed in duplicate.

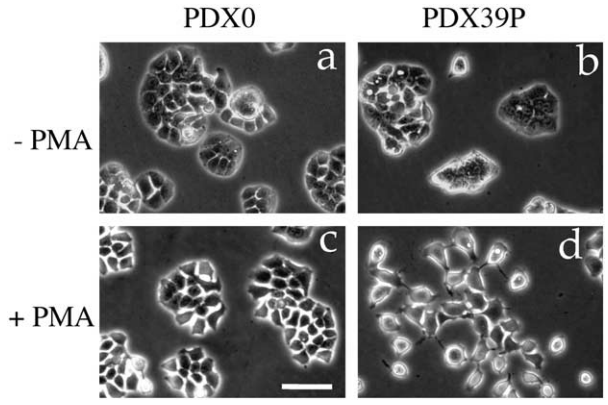

Fig. 3. Effect of α1-PDX expression on PMA-induced cell scattering. Adherent PDX0 (a,c) and PDX39P cells (b,d) were treated (c,d) or not (a,b) with 100 nM PMA. Images were taken by phase-contrast microscope after 4 h of treatment. Scale bar: 50 μm.

3.2. α1-PDX expression increases cell motility

Cell migration results from polarised changes in cell shape due to a continuous remodelling of the actin cytoskeleton. The changes in the cytoskeleton organisation observed by fluorescence staining in PMA-treated PDX39P cells could thus reflect an increased motility. To address this question, we first examined cell scattering in cell monolayers incubated for 4 h in the presence or absence of PMA. As shown in Fig. 3, in the absence of any treatment, both cell types formed tight colonies of adjacent epithelioid cells (Fig. 3a,b). When stimulated by PMA, PDX0 cells displayed enlarged intercellular spaces (Fig. 3c), suggesting that they migrated as coherent cell sheets (cohort-type migration), as already observed with the parental cells HT29-D4 [23]. The front cells of migrating cell sheets acquired a motile morphology with leading edges. Interestingly, results were drastically different on PDX39P cells, as PMA had a scattering-like activity. PDX39P cells dispersed from the cell islands into single cells or small clusters of cells with a marked motile morphology (Fig. 3d).

Because cell scattering is often accompanied by increased cell motility, we used haptotaxis assays towards attractive proteins in modified Boyden chambers. Cell motility was quantified by counting the number of cells that migrated to the underside of membrane. As matrix proteins, we used vitronectin, the sole ligand for integrin αvβ5 whose cleavage is important for its function, and laminin-1 as a cleavage-independent substrate [13]. Cells were unable to spontaneously

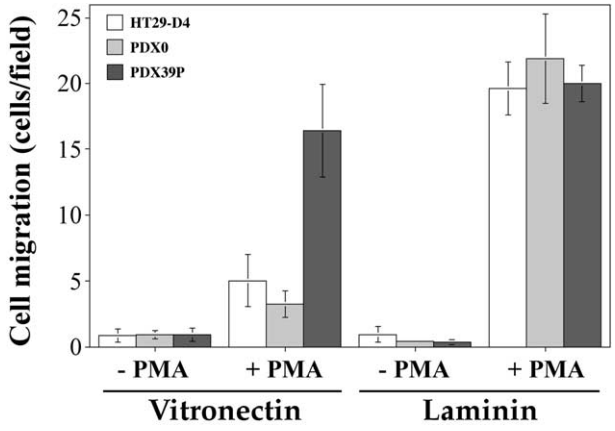

Fig. 4. Effect of α1-PDX expression on protein kinase C-dependent migration. Cell motility toward vitronectin or laminin-1 was determined using porous membranes precoated with ECM proteins. Cells were treated or not with 100 nM PMA prior to being added into the upper reservoir. After 5 h at 37°C, cells that migrated to the underside of the membrane were stained and enumerated. Results (±S.D.) are from three independent experiments performed in triplicate.

migrate towards ECM proteins (Fig. 4). However, both ECM proteins supported migration of cells upon PMA stimulation (Fig. 4), as previously reported for parental HT29-D4 cells [22]. Moreover, protein kinase C-dependent migration of PDX39P cells towards vitronectin was three- to four-fold higher than that observed with PDX0 or HT29-D4 cells. In contrast, laminin-1 supported cell migration with the same efficiency for all cell types (Fig. 4).

4. Discussion

Integrins confer different cell adhesive properties, particularly with respect to focal adhesion formation and motility. Cell migration towards vitronectin is supported by integrin αvβ5, the sole receptor for this ECM protein in the cells used in this study (see [22,24] and data not shown). We have previously shown that this integrin is not cleaved in α1-PDX-expressing PDX39P cells. This leads to alterations in signal transduction pathways activated by integrin ligation, resulting in a reduced attachment to vitronectin [13]. Here we demonstrate that migration of PDX39P cells towards vitronectin is markedly increased when compared to parental HT29-D4 or control PDX0 cells.

How does reduced adhesion lead to an enhanced motility? As cell migration requires the formation of new attachments at the cell front and the break of attachments at the rear, maximum cell speed is predicted to occur at an intermediate ratio of cell–substratum adhesiveness to intracellular contractile force [25,26]. The rate of cell migration versus ECM protein concentration is thus a bell-shaped curve in the case of HT29-D4 cells [22]. Therefore, any experimental manipulation that changes the adhesive state of the cell can potentially cause either an increase or a decrease in cell migration, depending upon where one is starting on the bell-shaped curve. In the case of PDX39P cells, the reduced attachment to vitronectin due to the unprocessing of αv integrin subunit results in a dramatically increased cell migration.

While the exact mechanism by which the absence of αvβ5 integrin cleavage leads to such a difference in the migratory behaviour is not understood, one can suspect that integrin-dependent signalling pathways might be involved. Thus, we have previously reported that activation of mitogen-activated protein kinase (MAPK) by protein kinase C is a prerequisite in the pathway leading to the integrin-dependent migration of HT29-D4 cells [22,23]. Moreover, immunolabelling experiments have showed that, upon inducing migration by PMA treatment, active MAPK is concentrated in lamellipodia-like extensions (data not shown). The enhanced MAPK activity in PDX39P cells [13] could therefore explain the higher motility to vitronectin observed with these cells.

Recent structural studies have revealed that integrin activation and signalling are, at the molecular level, one and the same process that needs large conformational changes propagated through the transmembrane domains of the integrin subunits [27–29]. The cleavage site of αv subunit is localised in the ectodomain nearby the plasma membrane, a region essential for the activity of β3 integrins [30]. It thus can be hypothesised that the endoproteolytic cleavage of α subunit may provide for the conformational flexibility required for integrin activation.

FAK phosphorylation, induced by αvβ5 integrin ligation, appears to contribute to the stabilisation of cell adhesion [31]. The phosphorylation level of FAK and its associated phosphoprotein paxillin, in response to ligation of αvβ5 integrin, is very low in the case of PDX39P [13]. The absence of cleavage of the αvβ5 integrin could thus lead to the impairment of signal transduction by FAK (or another molecule) upon cell adhesion, which in turn might result in a reduced efficiency of PDX39P cells attachment to vitronectin.

Our results clearly show that expression of the convertase inhibitor α1-PDX leads to a cytoskeleton remodelling and a marked increase in cell migration towards vitronectin when cells are stimulated by PMA. In support of these observations, we have established that this enhanced in vitro motility is associated with a highly invasive behaviour of cells upon inoculation into immunosuppressed newborn rats, and that the number of metastases is dramatically reduced when inoculation is performed in association with a function-blocking antibody against αv integrin subunit (Nejjari et al., submitted). Thus, the cleavage of αv integrin subunit appears to play an essential role in αvβ5 integrin-dependent events, especially those leading to cell migration.

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