Some integrin α subunits undergo a post-translational cleavage in their extracellular domain. However, the role of this cleavage in integrin function is unclear. Enzymes involved in this maturation belong to the subtilisin-like endoprotease family (convertases). To understand the role of the α subunit cleavage in integrin function, we have designed stable transfectants (PDX39P cells) expressing α1-PDX, a convertase inhibitor. Immunoprecipitation of cell surface proteins from PDX39P showed that αs, αg, and αv integrins lack endopeptidolytic cleavage. We have compared adhesion between PDX39P cells and mock-transfected cells on different extracellular matrix proteins. No difference in adhesion could be observed on laminin-1 and type I collagen, while attachment of PDX39P cells to vitronectin (ligand of the αvβ3 integrin) was dramatically reduced. The reduced adhesion of PDX39P cells was not due to changes in integrin affinity as determined by solid-phase receptor assay in a cell-free environment. Intracellular signaling pathways activated by αv integrin ligation were also affected in PDX39P cells. It thus seems that the absence of endopeptidolytic cleavage of αv integrins has important consequences on signal transduction pathways leading to alterations in integrin function such as cell adhesion.

Integrins are transmembrane glycoproteins, composed of noncovalently associated α and β subunits, that are involved in cell–extracellular matrix (ECM) and cell–cell interactions (1). Many integrin α chains undergo a post-translational endopeptidolytic cleavage. The αs, αg, αv, α3, α6, and αIIb subunits are cleaved in the membrane-proximal extracellular region, resulting in a heavy chain that is disulfide-linked to a membrane-spanning light chain (2). The αs and αg subunits can also be cleaved, but at unusual positions, near the middle and in the N-terminal region of the molecule, respectively (3, 4). Endopeptidolytic cleavage of integrin α subunits occurs at specific sites comprising pairs of basic amino acids.

Post-translational proteolysis is a common mechanism required for the synthesis of biologically active proteins in bacteria, fungi, yeast, invertebrates, and mammals (5). However, the role of endopeptidolytic cleavage of integrin α subunits is not clear. The cleavage is conserved, not only in different α chains but also across species, suggesting that it might be of functional importance. It has been established, by site-directed mutagenesis of cleavage sites, that uncleaved αIβ1 and αvβ5 are able to mediate cell adhesion to their ligands (3, 6). However, it has been reported that a defect in αv cleavage impairs the inside/out signaling of uncleaved αvβ3 integrins induced by phorbol ester, indicating that cleavage is necessary for proper integrin function (7).

The search for mammalian analogues of kexin, a yeast endoprotease, has led to the discovery of the subtilisin/kexin-like family of proprotein convertases. These calcium-dependent serine proteases can be subdivided into four groups according to their cellular localization and tissue distribution (for reviews and updates, see Refs. 8–11). Furin and PC7, belonging to the first group, are widely expressed in tissues and mainly localized to the trans-Golgi network. PC1 and PC2 are primarily expressed in neural and endocrine cells and are found essentially within secretory granules. The third group includes PC5A and PACE4, widely distributed in tissues and localized within the trans-Golgi network and secretory granules, and PC5B (also called PC6), mostly found within the trans-Golgi network. PC4, exclusively expressed in germ cells, constitutes the fourth group. The general consensus sequence cleaved by these enzymes contains the motif (K/R)-Xn-(K/R), where n = 0, 2, 4, or 6. In vitro and phage display data revealed that the X residue and those following the cleavage site define the fine specificity of each enzyme (12, 13).

Due to their potential pharmacological interest, several inhibitors of proprotein convertases have been designed (14). In humans, a naturally occurring mutation of α1-antitrypsin (α1-AT), known as α1-AT Pittsburgh, changes the specificity of this serpin from an inhibitor of elastase into a potent inhibitor of thrombin (15). Another variant of α1-AT, called α1-AT Portland (α1-PDX), has been engineered by Anderson et al. (16) and described as a potent inhibitor of convertases. Recent findings show that α1-PDX is a selective inhibitor for furin and, to a lesser extent, for PC5B (14). This inhibitor blocks the convertases dependent processing of various precursors (17–21).

In a previous report (22), we showed that αs, αg, and αv integrin subunits are not processed in the furin-deficient LoVo-C5 cell. Moreover, pro-forms of α integrin subunits are cleaved both by recombinant convertases in vitro and ex vivo.
after overexpression in LoVo cells (22, 23). In the present study, we investigated the functional importance of the α subunits cleavage during the interaction of integrins with ECM proteins. We have designed stable transfectants of α1-PDX in HT29-D4 adenocarcinoma cells. These transfected cells, bearing uncleaved integrin α subunits, displayed a reduced attachment to vitronectin. This alteration was correlated with defects in the intracellular signaling pathways activated by α integrin ligation.

MATERIALS AND METHODS

Reagents—Dulbecco’s modified Eagle’s medium was purchased from Life Technologies, Inc. (Cergy-Pontoise, France) and fetal bovine serum from BioWhittaker (Fontenay-sous-Bois, France). PBS was from Oxoid (Basingstoke, United Kingdom). The synthetic peptides RGDS and RGDG were from R&D Systems, Inc. (Minneapolis, MN). Pseudomonas exotoxin A, BSA, type I collagen, poly-L-lysine, and crystal violet were obtained from Sigma (St-Quentin Fallavier, France). Sulfo-Succinimidyl-6-(biotinamido) hexanote (NHS-LEC-biotin) was from Pierce. TMB peroxidase EIA substrate kit was obtained from Bio-Rad (Irvine, CA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep anti-mouse and rat mAb GoH3 (anti-

RESULTS

Cell Surface α5, α6, and α1 Integrin Subunits Are Uncleaved in PDX39P Cells—α1-PDX has been described as a potential inhibitor of convertases (16), which are responsible for the cleavage of α integrin subunits (22, 23). We therefore expressed the α1-PDX inhibitor in HT29-D4 cells, and we selected stable transfectants. The clone PDX39 was further selected on the basis of its resistance to PEA, a toxin activated by convertases (27), to obtain a population (PDX39P cells) expressing high levels of the α1-PDX inhibitor (data not shown). Among the cullable integrins, HT29-D4 cells express α1, α5, α6, and α1-PDX expression impairs the endoproteolytic processing of α1α5 subunits, as confirmed by flow cytometry and by immunoprecipitation of biotinylated cell surface proteins as described elsewhere (28). Cell Adhesion Assay—Adhesion assays were performed as described previously (28, 29). Briefly, cells were harvested in single cell suspension by treatment with 0.53 mM EDTA in PBS, added to wells coated with purified ECM proteins, and allowed to adhere to the substrate for 2 h at 37 °C. After washing, attached cells were stained with 0.1% crystal violet and lysed with 1% SDS. Absorbance was then measured at 600 nm.

Solid-phase Receptor Assay—Serum-starved cells in suspension were washed twice with 10 mM Tris, pH 7.4, and resuspended in the same buffer containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 500 units/ml aprotinin, 1 μg/ml leupeptin, 1 μM pepstatin, 1 mM iodoacetamide, and 1 mM o-phenanthroline). Cells were disrupted with a Potter homogenizer and homogenate was centrifuged at 600 × g for 10 min at 4 °C. Plasma membranes were recovered by centrifuging the supernatant at 50,000 × g for 45 min and then solubilized for 45 min at 4 °C with 50 mM Tris, pH 7.4, 150 mM NaCl (TBS) containing 1% n-octyl-β-D-glucopyranoside, 1 mM MgCl2, 1 mM CaCl2, and the mixture of protease inhibitors. The extract was clarified by centrifugation at 15,000 × g for 10 min at 4 °C, and the protein concentration was determined by the Bio-Rad DC protein assay.

MaxiSorbTM microtiter plate wells (Nunc) were coated for 2 h at 37 °C with 10 μg/ml vitronectin in 50 μl of TBS and blocked with 1% BSA for 1 h at room temperature. Vitronectin-coated wells were incubated with 100 μl of solubilized membranes (300 μg of protein) overnight at 4 °C. Unbound proteins were removed by four washes with TBS containing 0.2% Tween 20 and 1% BSA. α6 binding was detected by sequential incubations for 1 h at 37 °C with 100 μl of 10 μg/ml mouse anti-human (anti-MKH2) or rat anti-human (anti-MKH1) integrin subunit antibody, 200 μg/ml aprotinin, 1 mM AMF-7 and at room temperature with rabbit anti-mouse IgG (1/500) and anti-rabbit IgG (1/500) Fab′-conjugated antibodies. Finally, 100 μl of peroxidase substrate was added for 10 min at room temperature, the reaction was stopped by adding 100 μl of 0.5 mol/liter H2SO4, and absorbance was measured at 450 nm.

Detection of Activated MAPK and Tyrosine Phosphorylation of FAK and Paxillin—Confluent cells were washed twice with serum-free medium and incubated in Dulbecco’s modified Eagle’s medium containing 0.1% BSA for 24 h at 37 °C. Serum-starved cells were harvested in single cell suspension, added (5 × 106 cells in 1 ml) to 9.6-cm2 wells coated with 10 μg/ml poly-L-lysine or vitronectin, and allowed to adhere to the substrata for the indicated periods of time at 37 °C. After three washes with PBS, attached cells were lysed with 20 μl Tris-HCl, pH 8, 200 mM NaCl, 1 mM EDTA, and 1% Triton X-100 (RIPA buffer) containing 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM NaF, and the mixture of protease inhibitors. Lysates were clarified by centrifugation and analyzed by immunoblot, after immunoprecipitation, or directly with an anti-active MAPK polyclonal antibody as described previously (30). For immunoprecipitation, 600–900 μg of proteins were incubated with 1 μg of anti-FAK or 2 μg of anti-paxillin antibody overnight at 4 °C and then with protein G-agarose for 45 min. Pellets were washed three times with RIPA buffer, three times with RIPA buffer, 500 mM NaCl, and once with PBS. Immunoprecipitated proteins were resolved by SDS-PAGE and blotted onto a nitrocellulose sheet. Membranes were blocked in PBS, 4% BSA, 0.2% Tween 20 and probed overnight at 4 °C with FY20 antibody in PBS, 0.8% BSA, 0.2% Tween 20. Blots were then visualized with horseradish peroxidase-conjugated secondary antibodies.

When necessary, nitrocellulose membranes were stripped and re-probed with anti-FAK (1/1000), anti-FR (0.2 μg/ml), or anti-paxillin (0.1 μg/ml) antibodies.

Endoproteolytic Cleavage Controls Integrin α5β6 Function

Flow Cytometric Analysis and Cell Surface Labeling—The cell surface expression of integrin subunits was determined by flow cytometry and by immunoprecipitation of biotinylated cell surface proteins as described elsewhere (28).
Endoproteolytic Cleavage Controls Integrin α5β3 Function

Fig. 1. Cleavage status of α integrin subunits in transfected cells. HT29-D4 cells (D4) were transfected with a pBSK-CMV expression vector containing (clone PDX39) or not (clone PDX0) the full-length α5-PDX cDNA. PDX39P cells represent a subpopulation of the clone PDX39 resistant to PEA and expressing high levels of α5-PDX. Cells were surface-biotinylated, and integrins were immunoprecipitated with specific antibodies against α5 (A), α6 (B), or αv (C) subunits. Immunopurified proteins were then analyzed by SDS-PAGE under nonreducing (NR) or reducing (R) conditions. The migration of β3 (NR: 190 kDa; R: 210 kDa), noncleaved α5 (α5NC; R: 150 kDa), cleaved α5 (α5c; R: 120 kDa), β5c (NR: 95 kDa; R: 105 kDa), noncleaved αv (αvNC; R: 155 kDa), cleaved αv (αvC; R: 130 kDa), β1 (NR: 110 kDa; R: 130 kDa), noncleaved α3 (α3NC; R: 160 kDa) and cleaved α3 (α3c; R: 130 kDa) are emphasized.

concentration of PEA increased α5-PDX expression and led to a strong impairment of α6 cleavage.

These observations were extended to the α5 and α6 subunits using the same experimental approach. As seen on Fig. 1B, most α5 chain, that associated with β3 and β6 subunits, failed to be cleaved in PDX39P cells, as observed with α6 subunit. The amount of normally processed α5 is more difficult to evaluate because cleaved form (α5C) comigrated with reduced β3 (Fig. 1C). However, the bands corresponding to noncleaved (α5NC) and nonreduced α5 chains from PDX39P have the same intensity, suggesting that virtually all α5 subunits are in an uncleaved form. Thus, the expression of high levels of α5-PDX inhibitor blocked almost totally the endoproteolytic maturation of all the cleavable α integrin subunits expressed in PDX39P cells. Moreover, the ratio cleaved/uncleaved form correlated with the expression level of α5-PDX (data not shown).

α5-PDX Does Not Alter Cell Surface Expression of Integrins—As observed above after cell surface biotinylation and immunoprecipitation, the expression of α5-PDX does not seem to affect the labeling intensity of integrin subunits. To confirm these results by a more quantitative approach, cells were examined for cell surface expression of integrin subunits by indirect immunofluorescence using specific mAbs. Flow cytometry analysis indicated that the extent of integrin expression at the cell surface is quite similar whatever the cell population studied (Fig. 2). This demonstrates that the defect in α5 chains processing did not impair the exportation of the heterodimers to the plasma membrane.

Expression of α5-PDX Decreases Adhesion of PDX39P Cells on Vitronectin—To examine the importance of the α chain cleavage for integrin function, we first measured the adhesion of transfected cells to purified ECM proteins and to poly-L-lysine as an integrin-independent substrate. Adhesion of PDX39P cells to laminin-1 and collagen type I was quite similar to control cells, as was the attachment to poly-L-lysine (Fig. 3A). On the contrary, adhesion of PDX39P cells to vitronectin was significantly reduced when compared with HT29-D4 or PDX0 cells.

The integrin α5β3, the unique receptor for vitronectin in HT29-D4 (26) and in PDX0 and PDX39P cells (not shown), does not require activation to mediate ligand binding. However, the absence of cleavage of α subunits could lead to the suppression of the constitutive integrin activation. We, therefore, assessed whether PMA or the divalent cation Mn2+ could restore a normal attachment of α5-PDX-expressing cells on vitronectin. As illustrated in Fig. 3B, stimulation of PDX39P cells by PMA failed to restore cell adhesion to vitronectin. Moreover, treatment of cells by 1 mM (Fig. 3B) or higher concentrations (not shown) of Mn2+ does stimulate cell adhesion, but in the same proportion for all cell types. These data indicate that uncleaved α5β3 integrin present on PDX39P cells seems to be constitutively active, but it can be further activated by divalent ions as does cleaved integrin.

Cleavage Does Not Affect Binding of Soluble α5β3 Integrins—Integrin-mediated cell adhesion may be altered by changes either in affinity of individual receptors for ligand or in integrin avidity (32). It has been proposed that integrin interaction with cytoskeletal proteins that serve to anchor and cluster integrins regulates receptor avidity. We therefore used a solid-phase binding assay to test the interaction of the α5β3 integrins with
Microtiter plates were coated with ECM proteins or poly-L-lysine and then examined whether signaling pathways that can be affected by cleavage. We therefore assessed after immunoprecipitation. We observed a time-dependent increase in tyrosine phosphorylation of FAK from PDX0 cells (data not shown). As observed with FAK, paxillin from PDX0 cells was phosphorylated after stimulation of PDX39P cells by integrin ligation. Protein tyrosine phosphorylation is one of the earliest events detected in response to cell attachment to an ECM protein-coated surface. Adhesion of PDX0 cells to vitronectin led to a time-dependent increase in tyrosine phosphorylation of proteins in the molecular mass range of 110–125 kDa and around 70 kDa, whereas no enhanced phosphorylation could be observed with PDX39P cells (data not shown).

In most cell types, the FAK accounts for a large proportion of the tyrosine phosphorylation of proteins in the molecular mass range of 110–125 kDa and around 70 kDa, whereas no enhanced phosphorylation could be observed with PDX39P cells (data not shown).

In most cell types, the FAK accounts for a large proportion of the tyrosine phosphorylation of proteins in the molecular mass range of 110–125 kDa and around 70 kDa, whereas no enhanced phosphorylation could be observed with PDX39P cells (data not shown).
Endoproteolytic Cleavage Controls Integrin $\alpha_5\beta_3$ Function

**Fig. 6. Activation of MAPK pathway during cell adhesion to vitronectin.** A, serum-starved cells were harvested in single cell suspension and allowed to adhere for the indicated periods of time to wells coated with 10 µg/ml vitronectin. Attached cells were lysed and 10 µg of proteins were resolved by SDS-PAGE. The nitrocellulose membrane was probed with an anti-active MAPK antibody (upper panel). After stripping, the membrane was reprobed with anti-MAPK antibodies (lower panel). B, serum-starved cells were pretreated or not for 90 min with 10 µM PD98059 before performing the cell adhesion assay in wells coated with 10 µg/ml vitronectin in the absence or the presence of 10 ng/ml epidermal growth factor. At the end of the adhesion assay (2 h at 37 °C), the activation status of MAPK was checked as described above. Results are representative experiments of three performed.

**A**

| Cell Line | Adhesion (O.D.) |
|-----------|-----------------|
| PD98059   | -               |
| EGF       | +               |

**B**

| Cell Line | MAPK |
|-----------|------|
| PD90059   | -    |
| PDX0      | +    |

**DISCUSSION**

In the present work we have examined the importance of $\alpha$ subunit cleavage for integrin function. We have generated stable transfectants (PDX39P cells) expressing high levels of $\alpha_5$-PDX, a potent and selective inhibitor of convertases and especially of furin and PC5B (14). The $\alpha_5$-PDX-expressing cells displayed plasma membrane integrins mainly under an uncleaved form, although a minor cleavage could be observed. This result confirms that the convertase family of serine proteases is involved in the post-translational processing of integrin $\alpha$ chains, as we reported previously (22, 23).

Endoproteolytic cleavage is often required to generate active proteins from inactive precursors. Here we report that PDX39P cells, which display uncleaved integrins on cell surface, showed a reduced adhesion to vitronectin through the $\alpha_5\beta_3$ integrin. However, we found no evidence that cleavage of the $\alpha$ subunits has any major effect on cell adhesion to other ECM proteins, such as laminin-1 and collagen type I. This absence of effect is likely due to the involvement of noncleavable $\beta_1$ integrins, such as $\alpha_5\beta_1$ or $\alpha_6\beta_1$, which support HT29-D4 cell adhesion to these ECM proteins (26, 28). We neither observed any difference between cells when using the integrin-independent substrate poly-L-lysine.

The reduced adhesion of PDX39P cells to vitronectin was not due to changes in $\alpha_5\beta_3$ expression, as neither transfection nor PEA selection altered the amount of integrin subunits at the cell surface. For several reasons, abolishing $\alpha$ subunit cleavage neither seems to cause important alterations in integrin structure. (i) $\alpha/\beta$ association still occurred because heterodimers containing the uncleaved $\alpha_5$, $\alpha_6$, or $\alpha_6$ subunits were expressed on the cell surface, as also reported for the furin-deficient cell line LoVo (22) and mutated $\alpha_{1b}$ and $\alpha_6$ subunits (3, 6). (ii) PDX39P cells attached with the same efficiency than parental cells or empty vector transfected cells to two mAbs (69.6.5 and AMF-7) recognizing distinct $\alpha_i$ epitopes (not shown). (iii) The function-blocking mAb 69.6.5 inhibited adhesion to vitronectin with similar dose-effect profiles whatever the cell type (not shown).

The integrin adhesive function can be regulated by intracellular signals, a phenomenon known as inside-out signaling (reviewed in Refs. 34 and 35). This can be done either by altering the affinity of the individual integrins or by integrin clustering, which allows more efficient interaction and increased binding between cells and ECM. The reduced adhesion of PDX39P cells to vitronectin is most likely not due to changes in integrin affinity, as we noticed no significant difference of interaction between cleaved and uncleaved integrins in a solid-phase receptor assay. This result is in agreement with previous reports showing that the absence of cleavage of the $\alpha_5$ and $\alpha_{1b}$ subunits did not affect ligand binding of the $\alpha_5\beta_3$ and $\alpha_{1b}\beta_3$ integrins (3, 6). However, these studies have not examined the role of integrin cleavage on intracellular signaling pathways. In the present study we show that the phosphorylation level of FAK and its associated phosphoprotein paxillin, in response to ligation of $\alpha_5\beta_3$ integrin, was very low in $\alpha_5$-PDX-expressing cells.

Various signaling proteins have been involved in the inside-out modulation of integrin function. Thus, FAK phosphorylation induced by integrin ligation appears to contribute to stabilization of cell adhesion (36). Such a positive feedback loop is also thought to occur in the context of protein kinase C activation during adhesion and spreading (37). It thus can be hypothesized that the absence of cleavage of the $\alpha_5\beta_3$ integrin leads to the impairment of signal transduction by FAK (or another molecule) upon cell adhesion, that in turn might result in a reduced efficiency of PDX39P cells attachment to vitronectin.

In support of this hypothesis, Delwel et al. (7) have shown that the cleavage of the $\alpha_4$A subunit is essential for activation of the integrin by the phosphor ester PMA, a protein kinase C activator.

MAPKs (ERK1 and ERK2) are activated in response to a variety of extracellular signals and thus represent a conver-
gence point for many signaling pathways. It is now clear that integrin-mediated cell adhesion can also lead to the activation of the MAPK cascade (for review, see Ref. 1). In this work we show that ERK2 and, to a lesser extent, ERK1 are activated upon interaction of the αvβ3 integrin with vitronectin. We also reported that ERK2 was already activated in PDX39P cells kept in suspension and that the enzyme was further activated during adhesion. There is increasing evidence that suppression of integrin activation may be a physiological mechanism to control integrin-dependent cell adhesion and migration. The existence of signaling pathways acting as inhibitors of integrin activation has been proposed, and recently Ras/Raf-1-initiated activation of the MAPK pathway has been shown to suppress the activation of αv3 integrins (33). Thus, one possibility is that the reduced adhesion of PDX39P cells to vitronectin was the result of the high activity level of MAPK in these cells. However, this is likely not the case, because neither MAPK activation nor impediment of MAPK activation had any effect on adhesion to vitronectin. An alternative is the suppression of the constitutive integrin activation due to the absence of α subunits cleavage. Nevertheless, the integrin αvβ3 was already present as an active form in αv1-PDX-expressing cells, as neither PMA nor the divalent cation Mn2+, two integrin activators, could restore a normal attachment of αv1-PDX-expressing cells on vitronectin.

It thus appears that the absence of endoproteolytic cleavage of the αv subunit affects integrin function by altering αvβ3-dependent signaling pathways. The molecular mechanism responsible of this dysfunction remains to be determined.

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