Lack of integrin α -chain endoproteolytic cleavage in furin-deficient human colon adenocarcinoma cells LoVo

Maxime LEHMANN*, Véronique RIGOT*, Nabil G. SEIDAH†, Jacques MARVALDI*§ and Jean-Claude LISSITZKY‡

*URA CNRS 1924, Laboratoire de Biologie Cellulaire, Université Aix-Marseille II, Faculté de Pharmacie, 27 Bd J. Moulin, 13387 Marseille, France, †Laboratory of Biochemical Neuroendocrinology, IRCM and Protein Engineering Network of Centers of Excellence, 110 Pines Avenue, Montreal, Quebec, Canada H3W 1R7, and ‡INSERM U387, 270 Bvd St Marguerite, 13009 Marseille, France

In the present report the biosynthesis of the integrin α -chains endowed with constitutive endoproteolytic cleavage was evaluated in LoVo cells where furin, a subtilisin-like convertase involved in post-translational endoproteolytic processing, is not functional. It was found that cell-surface $\alpha 3$, $\alpha 6$ and αv subunits were not processed endoproteolytically into heavy and light chains as they were in HT29-D4 cells, a furin-competent cell line. Complete removal of N-linked oligosaccharides and pulse-chase experiments confirmed that the cleavage of the $\alpha 6$ integrin subunit occurring 45 min after translation in HT29 cells did not

take place in LoVo cells. Apart from cleavage deficiency, $\alpha 6$ subunit glycosylation, association with $\beta 4$ subunits and targeting to the plasma membrane seemed comparable in LoVo and HT29 cells. The pro- $\alpha 6$ and the pro- $\alpha 3$ subunits immunopurified from LoVo cells were highly sensitive to endoproteolysis by recombinant furin. Furin cleavage was calcium dependent and resulted in the conversion of the 140 kDa pro- $\alpha 6$ into a 120 kDa heavy chain. These results suggest strongly that furin is involved in the endoproteolytic processing of cleavable integrin α subunits.

INTRODUCTION

Integrins are transmembrane cell-surface α/β heterodimeric receptors [1], which may connect internally to the cytoskeleton [2–4]. The binding of integrins with ligands in the extracellular matrix or on the cell surface of neighbouring cell results in control of cell locomotion (adhesion and migration), cell shape, differentiation and tissue structure. As many as 16 different α and 8 β subunits have been identified so far. Except for $\alpha 4$, $\alpha 6$ and αv , α subunits associate with only one of the different β subunits, resulting in the production of 21 different heterodimers [1]. The genetic structural diversity of integrins is amplified by post-transcriptional alternative splicing and post-translational glycosylation and phosphorylation. Some integrin α -chains ($\alpha 3$, $\alpha 5$, $\alpha 6$, $\alpha 7$, αv and αIIb) also show a constitutive post-translational endoproteolytic cleavage occurring at a site comprising pairs of basic amino acids. This site is located extracellularly near to the transmembrane domain so that the molecule is constituted by a membrane-bound 25–30 kDa C-terminal light chain that is disulphide bonded to the N-terminal extracellular heavy chain.

Endoproteolytic cleavages at sites comprising pairs of basic amino acids participate in the post-translational processing of many proteins [5,6], hormones, growth factors and neuropeptides, and also in function-activation of several cell-surface receptors, adhesion molecules [7] and extracellular matrix proteins [8]. The search for the mammalian homologues of KEX2, a yeast cellular processing endoprotease that cleaves at basic amino acid sites, has resulted in the discovery of a family of calcium-dependent serine endoproteases: the subtilisin-related proprotein convertases [9–13]. These enzymes share between 50 and 74% amino acid sequence identity in the subtilisin-like

catalytic domain. However, two main subsets of enzymes can be distinguished. The first includes convertases such as PC2 and PC1/3 [14,15], which are expressed mainly in neuroendocrine tissues and brain, where they are located in secretory vesicles. These enzymes play a key role in allowing the release of hormones or neuropeptides from biologically inactive precursors. The second subset of convertases, which includes furin, PACE 4 and PC5/6, shows wide tissue expression in the body (reviewed in [16]). These enzymes are equipped with a C-terminal cysteine-rich region not found in PC2 or PC1/3. Furin and PC6B, a splice variant of PC6, are type I membrane-bound convertases with a C-terminal transmembrane domain and a cytosolic tail. PACE 4, PC5/6B and especially furin are believed to be involved in precursor processing of constitutively secreted proteins such as growth factors and their receptors [17,18] and in cleavage of viral polyproteins [19]. In the present paper, the hypothesis that furin is involved in endoproteolytic processing of integrin α -chains was analysed by studying integrin biosynthesis and processing in the LoVo cell line where furin is not functional because of a nucleotide deletion in the coding region of the gene [20].

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, NY, U.S.A.) and Dulbecco's PBS was from Oxoid (Basingstoke, Hampshire, U.K.). Fetal calf serum (FCS) was from Sera-Lab (France). Trypsin, BSA, McCoy's 5A modified medium, mouse anti-rat, fluorescein isothiocyanate (FITC)-conjugated goat anti-rat and FITC-goat–

Abbreviations used: AMC, amino-4-methylcoumarin; DMEM, Dulbecco's modified Eagle's medium; Endo H, Endoglycosidase H; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; mAb, monoclonal antibody; NHS-LC biotin, sulphosuccinimidyl-6-(biotinamido)hexanoate; PEA, *Pseudomonas* exotoxin A; PNGase F, peptide N-glycanase.

§ To whom correspondence should be addressed.

mouse antibodies were purchased from Sigma (La Verpillière, France). Endoglucosidase H (Endo H), protein G-agarose and protein A-agarose were purchased from Boehringer (Mannheim, Germany). Peptide N-glycanase (PNGase F) was prepared from *Flavobacterium meningosepticum* and was kindly provided by Dr. Karamanos (Limoges, France). Sulphosuccinimidyl-6-(biotin-amido)hexanoate (NHS-LC biotin) was obtained from Pierce (Rochford, IL, U.S.A.). Enhanced chemiluminescence reagent and horseradish peroxidase-streptavidin complex (HRP-streptavidin) were from Amersham (Les Ulis, France). Free amino-4-methylcoumarin (AMC) and pRTKR-MCA were from the Peptide Institute (Osaka, Japan).

Antibodies

Rat monoclonal antibody (mAb) 69-6-5 against the αv integrin subunit was obtained as previously described [21]. Rat mAb GoH3 anti- $\alpha 6$ was purchased from Immunotech (Marseille, France). Mouse mAb P1B5 anti- $\alpha 3$ was from Telios (La Jolla, CA, U.S.A.).

Cell cultures

The human colon adenocarcinoma cell lines HT29-D4 and LoVo-C5 were cloned by limiting dilution of parental cell lines and were routinely cultured as described [22,23].

Cell toxicity evaluation of *Pseudomonas* exotoxin A (PEA)

Subconfluent cells grown in 48-well cell culture plates were incubated for 36 h in medium with or without PEA at the indicated concentrations before labelling for 30 min with 100 μ Ci/ml 35 S-Translabel® (ICN, Orsay, France) in Met-, Cys-deficient medium containing 10% (v/v) dialysed FCS. Cells were then washed with cold PBS and solubilized with 100 μ l of 0.25 M NaOH containing 0.1% SDS at 110 °C. BSA (2 mg/ml) was added to 10 μ l of cell lysate and proteins were precipitated in cold 15% (w/v) trichloroacetic acid solution containing Met and Cys (1 mg/ml each). Insoluble proteins were recovered on glass fibre filters and radioactivity was determined by β -scintillation counting. Results are expressed as the means $\pm$ S.D. for triplicate wells.

Flow cytometry analysis of cell-surface expression of integrin subunits

Cell-surface expression of $\alpha 6$, $\alpha 3$ and αv integrin subunits in HT29-D4 and LoVo-C5 cells was determined by flow cytometry. Subconfluent cells were harvested and resuspended in medium containing 1% (v/v) BSA. The single-cell suspension (10^6 cells/ml) was incubated for 90 min at 4 °C in the presence of anti-integrin mAbs or non-immune immunoglobulin (10 μ g/ml each). Cells were rinsed four times with ice-cold PBS containing 1% (v/v) BSA and then incubated for 45 min at 4 °C with an excess of appropriate secondary FITC-conjugated anti-mouse or anti-rat antibodies. After washing, cells were fixed in 1% para-formaldehyde, and cell-bound fluorescence was analysed by injection on an EPICS® flow cytometry device. Gating parameters were set so that 90% of injected cells were analysed. Results are expressed as the means $\pm$ S.D. of fluorescence in arbitrary units.

Cell-surface labelling

After washing with PBS, surface proteins of exponentially growing cells were labelled with NHS-LC biotin (0.5 mg/ml in ice-cold PBS) for 30 min at 4 °C. After being washed with PBS,

labelled cells were lysed in RIPA buffer [50 mM Tris/HCl, pH 8, 150 mM NaCl, 0.5% BSA, 1 mM EDTA, 1% (w/v) Triton X-100] containing protease inhibitors: aprotinin, leupeptin, iodoacetamide, pepstatin and $\alpha 2$ -microglobulin (1 μ g/ml each) and 1 mM PMSF. After centrifugation at 15000 *g* for 10 min, extracted proteins were incubated with anti-integrin mAbs (2 μ g) for 18 h at 4 °C and with protein G-agarose for a further 2 h. After being washed, immunoprecipitates were analysed by SDS/PAGE under non-reducing or reducing (20 mM dithiothreitol) and alkylating (50 mM iodoacetamide) conditions. After electro-transfer of gel content to nitrocellulose membranes (Hybond C+; Amersham, les Ulis, France) in accordance with the supplier's instructions, membranes were blocked with streptavidin buffer [50 mM Tris buffer, pH 8, 10% (w/v) glycerol, 1 M glucose, 0.5% BSA and 0.1% Tween 20] for 1 h at room temperature. Biotinylated proteins were detected with HRP-streptavidin in streptavidin buffer for 1 h at room temperature. HRP-streptavidin was revealed with the light-based ECL System (Amersham), in accordance with the supplier's instructions.

Cell metabolic labelling and pulse-chase experiments

Exponentially growing cells were depleted for 1 h at 37 °C in Met-, Cys-deficient DMEM containing 10% (v/v) dialysed FCS. Cells were then pulsed for 15 min in the same medium containing 1 mCi/ml [35 S]-Translabel®. Cells were washed three times with culture medium containing 200 mg/l cystine and methionine and 10% (v/v) FCS and were chased for various periods in the same medium. Cells were then placed on ice, solubilized in RIPA buffer and lysates were submitted to immunoprecipitation as described above. Immunopurified integrins were reduced and alkylated and analysed by SDS/PAGE [7.5% (w/v) gel]. Gels were impregnated with 1 M sodium salicylate and fluorographed at -80 °C.

Steady-state metabolic labelling was performed for 18 h at 37 °C with 150 μ Ci/ml 35 S-Translabel® in DMEM containing 10% (v/v) FCS.

Glycosidase treatments of immunoprecipitated integrins

Integrins were immunoprecipitated from solubilized biotinylated cells. After recovery with 1% (w/v) SDS, immunoprecipitates were digested at 37 °C either by 20 m-units/ml PNGase F for 120 min in PNGase F buffer [Tris/HCl buffer, pH 8.6, containing 1% (w/v) Triton X-100, 0.1% β -mercaptoethanol, 0.25% SDS and protease inhibitors] or by 10 m-units/ml Endo H for 6 h in Endo H buffer (50 mM citrate/phosphate buffer, pH 5.5, containing 1% Triton X-100, 0.1% β -mercaptoethanol and protease inhibitors). Controls were incubated in the appropriate buffer without enzymes. Reactions were stopped by addition of 5 $\times$ SDS/PAGE sample buffer. Proteins were analysed by SDS/PAGE and revealed after transfer to nitrocellulose membrane with HRP-streptavidin as described above.

Production of recombinant furin

Recombinant human furin was obtained from the medium of infected sf9 cells with a baculovirus recombinant expressing soluble human furin where a stop codon was introduced in place of the codon for furin amino acid 586 located before the cysteine-rich domain (BCRD-furin). The specific activity of the enzyme preparation obtained was measured with a fluorogenic peptide P-Glu-Arg-Thr-Lys-Arg-AMC, as previously described [24], based on the increase of fluorescence generated by the cleavage of this substrate (0.1 mM) in 50 mM sodium acetate, pH 6.8. After incubation with or without furin, in the presence of 2 mM

calcium or of 5 mM EDTA, the fluorescence of released AMC was measured at 460 nm after excitation at 370 nm and evaluated by plotting on a standard curve obtained with known amounts of free AMC.

Integrin cleavage by furin or trypsin

Integrins $\alpha 6$ or $\alpha 3$ were immunoprecipitated by mAb GoH3 from the cell lysate of LoVo cells labelled for 4 h with [35 S]methionine in methionine-free DMEM. Immunocomplexes were recovered by incubation for 2 h with magnetic beads coated with anti-mouse or anti-rat antibody (Dyna, Compiègne, France). After washing, beads were resuspended in 62 mM Tris, pH 6.8, containing 1% (w/v) n-octyl glucoside. Beads were incubated with or without furin or trypsin at the indicated concentrations in 62 mM Tris, pH 6.8, containing 1% (w/v) N-octyl glucoside in the presence or absence of 1 mM CaCl_2 as indicated, for 4 h at room temperature with constant shaking. The reaction was stopped by freezing after the addition of 5 $\times$ reducing SDS/PAGE sample buffer. The extent of integrin cleavage was measured by SDS/PAGE and fluorography.

RESULTS

HT29 cells are 1000 times more sensitive to PEA than LoVo cells

Furin has been shown to be necessary for cleavage and activation of PEA, a toxin that inhibits protein synthesis by catalysing the ADP-ribosylation of elongation factor 2 [25]. Cell toxicity of PEA appears as an indicative bioassay for furin activity and was assessed with our cell strains.

To this end, HT29-D4 or LoVo-C5 cells were treated with increasing concentrations of PEA and protein synthesis was assessed by measuring the incorporation of [35 S]methionine into protein during pulse-labelling. The results in Figure 1 show that a 50% inhibition of incorporation of [35 S]methionine into protein was obtained with as little as 20 ng/ml PEA for HT29-D4 cells, whereas at least 1000 times this concentration was needed to reach the same inhibition level for LoVo cells. Thus the high resistance to PEA, assigned to a furin functional defect in LoVo cells, was also found in our LoVo-C5 cells.

Figure 1 Cytotoxicity of PEA for HT29-D4 and LoVo-C5 cells

Cells were incubated for 36 h in the presence of the indicated concentrations of PEA, and protein synthesis was measured as described in the Materials and methods section. Symbols: ●, LoVo-C5 cells; ○, HT29-D4 cells.

Table 1 Cell-surface expression of $\alpha 6$, $\alpha 3$ and αv integrin subunits in HT29-D4 and LoVo-C5 cells

Expression of cell-surface integrin was analysed by flow cytometry as described in the Materials and methods section with G0H3 mAb anti- $\alpha 6$, P1B5 mAb anti- $\alpha 3$ and 69-6-5 mAb anti- αv . Results are means $\pm$ S.D. for three experiments.

Cells	mAb binding (fluorescence, arbitrary units)		
	Anti- $\alpha 6$	Anti- αv	Anti- $\alpha 3$
HT29-D4	120 $\pm$ 48	30 $\pm$ 12	66 $\pm$ 33
LoVo-C5	97 $\pm$ 47	7 $\pm$ 8	15 $\pm$ 10

Cell-surface $\alpha 3$, $\alpha 6$ and αv integrin subunits lack intra-chain cleavage in LoVo cells

Some of the integrin α -chains, which show a constitutive post-translational endoproteolytic cleavage, might be expressed in epithelial tissues. Table 1 shows that the cell-surface expression of the $\alpha 3$, $\alpha 6$ and αv chains was significant in LoVo and HT29 cells. Therefore the molecular status of these subunits was compared in the two cell lines to establish whether furin participated in the endoproteolytic processing of integrin α -chains.

Cells were surface-labelled with biotin and solubilized, and integrins were immunoprecipitated with anti- $\alpha 3$ (P1B5), anti- $\alpha 6$ (GoH3) or anti- αv (69-6-5) mAbs. Immunoprecipitates were analysed by SDS/PAGE under reducing or non-reducing conditions, followed by blotting and detection of biotinylated adducts by streptavidin-peroxidase-dependent chemiluminescence.

SDS/PAGE analysis of $\alpha 3$, $\alpha 6$ and αv immunoprecipitates under non-reducing conditions indicated that similar integrins were found in LoVo and HT29 cells. The unreduced $\alpha 6$ -immunoprecipitates were composed of an $\alpha 6$ chain (140 kDa) non-covalently associated with $\beta 4$ chains (200 kDa) (Figure 2, $\alpha 6$, lanes 1 and 2). A 130 kDa polypeptide ($\alpha 3$ subunit) co-precipitating with a 120 kDa species ($\beta 1$ subunit) was obtained with P1B5 mAb (Figure 2, $\alpha 3$, lanes 1 and 2), and a 140 kDa band (αv subunit) co-precipitating with 95/100 kDa proteins (β subunits) was found with 69-6-5 mAb (Figure 2, αv , lanes 1 and 2).

In HT29 cells the SDS/PAGE migration pattern of reduced and unreduced α -chains was obviously different. Comparison of lanes 1 and 3 in Figure 2 indicates that reduced α -chains migrated with a higher velocity than their unreduced counterparts. The smaller molecular mass of reduced α -chains was easily distinguished for $\alpha 6$ and αv immunoprecipitates. For $\alpha 3$ immunoprecipitates, the decrease in molecular mass of reduced α -chains resulted in co-migration with the $\beta 1$ chain. In all cases a 30 kDa polypeptide was detected in the reduced immunoprecipitates, which most probably corresponds to the light chains released on reduction.

In LoVo cells the SDS/PAGE migration pattern was quite similar for reduced and unreduced $\alpha 3$, $\alpha 6$ and αv integrin subunits (compare lanes 2 and 4 in Figure 2). In particular, reduced $\alpha 3$ and αv chains did not display the decrease in molecular mass found in HT29 cells. The reduced and alkylated $\alpha 6$ subunit migrated with an even higher apparent molecular mass than its unreduced counterpart, a feature that is commonly observed when proteins are unfolded by reduction. Furthermore the 30 kDa light chains found after reduction of $\alpha 3$, $\alpha 6$ or αv immunoprecipitates in HT29 cells were not detected in LoVo cells. All these observations indicate that endoproteolytic cleavage of the α integrin subunit in LoVo cells was defective.

Figure 2 Molecular status of cell-surface $\alpha 6$, $\alpha 3$ and αv integrins in HT29-D4 and LoVo-C5 cells

HT29-D4 (lanes 1 and 3) and LoVo-C5 cells (lanes 2 and 4) were surface-biotinylated. Extracted proteins were immunoprecipitated with mAb GoH3 (anti- $\alpha 6$), mAb P1B5 (anti- $\alpha 3$) or mAb 69-6-5 (anti- αv) and analysed by SDS/PAGE under non-reducing (lanes 1 and 2) or reducing (lanes 3 and 4) conditions. Biotinylated products on the blot were revealed with HRP-streptavidin and enhanced chemiluminescence as described in the Materials and methods section. Molecular mass standards are shown (MW) at the left of gels.

Figure 3 Glycosylation state of the cell-surface $\alpha 6$ integrin subunit in HT29-D4 and LoVo-C5 cells

$\alpha 6$ integrins immunoprecipitated from surface-biotinylated cells were incubated without enzyme (lanes C) or in the presence of Endo H (lanes EH) or PNGase F (lanes G). Proteins were then separated by SDS/PAGE under reducing conditions (6% acrylamide) and biotinylated integrins were revealed with HRP-streptavidin as described in the Materials and methods section. The positions of molecular mass standards are shown (MW) at the left of the autoradiogram.

Digestion with PNGase F confirms the lack of endoproteolytic processing of the $\alpha 6$ subunit in LoVo cells

Immunoprecipitated cell-surface $\alpha 6$ subunits were treated by PNGase F, which hydrolyses all N-linked carbohydrate side-chains, to establish that the molecular mass difference of $\alpha 6$ subunits in HT29 and LoVo cells was due to the protein core. The reduced PNGase-treated $\alpha 6$ subunits in LoVo cells remained 30 kDa larger than in HT29 cells (Figure 3, lanes G), which was additional evidence that they were uncleaved. In contrast, the carbohydrate side chains of $\alpha 6$ chains in both HT29 and LoVo cells were resistant to hydrolysis by Endo H, which removes high-mannose-type oligosaccharides (Fig. 3, lanes EH). The result indicated that, in LoVo cells, the $\alpha 6$ subunit was normally routed

Figure 4 Pulse-chase study of the biosynthesis of the $\alpha 6$ integrin subunit in HT29-D4 and LoVo-C5 cells

(A) HT29-D4 and LoVo-C5 cells were metabolically labelled for 15 min with [35 S]-Translabel[®] and chased for the indicated periods of time. Cells were then lysed and subjected to immunoprecipitation with GOH3 mAb as described in the Materials and methods section. Immunopurified integrins were analysed by SDS/PAGE under reducing conditions [7.5% (w/v) acrylamide]. (B) Cells were labelled for 18 h in the presence of 150 μ Ci/ml [35 S]-Translabel[®] and $\alpha 6$ integrins were immunopurified and analysed as described above.

through the Golgi apparatus before being targeted to the cell surface.

Endoproteolytic processing is a late feature in integrin $\alpha 6$ chain processing that never occurs in LoVo cells

Pulse-chase experiments were performed to study the endoproteolytic processing of integrin $\alpha 6$ subunit precursors and to define the role of post-translational cleavages in integrin biosynthesis. $\alpha 6$ integrins from LoVo and HT29 cells were immunoprecipitated during the course of the chase and analysed by SDS/PAGE under reducing conditions, and then subjected to autoradiography (Figure 4).

Figure 5 Cleavage of pro- $\alpha 6$ and pro- $\alpha 3$ subunits *in vitro* by furin and trypsin

Pro- $\alpha 6$ and pro- $\alpha 3$ subunits were immunoprecipitated from LoVo cells metabolically labelled with [^{35}S]methionine with mAb GoH3 and P1B5 respectively and incubated for 4 h at 25 °C with enzymes in 50 μ l of 62 mM Tris buffer, pH 6.8, containing 1% (w/v) n-octyl glucoside, with or without 1 mM calcium. Reduced and unreduced incubates were then analysed by SDS/PAGE and fluorography. (A) Digestion of pro- $\alpha 6$ subunit with 5 μ l (lanes 1–4), 2.5 μ l (lane 5), 0.5 μ l (lane 6), 0.25 μ l (lane 7) or 0.02 μ l (lane 8) of furin without (lanes 1 and 2) or with (lanes 3–8) 1 mM calcium. Incubates were analysed unreduced (lanes 1 and 3) or reduced and alkylated (lanes 2, 4, 5, 6, 7 and 8) by SDS/PAGE [4.75% (w/v) acrylamide] and fluorographed for 2 days. (B) $\alpha 3$ immunoprecipitates untreated (lanes 1 and 2) or digested with 5 μ l of furin (lanes 2, 4, 5 and 6) with (lanes 3 and 4) or without (lanes 5 and 6) 1 mM calcium. Incubates were analysed unreduced (lanes 1, 3 and 5) or reduced and alkylated (lanes 2, 4 and 6) by SDS/PAGE [6% (w/v) acrylamide] and fluorographed for 7 days. (C) Digestion of pro- $\alpha 6$, in the presence of 1 mM CaCl_2 , without trypsin (lane 6) or with 10 ng (lane 1), 1 ng (lane 2), 100 pg (lane 3), 10 pg (lane 4) or 1 pg (lane 5) of trypsin. Reduced and alkylated incubates were analysed by SDS/PAGE [6% (w/v) acrylamide] and fluorographed for 3 days.

In HT29 cells the main species detectable after 20 min of chase corresponded to uncleaved 140 kDa $\alpha 6$ subunit precursor (pro- $\alpha 6$). Association of pro- $\alpha 6$ with newly synthesized $\beta 4$ subunit was not detected until 1 h of chase. At that time a substantial amount of pro- $\alpha 6$ subunit was affected by endoproteolysis, a process that was fully completed after 2 h of chase. Thus the pro- $\alpha 6$ subunit was a short-lived product in HT29 cells. This conclusion is in agreement with the finding that the pro- $\alpha 6$ subunit was not detected after long-term (18 h) labelling (Figure 4), indicating that it accounted for a minor fraction of the $\alpha 6$ subunit pool.

In LoVo cells, the $\alpha 6$ subunit remained uncleaved during the course of pulse-chase experiments and only the pro- $\alpha 6$ species was detected after 2 h of chase or after long-term (18 h) labelling (Figure 4). Despite defective cleavage in LoVo cells, the kinetics of $\alpha 6$ subunit biosynthesis and of pairing with $\beta 4$ subunits seemed to be similar in the two cell lines.

High-efficiency cleavage of LoVo pro- $\alpha 6$ and pro- $\alpha 3$ subunits *in vitro* by recombinant soluble furin

The hydrolysis of pro- $\alpha 6$ and pro- $\alpha 3$ subunit *in vitro* by furin was analysed to provide direct proof that furin might be involved in the endoproteolytic of integrin α subunits. The results in Figure 5(A) show that the immunopurified 140 kDa pro- $\alpha 6$ subunit was dose-dependently cleaved into a 120 kDa high-molecular-mass chain (Figure 5A, lanes 5–8) by a recombinant soluble form of furin (hydrolysis activity of pERTKR-AMC furin-specific substrate peptide, 150 nmol/h per ml). Furin-mediated endo-

proteolysis produced by furin was only apparent under reducing conditions, indicating that cleavage occurred within a disulphide-bridged region. In addition, cleavage was not obtained when digestions were performed in calcium-free conditions (Figure 5A, lane 2), and the protease-sensitive $\beta 4$ subunit remained unaffected by furin.

A significant heavy-chain production was also obtained when immunopurified pro- $\alpha 3$ subunit was digested with recombinant furin. Although the signal arising from the pro- $\alpha 3$ subunit was faint due to its low expression in LoVo cells, reduced pro- $\alpha 3$ (Figure 5B, lane 2) migrated slightly more slowly than its unreduced counterpart (Figure 5B, lane 1) and distinct from the $\beta 1$ subunit. By contrast, furin-digested pro- $\alpha 3$ chain could no longer be distinguished from co-migrating $\beta 1$ chain (Figure 5B, lane 4). This decrease in molecular mass was not observed after incubation in calcium-free conditions (Figure 5B, lane 6).

Hydrolysis of the pro- $\alpha 6$ subunit *in vitro* by trypsin was also analysed to find whether the cleavage site involved in $\alpha 6$ subunit processing was constitutively sensitive to proteolysis. The pro- $\alpha 6$ subunit was found to be much more resistant to hydrolysis by trypsin than the $\beta 4$ subunit, which was dose-dependently degraded (Figure 5C, lanes 1–3). Furthermore the 120 kDa $\alpha 6$ heavy chain was not specifically produced when pro- $\alpha 6$ subunit was strongly hydrolysed by trypsin (Figure 5C, lane 1), although at 1 ng of trypsin some formation of the 120 kDa form was barely detectable. Instead, many fragments [too faint to be observed on the high-contrast picture (Figure 5C)] were detected, indicating that trypsin-mediated cleavage was multifocal when it occurred.

Taken together these results show that integrin pro- $\alpha 6$ and

pro- $\alpha 3$ subunits can be directly and specifically endoproteolytically processed by furin in a calcium-dependent manner.

DISCUSSION

Some integrin α subunits show constitutive endoproteolytic processing that divides the molecule into a 120 kDa heavy chain disulphide bonded to a 30 kDa light chain. The cleavage site occurs at sites containing basic amino acids located near the transmembrane domain. This was shown by direct sequencing of the light chain for the αv subunit [26] and by mutagenesis for the αIIb subunits, which failed to be endoproteolytically processed when Arg residues 856 or 858–859 were changed to Gly [27]. The protein convertase furin, which is considered to be the processing protease for a variety of proteins displaying the Arg-X-Arg/Lys-Arg consensus sequence also found in the αIIb subunit, was therefore suspected to participate in α integrin subunit endoproteolysis.

In this paper we have examined this hypothesis by looking at the molecular status of post-translationally cleaved α subunits in the cell line LoVo. As demonstrated recently, the furin gene in these cells presents a nucleotide deletion that results in a reading frame shift covering the Homo B (also called P-) domain that is essential for the endoproteolytic activity [19]. LoVo cells therefore constitute an attractive model for identifying potential substrates for furin. For example, the defective endoproteolytic processing of the HGF receptor, of stromelysin and of *Diphtheria* toxin in LoVo cells has been attributed to furin deficiency [17,28,29].

Our analysis showed that cell-surface $\alpha 3$ and $\alpha 6$ integrin subunits in LoVo cells were not converted into heavy and light chains after reduction, indicating that they were not endoproteolytically processed. The cleavage defect was confirmed by pulse-chase analysis of $\alpha 6$ subunit biosynthesis and by PNGase hydrolysis of $\alpha 6$ subunit carbohydrates. The demonstration that furin was directly involved in the endoproteolysis of integrin $\alpha 6$ and $\alpha 3$ subunits was established by showing that recombinant furin allowed highly efficient calcium-dependent cleavage of 140 kDa pro- $\alpha 6$ and pro- $\alpha 3$ subunits into a 120 kDa heavy chain at neutral pH. These results are in line with the observation that the tetrabasic amino acid sequence in $\alpha 6$ and $\alpha 3$ subunit, RKKR and RRRR respectively, with an Arg residue in position P4 upstream of the Arg cleavage site (P1), is a typical consensus motif for cleavage by this enzyme.

Besides defective furin, PACE 4 seems to be the only known convertase to be expressed in LoVo cells [16]. The partial cleavage of HIV GP 160 transfected into LoVo cells has been attributed to hydrolysis by PACE 4 [30]. Our observation that the $\alpha 6$ chain was mostly uncleaved in LoVo cells might indicate that PACE 4 or any other unknown convertase expressed in LoVo cells is not redundant with furin in endoproteolytic processing of integrins.

Altogether these results strongly suggest that furin is a processing endoprotease for the integrin $\alpha 3$ and $\alpha 6$ subunit precursor. The situation is less clear for the αv subunit, which was also uncleaved in LoVo cells although its putative cleavage site, ITKR, lacks an Arg residue in the P4 position and hence would not be ideal for hydrolysis by furin. Endoproteolysis at a pair of basic amino acids has been described for other proteins secreted along the constitutive pathway, e.g. albumin, complement pro-C3 [12] and the $\alpha 4$ integrin subunit [31]. Pro-albumin and pro-C3 were endoproteolytically processed when they were coexpressed with furin in COS cells [32]. Furthermore cleavage of the $\alpha 4$ integrin subunit occurs only in the activated lymphocyte, where up-regulation of furin mRNA has been found [19]. Thus it is possible that some structural properties of these substrates,

upstream or downstream of the cleavage site, enhances furin hydrolysis at pairs of basic amino acids. Alternatively it may be that these proteins are processed by another convertase endowed with a dedicated specificity for basic sites. However, the above observations, including our finding that the αv subunit was uncleaved in the furin-deficient LoVo cells, would suggest that the function of this putative protease would be highly dependent on endoproteolytic activation by furin.

The membrane-associated endoprotease furin, which is mainly located in the *trans*-Golgi network, may also be recycled from the cell surface. This dynamic distribution is in accordance with the observations that furin works in both exocytotic (endoproteolytic processing of host proteins) and endocytic (activation of bacterial toxins) pathways [33]. Pulse-chase experiments with HT29 cells, where endoproteolytic processing occurred normally, indicated that integrin $\alpha 6$ -subunit cleavage began 30 min after synthesis and was completed after a further 45 min. Because most of the cell-surface $\alpha 6$ subunits were cleaved and their endocytosis was a slow process (J.-C. Lissitzky, unpublished work), this time course suggested that pro- $\alpha 6$ endoproteolytic processing occurred along the exocytotic pathway. In any case, the comparison of integrin biosynthesis in HT29 and LoVo cells indicated that α -subunit cleavage did not play a critical role in the kinetics of $\alpha 6/\beta 4$ subunit pairing or in the targeting of integrins to the cell surface, which is similar to what was found in the case of $\alpha IIb/\beta 3$ integrins [27].

Ligated integrins transmit signals (outside-in signalling) that result in cell adhesion and thereby participate in the control of cell shape, cell locomotion, differentiation and tissue structure [3]. Also, integrins might be activated or inactivated by conformational changes. This functional modulation is important in adjusting the integrin response to the changing biological situations (inside-out signalling) [34]. At present the role of endoproteolytic cleavage in these two aspects of integrin biology remains elusive. Indeed, a similar efficiency of attachment to fibrinogen, whether basal or stimulated by a function-activating antibody to αIIb , was found in COS cells expressing cleaved or uncleaved $\alpha IIb/\beta 3$ integrins, which suggests that endoproteolytic cleavages of the α integrin subunit does not play a major role in outside-in adhesion signalling [27]. In line with these conclusions, LoVo cells were found to attach significantly to laminin-1 and vitronectin in an $\alpha 6$ - and αv -dependent manner respectively, and the attachment efficiency to laminin-1 was not different for HT29 and LoVo cells, which display similar levels of cell-surface expression of $\alpha 6$ -containing integrins. Nevertheless a direct comparison with LoVo cells transfected with a gene encoding active furin is needed before it can be decided whether or not endoproteolytic cleavage of $\alpha 3$, $\alpha 6$ or αv subunits plays a role in cell adhesion or in more sophisticated aspects of integrin biology.

We thank F. Gianellini and J. Secchi for technical assistance, and Dr. Scott Munzer (IRCM, Montreal, Canada) for help in the preparation of BRCD-furin. This work was supported by the Universities of Aix-Marseille I and II, and by grants from the ARC (Association de Recherche sur le Cancer), the FNLC (Fédération Nationale de Lutte Contre le Cancer), the Fondation de France, the Fondation de la Recherche Médicale, and a PENCE MRC grant (to N.G.S.).

REFERENCES

- 1 Hynes, R. O. (1992) *Cell* **69**, 11–25
- 2 Burridge, K. F., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988) *Annu. Rev. Cell Biol.* **4**, 487–525
- 3 Sastry, S. K. and Horwitz, A. F. (1993) *Curr. Opin. Cell Biol.* **5**, 819–831
- 4 O'Toole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., Loftus, J. C., Shattil, S. J. and Ginsberg, M. H. (1994) *J. Cell Biol.* **124**, 1047–1059
- 5 Docherty, M. and Steiner, D. F. (1982) *Annu. Rev. Physiol.* **44**, 625–638
- 6 Lindberg, I. (1991) *Mol. Endocrinol.* **5**, 1361–1365

- 7 Ozawa, M. and Kemler, R. (1990) *J. Cell Biol.* **111**, 1645–1650
- 8 Wise, R. J., Barr, P. J., Wong, P., Kieffer, N., Brake, A. and Kaufman, R. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9378–9382
- 9 Seidah, N. G. and Chrétien, M. (1992) *Trends Endocrinol. Metab.* **3**, 133–140
- 10 Seidah, N. G. and Chrétien, M. (1994) *Methods Enzymol.* **244**, 175–188
- 11 Steiner, D. F., Smeekens, S. P., Ohagi, S. and Chan, S. J. (1992) *J. Biol. Chem.* **267**, 23435–23438
- 12 Barr, P. J. (1991) *Cell* **66**, 1–3
- 13 Halban, P. A. and Irminger, J. C. (1994) *Biochem. J.* **299**, 1–18
- 14 Seidah, N. G., Gaspar, P., Mion, M., Marcinkiewicz, M., Mbikay, M. and Chrétien, M. (1990) *DNA* **9**, 415–424
- 15 Smeekens, S. and Steiner, D. (1990) *J. Biol. Chem.* **265**, 2997–3000
- 16 Seidah, N., Chrétien, M. and Day, R. (1994) *Biochimie* **76**, 197–209
- 17 Komada, M., Hatsuzawa, K., Shibamoto, S., Ito, F., Nakayama, K. and Kitamura, M. (1993) *FEBS Lett.* **328**, 25–29
- 18 Robertson, B., Moehring, J. M. and Moehring, T. J. (1993) *J. Biol. Chem.* **268**, 24274–24277
- 19 Decroly, E., Vandenbranden, M., Ruysschaert, J. M., Cogniaux, J., Jacob, G. S., Howard, S. C., Marshall, G., Kompelli, A., Basak, A., Jean, F. et al. (1994) *J. Biol. Chem.* **269**, 12240–12247
- 20 Takahashi, S., Kasai, K., Hatsuzawa, K., Kitamura, N., Misumi, Y., Ikehara, Y., Murakami, K. and Nakayama, K. (1993) *Biochem. Biophys. Res. Commun.* **195**, 1019–1026
- 21 Lehmann, M., Rabenandrasana, C., Tamura, R., Lissitzky, J. C., Quaranta, V., Pichon, J. and Marvaldi, J. (1994) *Cancer Res.* **54**, 2102–2107
- 22 Fantini, J., Abadie, B., Tirard, A., Remy, L., Ripert, J. P., El Battari, A. and Marvaldi, J. M. (1986) *J. Cell Sci.* **83**, 235–249
- 23 Remy, L., Jacquier, M., Daemi, N., Doré, J.-F. and Lissitzky, J.-C. (1993) *Differentiation* **54**, 191–200
- 24 Jean, F., Bask, A., DiMaio, J., Seidah, N. G. and Lazure, C. (1995) *Biochem. J.* **307**, 689–695
- 25 Moehring, J., Inosencio, N., Robertson, B. and Moehring, T. (1993) *J. Biol. Chem.* **268**, 2590–2594
- 26 Susuki, S., Argraves, W. S., Arai, H., Languino, L. R., Pierschbacher, M. D. and Ruoshtali, E. (1987) *J. Biol. Chem.* **262**, 14080–14085
- 27 Kolodziej, M., Vilaire, G., Gonder, D., Poncz, M. and Bennett, J. (1991) *J. Biol. Chem.* **266**, 23499–23504
- 28 Tsuneoka, M., Nakayama, K., Hatsuzawa, K., Komada, M., Kitamura, N. and Mekada, E. (1993) *J. Biol. Chem.* **268**, 26461–26465
- 29 Duanquin, P. and Weiss, S. (1995) *Nature (London)* **375**, 244–247
- 30 Ohnishi, Y., Shiodo, T., Nakayama, K., Iwata, S., Gotoh, B., Hamaguchi, M. and Nagai, Y. (1994) *J. Virol.* **68**, 4075–4079
- 31 Teixido, J., Parker, C. M., Kassner, P. D. and Hemler, M. E. (1992) *J. Biol. Chem.* **267**, 1786–1791
- 32 Misumi, Y., Oda, K., Fujiwara, T., Takami, N., Tashiro, K. and Ikehara, Y. (1991) *J. Biol. Chem.* **266**, 16954–16959
- 33 Molloy, S., Thomas, L., VanSlyke, J., Stenberg, P. and Thomas, G. (1994) *EMBO J.* **13**, 18–33
- 34 Diamond, M. S. and Springer, T. A. (1994) *Curr. Biol.* **4**, 506–517