Identification of the cleavage sites in the $\alpha 6A$ integrin subunit: structural requirements for cleavage and functional analysis of the uncleaved $\alpha 6A\beta 1$ integrin

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The $\alpha 6A$ and $\alpha 6B$ integrin subunits are proteolytically cleaved during biosynthesis into a heavy chain (120 kDa) that is disulphide-linked to one of two light chains (31 or 30 kDa). Analysis of the structure of the $\alpha 6A$ subunit on the carcinoma cell line T24 and human platelets demonstrated that the two light chains of $\alpha 6$ are not differentially glycosylated products of one polypeptide. Rather they possess different polypeptide backbones, which presumably result from proteolytic cleavage at distinct sites in the $\alpha 6$ precursor. Mutations were introduced in the codons for the R⁸⁷⁶K⁸⁷⁹, E⁸⁸³K⁸⁸⁴, R⁸⁹⁰K⁸⁹¹ and R⁸⁹⁸K⁸⁹⁹ sequences, the potential proteolytic cleavage sites, and wild-type and mutant $\alpha 6A$ cDNAs were transfected into K562 cells. The mutant $\alpha 6A$ integrin subunits were expressed in association with endogenous $\beta 1$ at levels comparable to that of wild-type $\alpha 6A\beta 1$. A single $\alpha 6$ polypeptide chain (150 kDa) was precipitated from transfectants expressing $\alpha 6A$ with mutations or deletions in the RKKR sequence. Mutations in the EK sequence yielded $\alpha 6A$ subunits that were cleaved once into a heavy and a light chain, whereas $\alpha 6A$ subunits with mutations in one of the two RK sequences were, like wild-type $\alpha 6A$, cleaved into one heavy and two light chains. Thus a change in the RKKR sequence prevents the cleavage of $\alpha 6$. The EK site is the secondary cleavage site, which is used only when the primary site (RKKR) is intact.

Microsequencing of the N-termini of the two $\alpha 6A$ light chains from platelets demonstrated that cleavage occurs after Arg⁸⁷⁹ and Lys⁸⁸⁴. Because $\alpha 6^{\text{RKKG}}$, $\alpha 6^{\text{GKKR}}$ and $\alpha 6^{\text{RGGK}}$ subunits were not cleaved it seems that both the arginine residues and the lysine residues are essential for cleavage of RKKR. $\alpha 6A$ mutants with the RKKR sequence shifted to the EK site, in such a way that the position of the arginine residue after which cleavage occurs corresponds exactly to Lys⁸⁸⁴, were partly cleaved, whereas $\alpha 6A$ mutants with the RKKR sequence shifted to other positions in the $\alpha 6A$ subunit, including one in which it was shifted two residues farther than the EK cleavage site, were not cleaved. In addition, $\alpha 6A$ mutants with an $\alpha 5$ -like cleavage site, i.e. arginine, lysine and histidine residues at positions -1 , -2 and -6 , were not cleaved. Thus both an intact RKKR sequence and its proper position are essential. After activation by the anti- $\beta 1$ stimulatory monoclonal antibody TS2/16, both cleaved and uncleaved $\alpha 6A\beta 1$ integrins bound to laminin-1. The phorbol ester PMA, which activates cleaved wild-type and mutant $\alpha 6A\beta 1$, did not activate uncleaved $\alpha 6A\beta 1$. Thus uncleaved $\alpha 6A\beta 1$ is capable of ligand binding, but not of inside-out signalling. Our results suggest that cleavage of $\alpha 6$ is required to generate a proper conformation that enables the affinity modulation of the $\alpha 6A\beta 1$ receptor by PMA.

INTRODUCTION

Integrins are transmembrane glycoproteins composed of non-covalently associated α and β chains. The extracellular domains of both α and β chains bind ligands, whereas the short cytoplasmic tails are connected to the cytoskeleton. Integrins participate in cell–extracellular matrix and cell–cell interactions, and they are involved in outside-in and inside-out signalling processes [1–3].

Many integrin α subunits undergo proteolytic cleavage. The $\alpha 3$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, αIIb and αv subunits are cleaved near the C-terminal part of the extracellular domain, which results in a heavy chain that is disulphide-linked to a membrane-spanning light chain [1,2]. The $\alpha 4$ and αE subunits are cleaved at unusual positions, in the middle region and the N-terminal region of the extracellular domain respectively [4,5]. The potential proteolytic cleavage sites in the α subunits are monobasic, dibasic and multibasic sequences. Microsequencing of the N-termini of the αv and $\alpha 4$ light chains has revealed that cleavage occurs after the dibasic KR⁴⁷⁸ and KR⁵⁵⁸ sequences respectively [4,6]. The αIIb heavy and two light chains are generated by cleavage at two

positions, RDRR⁸⁵⁹ and SR⁸⁷¹, as has been determined by microsequencing of the N-termini of the two light chains [7,8]. Microsequencing of the C-terminus of the αIIb heavy chain suggested a third cleavage site at KR⁸⁵⁶ [8] (Table 1).

The enzymes involved in cleavage remain to be identified, but they probably belong to the family of subtilisin-like serine proteases [9,10]. These enzymes frequently cleave after the basic amino acid arginine [11,12]. One of them, the ubiquitously expressed endoprotease furin, which cleaves proteins in the constitutive secretory pathway, recognizes the consensus sequence RXR/KR[↓] (in which X can be any amino acid) [13]. This sequence is present in the primary structures of $\alpha 3$, $\alpha 6$, $\alpha 7$, αIIb and αE (Table 1). For $\alpha 3$ and $\alpha 6$ it has recently been shown that their pro-forms, isolated from the furin-deficient human cell line LoVo, are cleaved by recombinant furin [14].

Endoproteolytic cleavage is often necessary to generate biologically active proteins. This has for example been reported for peptide hormones, serum proteins, cell adhesion proteins and growth factor receptors [9,10,15]. Cleavage of integrin α subunits is likely to be of functional importance, because proteolytic

Abbreviations used: endo F, endoglycosidase F; endo H, endoglycosidase H; mAb, monoclonal antibody; PTH, phenylthiohydantoin.

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Table 1 Amino acids present at the proteolytic cleavage sites in integrin α subunitsAll sequences are aligned to human $\alpha 6$, except for αE .

Source	Sequence
$\alpha 6$ human	HNSRKKREITEKQIDDN.RKFSLF AERKYQ
$\alpha 6$ mouse	HNSRKKRELPEKQIDDS.RKFSLF PERKYQ
$\alpha 6$ chick	HNSRKKREIAEKQLTDS.KTFSLF SERKYK
$\alpha 6$ <i>Xenopus</i>	GKTRHRREIGESQSGTSDKSFSLF SERKYM
$\alpha 3$	SPQRRRRQLDPGGDQGS.PPVTLAAAKKAK
$\alpha 7$	SRDRRRRELGPPEPQEPPEKVEPSTSWWPV
$\alpha 11b$	HHKRDRRQIFLPEPEQP.SRLQDPVLVSCD
$\alpha 5$	HHQQ.KREAPSRSS.....ASSGPQ
αv	DHLITKRD LAL.....SEGDIH
αE	NTARQRRRALEKEEEDKEEEDEEEEEEAGTE

cleavage sites in α subunits are conserved, not only in different α chains but also across species. A role in ligand binding has been proposed, although the integrins $\alpha 4\beta 1$ and $\alpha 11b\beta 3$ containing either cleaved or uncleaved α subunits are capable of ligand binding [4,16]. We have recently shown that proteolytic cleavage of the $\alpha 6A$ subunit is essential for the proper transduction of inside-out signals by the $\alpha 6A\beta 1$ integrin, whereas its ligand-binding capacity does not depend on cleavage [17]. Thus the cleavage of integrin α subunits might be necessary for generating a conformation that is required for modulating the affinity of the integrin receptor.

During biosynthesis the two cytoplasmic variants of $\alpha 6$, $\alpha 6A$ and $\alpha 6B$, are each synthesized as a single precursor of 150 kDa. Both are proteolytically cleaved into a disulphide-linked heavy (120 kDa) chain and a light chain of either 31 or 30 kDa before expression on the cell surface [18–20]. There are several potential proteolytic cleavage sites in the primary structure of $\alpha 6$ (see

Figure 4) [21,22], and we have recently shown that the $R^{876}KKR^{879}$ sequence is the primary cleavage site [17]. In the present study we have investigated the cleavage of $\alpha 6$ in detail. We used cDNA for the $\alpha 6A$ cytoplasmic variant to introduce mutations in the codons for the potential proteolytic cleavage sites: $R^{876}KKR^{879}$, $E^{883}K^{884}$, $R^{890}K^{891}$ and $R^{898}K^{899}$ (Table 2). The mutant $\alpha 6A$ cDNA species were transfected into K562 cells. We found that there is a second cleavage site in $\alpha 6A$, and we determined the sequence requirements for cleavage at the two cleavage sites, and the ligand binding of the mutant $\alpha 6A\beta 1$ integrins.

MATERIALS AND METHODS

Cell culture and antibodies

K562 transfectants were grown in RPMI-1640 medium supplemented with 7.5 % (v/v) fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and 1 mg/ml Geneticin (G-418 sulphate; Life Technologies, Grand Island, NY, U.S.A.). The human bladder carcinoma cell line T24 was grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. The following monoclonal antibodies (mAbs) to integrin subunits were used: the rat mAb GoH3 [18] and the mouse mAb J8H [23] to $\alpha 6$; the rabbit polyclonal antibody $\alpha 6A$ directed against the C-terminal sequence of $\alpha 6A$ [24]; the mouse mAb 1A10 [25] to the cytoplasmic domain of $\alpha 6A$. The mouse mAbs K20 [26] and TS2/16 [27] directed against the $\beta 1$ subunit were purchased from Immunotech (Marseilles, France) and the American Type Culture Collection (Rockville, MD, U.S.A.) respectively.

Construction of mutant $\alpha 6A$ cDNA species

The overlap extension method [28] was used for site-directed mutagenesis of the sequences encoding the potential proteolytic

Table 2 List of $\alpha 6A$ mutants

Abbreviation: n.d., not done

$\alpha 6A$ mutant		Number of light chains	Activation by PMA
	A B C		
$\alpha 6RKKR$	873-HNSRKKREITEKQIDDNRKFSLF AERKYQTL-903	2	+
$\alpha 6GKKR$	... GKKR	0	—
$\alpha 6RKKG$	... RKKG	0	—
$\alpha 6RGGR$	... RGGR	0	—
$\alpha 6RKKR/A1$	... RKKR ... EG	1	+
$\alpha 6RKKR/A2$	... RKKR ... GK	1	+
$\alpha 6RKKR/B1$	... RKKR GS	2	+
$\alpha 6RKKR/C1$	... RKKR GG	2	+
$\alpha 6RGGR/B2C2$	... RGGR RI RQ	0	—
$\alpha 6RK$	... RK ... EITEK	0	—
$\alpha 6RK/A4$	... RKEITRKKR	1	+
$\alpha 6RK/A4(E)$	... RKEITRKKRE	1	+
$\alpha 6RG/A4$	... RGEITRKKR	1	+
$\alpha 6RKKR(D)$	... RKKRD	2	+
$\alpha 6RGGR(D)/A3$	... RGGRD ... RKKR	0	—
$\alpha 6RGGR(D)/B3$	... RGGRD RKKR	0	±
$\alpha 6RGGR(D)/C3$	... RGGRD RKKR	0	—
$\alpha 6RKKR/A5$	... RKKR ... EKG	1	+
$\alpha 6KKR$	... -KKR ... EK	0	n.d.
$\alpha 6KKR/A1$	... -KKR ... EG	0	n.d.

cleavage sites in $\alpha 6$. Briefly, for each mutant two completely overlapping sense and anti-sense primers were synthesized encoding the appropriate mutations (see Table 2 for all $\alpha 6A$ mutants). These primers were used in first-round PCRs together with two flanking primers. The two products of the first-round PCR were gel-purified and used in a second-round PCR with the two flanking primers. The different primers were: for $\alpha 6^{RKKR/A1}$ positions 2692–2717, 5'-ATTACTGAAGGACAGATAGATG-ATAA-3'; for $\alpha 6^{RKKR/A2}$ positions 2689–2717, 5'-GAAATTAC-TGGAAAACAGATAGATGATAA-3'; for $\alpha 6^{RKKR/A5}$ positions 2696–2720, 5'-CTGAAAAAGGGATAGATGATAACAG-3'; for $\alpha 6^{RKKR/B1}$ positions 2710–2738, 5'-GATGATAACGGAT-CCTTTTCTTTATTTGC-3'; for $\alpha 6^{RKKR/C1}$ positions 2731–2766, 5'-TTATTTGCTGAAGGAGGGTACCAGAC-3'; for $\alpha 6^{RGGR/B2C2}$ positions 2719–2751, 5'-AGAATATTTCTTTAT-TTGCTGAAAGACAATAC-3'; for $\alpha 6^{RKKR(D)}$ positions 2680–2704, 5'-AAGAAACGGGATATCACTGAAAAAC-3'; for $\alpha 6^{RK}$ positions 2666–2706, 5'-CTCACAACCAAGAAAGGA-AATTACTGAAAAACAG-3' (these primers lack the codons for K⁸⁷⁸R⁸⁷⁹); for $\alpha 6^{RK/A4}$ positions 2674–2721, 5'-TCAAGAA-AGGAAATTACTAGAAAAAAGAGACAGATAGATGAT-AACAGA-3' [these primers lack the codons for K⁸⁷⁸R⁸⁷⁹, and the codons for K⁸⁸³R⁸⁸⁴ (underlined) have been added to these primers]; for $\alpha 6^{RK/A4(E)}$ positions 2695–2712, 5'-ACTA-GAAAAAAGAGAGAGATAGAT-3' [the codons for K⁸⁸³R⁸⁸⁴ (underlined) have been added to these primers]; for $\alpha 6^{RG/A4}$ positions 2668–2697, 5'-CACAACTCAAGAGGGGAAATTACT-3' (these primers lack the codons for K⁸⁷⁸R⁸⁷⁹); for $\alpha 6^{KKR}$ and for $\alpha 6^{KKR/A1}$ positions 2661–2697, 5'-GGAGTCTCACA-CTCAAAGAAACGGGAAATTACT-3' (these primers lack the codon for R⁸⁷⁶). Two flanking primers were a sense primer for positions 2425–2449, 5'-GTTGGCGAGCAAGCTATGA-AATCTG-3', and an anti-sense primer for positions 2896–2917, 5'-TGAAGGCTCGCATGAGAATGTC-3'. Primer positions for $\alpha 6$ correspond to the sequence published by Hogervorst et al. [21]. First-round PCRs were performed with the appropriate primer sets using *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.) and pUC18- $\alpha 6A$ cDNA as template. Templates for the $\alpha 6^{RGGR/B2C2}$, $\alpha 6^{RK/A4}$ and $\alpha 6^{KKR/A1}$ mutants were the $\alpha 6^{RGGR}$ [17], $\alpha 6^{RK}$ and $\alpha 6^{RKKR/A1}$ cDNA species respectively. For the $\alpha 6^{RK/A4(E)}$ and $\alpha 6^{RG/A4}$ mutants, $\alpha 6^{RK/A4}$ cDNA was used as template. First-round PCR conditions were 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 90 s at 55 °C, 30 s at 72 °C and 5 min at 72 °C. Conditions for second-round PCRs were identical except for extension, which was 45 s at 72 °C. The PCR products from the second-round PCRs were digested with *Bst*XI and *Xba*I and subcloned into pUC18- $\alpha 6A$ cDNA. Mutant clones were identified by sequence analysis. $\alpha 6A$ cDNA fragments were isolated after digestion with *Hind*III and cloned into the *Hind*III site of pRc/CMV (Invitrogen, San Diego, CA, U.S.A.).

The $\alpha 6^{RGGR(D)}$ mutant was generated for the construction of the $\alpha 6^{RGGR(D)/A3}$, $\alpha 6^{RGGR(D)/B3}$ and $\alpha 6^{RGGR(D)/C3}$ mutants. In these mutants a glutamic acid residue was replaced by aspartic acid, which yields a unique *Eco*RV restriction site that was used for cloning. Primers for $\alpha 6^{RGGR(D)}$ and $\alpha 6^{RGGR(D)/A3}$ were: positions 2671–2701, 5'-AACTCAAGAGGGGGACGGGATATCACT-GAAA-3', and positions 2682–2715, 5'-GAAACGGGATAT-CACTCGAAAAAAGAGAGATGAT-3' respectively. Flanking primers and PCR for these two mutants were as described above. Primers for $\alpha 6^{RGGR(D)/B3}$ and $\alpha 6^{RGGR(D)/C3}$ were: positions 2713–2742, 5'-GATAACAGAAAAAACGTTTATTTGCTG-AA-3', and positions 2737–2766, 5'-GCTGAAAGAAAAA-ACGGAATCTTAAGT-3' respectively. A sense flanking primer positions 2695–2715, 5'-GCACGATATCACTGAAA-AACAGATAGATGAT-3', containing 10 additional nucleotides

encoding an *Eco*RV restriction site (underlined) and the anti-sense primer positions 2896–2917 were used for these two mutants. PCR was as described above. The second-round PCR products of the $\alpha 6^{RGGR(D)/A3}$, $\alpha 6^{RGGR(D)/B3}$ and $\alpha 6^{RGGR(D)/C3}$ mutants were digested with *Eco*RV and *Xba*I and subcloned into $\alpha 6^{RGGR(D)}$ cDNA. Mutant clones were identified by sequence analysis. $\alpha 6A$ cDNA fragments were isolated after *Hind*III digestion and cloned into the *Hind*III site of pRc/CMV (Invitrogen). Construction of wild-type $\alpha 6A$ cDNA [24] and mutant $\alpha 6A$ cDNA species $\alpha 6^{GKKR}$, $\alpha 6^{RKKG}$ and $\alpha 6^{RGGR}$ [17] in pRc/CMV have been described.

Sequence analysis of $\alpha 6$ proteolytic products

The $\alpha 6A$ subunit was isolated from human platelets. Platelets were isolated from donor blood as described [18] and solubilized in lysis buffer [1 % (w/v) NP40/20 mM Tris/HCl (pH 7.5)/100 mM NaCl/4 mM EDTA/1 mM PMSF/10 μ g/ml soybean trypsin inhibitor/10 μ g/ml leupeptin] for 30 min at 4 °C. Lysates were clarified at 10000 *g* and incubated with anti- $\alpha 6A$ mAb (1A10) coupled to CNBr-activated Sepharose 4B beads (Pharmacia LKB Biotechnology, Uppsala, Sweden) for 3 h at room temperature. Beads containing the immunoprecipitated material were washed, diluted with SDS sample buffer and heated for 3 min at 100 °C. The beads were centrifuged, after which 2-mercaptoethanol [10 % (v/v) final concentration] was added to the immunoprecipitated material. Immunoprecipitates were separated by SDS/PAGE [10 % (w/v) gel] and blotted on PVDF membrane in blotting buffer [10 mM 3-(cyclohexylamino)-propane-1-sulphonic acid (pH 11.0)/10 % (v/v) methanol]. Proteins were revealed with Coomassie Brilliant Blue and the bands containing the $\alpha 6A$ light chains (31 and 30 kDa) were excised. N-terminal amino acid sequence analysis was performed by automated Edman degradation with an Applied Biosystems 473 pulsed-liquid-phase protein sequencing system. The chromatography system was calibrated before each analysis with phenylthiohydantoin (PTH)-amino acid standards.

Transfection and flow cytometry

$\alpha 6A$ cDNA constructs were transfected into K562 cells by electroporation with the Bio-Rad (Richmond, CA, U.S.A.) Gene Pulser, and cells strongly expressing the transfected $\alpha 6A$ subunit were sorted on the FACSscan (Becton Dickinson, Mountain View, CA, U.S.A.) three times with mAb J8H, as previously described [24].

Immunoprecipitation of ¹²⁵I-labelled cells

Transfectants were surface-labelled with ¹²⁵I by the lactoperoxidase/hydrogen peroxide method as previously described [18], washed and solubilized in lysis buffer (see above). The lysates were clarified at 10000 *g* and precleared by incubation with Protein A-Sepharose CL4B (Pharmacia LKB Biotechnology, Uppsala, Sweden). After incubation with antibodies and rabbit anti-mouse or anti-rat antibodies, immune complexes were collected with Protein A-Sepharose. Immunoprecipitated material was analysed by SDS/PAGE.

Enzyme treatment

Treatment with enzyme was performed directly on the immunoprecipitates. For digestion with endoglycosidase H (endo H) the precipitated proteins were boiled for 2 min in 5 μ l of 50 mM sodium acetate (pH 5.5)/0.5 M 2-mercaptoethanol/0.1 % SDS. After cooling and dilution to decrease the concentration of 2-

mercaptoethanol and SDS to 0.1 M and 0.02 % respectively, endo H (Boehringer Mannheim) was added at 10 m-units/ml. To inhibit potential protease activity, 1 mM PMSF was added to the samples. The mixtures were incubated for 4 h at 37 °C. For endoglycosidase F (endo F) treatment, the immunoprecipitates were resuspended in 25 μ l of 100 mM sodium phosphate (pH 6.1)/50 mM EDTA/1 % 2-mercaptoethanol/0.1 % SDS and boiled for 2 min. NP40 was added to 1 %, and the samples were incubated with 0.5 unit of endo F (Du Pont–New England Nuclear) in the presence of 1 mM PMSF for 2 h at 37 °C.

Cell adhesion assays

Microtitre plates (96-well; Greiner, Trickenhausen, Germany) were coated with 20 μ g/ml laminin-1 (Collaborative Biomedical Products, Bedford, MA, U.S.A.) for 16 h at 4 °C. Plates were washed with PBS and incubated in PBS containing 1 % BSA for 60 min at room temperature to block non-specific adhesion. Cells were labelled with ^{51}Cr (400 μ Ci), washed in Iscove's modified Dulbecco's medium containing 0.35 % BSA and resuspended at 10^6 cells/ml. Cells were stimulated either by PMA (dissolved at various concentrations in DMSO; Sigma) or TS2/16 (1:5 hybridoma supernatant). Cell adhesion was performed as described previously [24].

RESULTS

The two light chains of the $\alpha 6$ subunit result from differences in length of the polypeptide backbone

During biosynthesis the $\alpha 6$ subunit is proteolytically cleaved into a heavy chain and a light chain of either 31 or 30 kDa. The heavy chain and either of the two light chains remain linked to each other by disulphide bonds [19]. In Figure 1 the $\alpha 6$ heavy chain and light chains present on platelets and the T24 cell line are shown (lanes 1 and 2). Although only $\alpha 6\text{A}$ is expressed on both cell types [25], light chains of both 31 and 30 kDa were detected. Furthermore the two $\alpha 6$ light chains of T24 cells migrated slightly faster than those of platelets. By digestion with endo H or endo F, we investigated whether the differences in size between the two $\alpha 6$ light chains present on each cell type and between the two $\alpha 6$ light chains of platelets and those of T24 are due to differential addition and/or processing of the two N-linked glycans that are present on the $\alpha 6$ light chains [21,22]. Endo H cleaves N-linked oligosaccharides of the high-mannose type, whereas endo F cleaves both high-mannose and complex-type N-linked oligosaccharides. Endo H digestion decreased the molecular mass of the $\alpha 6$ light chains of platelets and T24 cells (Figure 1, lanes 3 and 4). The digestion products of the platelets contained two polypeptides of 27/28 kDa and two of 24/25 kDa; those of T24 cells only contained the latter two polypeptides. Digestion with endo F revealed the presence of the 24/25 kDa doublet in both platelets and T24 cells (Figure 1, lanes 5 and 6). Thus platelets possess a mixture of $\alpha 6$ light chains, most of which contain one high-mannose and one complex-type N-linked glycan, whereas a minority contain two high-mannose type N-linked glycans. The $\alpha 6$ light chains from the T24 cells contain two high-mannose type N-linked glycans. The existence of O-linked glycans on the $\alpha 6$ subunit was ruled out by simultaneous digestion with neuraminidase and O-glycanase (results not shown). These results demonstrate that the differences in the size of the $\alpha 6$ light chains of platelets and of T24 cells are the result of differences in N-linked glycosylation. The existence of two $\alpha 6$ light chains (31 and 30 kDa) on these cell types is a result of neither differential glycosylation nor the expression of different

Figure 1 Carbohydrate analysis of $\alpha 6$ from platelets and the human bladder carcinoma cell line T24

Lysates of ^{125}I -labelled platelets (lanes 1, 3 and 5) and T24 cells (lanes 2, 4 and 6) were immunoprecipitated with GoH3 (anti- $\alpha 6$). Precipitates were subjected to digestion with endo H or endo F and then analysed by SDS/PAGE (10% gel) under reducing conditions. The positions of molecular mass markers are shown at the left.

$\alpha 6$ variants ($\alpha 6\text{A}$ and $\alpha 6\text{B}$): rather it results from differences in the length of the polypeptide backbone.

The RKKR and EK sites in the $\alpha 6$ subunit are required for cleavage

The two $\alpha 6$ light chains presumably arise from proteolytic cleavage at distinct sites in the $\alpha 6$ precursor polypeptide. We recently showed that the primary cleavage site in $\alpha 6$ is the $\text{R}^{876}\text{KKR}^{879}$ sequence [17]. To analyse whether a second sequence in $\alpha 6$ is used for cleavage, we mutated the potential proteolytic cleavage sites $\text{E}^{883}\text{K}^{884}$, $\text{R}^{890}\text{K}^{891}$ and $\text{R}^{898}\text{K}^{899}$, present in the primary structure of $\alpha 6$ [21,22] (see Figure 4). We also generated a mutant with amino acid substitutions in the RKKR and the two RK sequences. Mutations were introduced into wild-type $\alpha 6\text{A}$ cDNA ($\alpha 6^{\text{RKKR}}$) by site-directed mutagenesis with PCR (see Table 2 for all $\alpha 6\text{A}$ mutants). The previously described $\alpha 6\text{A}$ mutants with mutations in the RKKR site ($\alpha 6^{\text{GKKR}}$, $\alpha 6^{\text{RKKG}}$ and $\alpha 6^{\text{RGGG}}$) [17] were also included in the analysis. Note that in the nomenclature of the $\alpha 6\text{A}$ mutants the RKKR sequence or mutations therein are used, whereas the EK and the first and second RK sequences or mutations therein are referred to as A, B and C respectively. The wild-type and mutant $\alpha 6\text{A}$ cDNA species were transfected into K562 cells, which express only the $\alpha 5\beta 1$ integrin. Analysis of the different $\alpha 6\text{A}$ transfectants by fluorescence-activated cell sorting shows that all mutant $\alpha 6\text{A}$ subunits were expressed at the cell surface, like the wild-type $\alpha 6\text{A}$ subunit on the $\alpha 6^{\text{RKKR}}$ cells (Figure 2). The expression levels of $\alpha 6$ were approximately similar for all transfectants except $\alpha 6^{\text{GKKR}}$ and $\alpha 6^{\text{RGGG/B2C2}}$, on which the level was somewhat lower and higher respectively. The levels of the endogenous $\alpha 5$ and $\beta 1$ subunits were comparable in each transfectant (results not shown).

Next we investigated which mutations in $\alpha 6\text{A}$ affect its cleavage. The integrin subunits were immunoprecipitated from lysates of ^{125}I -labelled cells by using antibodies to the extracellular (GoH3) and cytoplasmic ($\alpha 6\text{A}$) domains of $\alpha 6$ and to $\beta 1$ (K20),

Figure 2 Cell surface expression of the $\alpha 6$ integrin subunit on the different transfectants

Flow cytometry was performed with the anti- $\alpha 6$ mAb J8H (solid line) or PBS (dotted line), followed by incubation with fluorescein isothiocyanate-labelled goat anti-mouse IgG.

and they were subsequently analysed by SDS/PAGE (10% gel) in conditions under which the intrachain disulphide bond linking the $\alpha 6$ heavy and light chains is disrupted. As shown in Figure 3, the $\alpha 6$ heavy chain (120 kDa) and the two $\alpha 6$ light chains

(31 and 30 kDa) were precipitated from lysates of cells expressing wild-type $\alpha 6$ A ($\alpha 6^{\text{RKKR}}$), consistent with previous results [17]. The $\beta 1$ subunit (120 kDa) and the $\alpha 5$ heavy (130 kDa) and light chains (17 kDa) were also precipitated. The $\alpha 6$ heavy chain (120 kDa) and one $\alpha 6$ light chain of approx. 31 kDa were precipitated from lysates of the $\alpha 6^{\text{RKKR/A1}}$ and $\alpha 6^{\text{RKKR/A2}}$ mutants (Figure 3) and, as from cells expressing wild-type $\alpha 6$ A ($\alpha 6^{\text{RKKR}}$), the $\alpha 6$ heavy (120 kDa) and two light chains (31 and 30 kDa) were precipitated from the $\alpha 6^{\text{RKKR/B1}}$ and $\alpha 6^{\text{RKKR/C1}}$ mutants. No $\alpha 6$ heavy or light chains were precipitated from the $\alpha 6^{\text{RGGR/B2C2}}$ mutant, but instead one polypeptide chain of 150 kDa, representing the uncleaved $\alpha 6$ precursor (Figure 3B). Consistent with our previous findings this 150 kDa polypeptide chain was also precipitated from the $\alpha 6^{\text{GKKR}}$, $\alpha 6^{\text{RKKG}}$ and $\alpha 6^{\text{RGGR}}$ mutants (Figure 3B). The K20 antibody precipitated $\beta 1$ and co-precipitated the $\alpha 5$ and mutant $\alpha 6$ A subunits, and the mAb GoH3 and p $\alpha 6$ A co-precipitated the $\beta 1$ subunit from all transfectants. These results show that the EK site is the second cleavage site in $\alpha 6$: mutation of a glutamic acid or lysine residue prevents cleavage so that only the larger of the two light chains is formed. The two RK sequences do not have a role in the cleavage of $\alpha 6$. In addition, we confirm that the intact RKKR site is the primary cleavage site in $\alpha 6$. Furthermore all mutant $\alpha 6$ A subunits, cleaved or uncleaved, were expressed at the cell surface in association with endogenous $\beta 1$.

Cleavage occurs after Arg⁸⁷⁹ and Lys⁸⁸⁴

It is not clear from the results with the $\alpha 6$ A mutants after which residue of the RKKR and EK sequences cleavage occurs. We assume that cleavage at the RKKR site occurs after Arg⁸⁷⁹ because the RKKR site is a consensus sequence for the endoprotease furin [13]. To examine the cleavage positions in detail, the $\alpha 6$ A light chains from human platelets were isolated and their N-termini were subjected to microsequencing. The amino acid sequence of the N-terminus of the 31 kDa chain was E(11)I(14)T(6)E(9)K(3)Q(11)I(10)D(14)D(16)N(9)R(3)K(2) [yields in pmol of the PTH-amino acids are shown in parentheses].

Figure 3 Identification of the cleavage sites in the $\alpha 6$ A subunit

(A) ^{125}I -labelled cell lysates of the wild-type and mutant $\alpha 6$ A-expressing transfectants were immunoprecipitated with GoH3 (anti- $\alpha 6$, lanes 1), K20 (anti- $\beta 1$, lanes 2) or p $\alpha 6$ A (anti- $\alpha 6$ A, lanes 3). Precipitates were analysed by SDS/PAGE (10% gel) under reducing conditions. (B) Shorter exposure of part of the autoradiogram shown in (A), revealing the $\alpha 6$ heavy chain (120 kDa) and the uncleaved $\alpha 6$ polypeptide chain (150 kDa). The positions of molecular mass markers are shown at the left.

Figure 4 Localization of the two proteolytic sites in the primary sequence of $\alpha 6$

The sequence of $\alpha 6$ from His-873 to Leu-903 and the amino acid sequences of the N-termini of the 31 and 30 kDa $\alpha 6$ light chains are shown. Areas labelled I-VII are homologous repeat domains; TM is the transmembrane region.

This is consistent with cleavage after Arg⁸⁷⁹ (Figure 4). Sequencing of the 30 kDa band gave two PTH residues of similar yields per cycle. One of them was the 31 kDa protein described above, which slightly contaminated the 30 kDa protein population. The N-terminal sequence of the 30 kDa protein was found to be Q(1.3)I(3.3)D(1.8)D(1.8)N(0.4)R(0.3)K(0.2)F(0.6)-S(0.2)L(1.7)F(0.9)A(0.4), which points to cleavage at the EK sequence. The very clear background allowed us to detect very small amounts of the PTH-amino acids. It is of interest that the 30 kDa protein yielded an unexpectedly small amount of PTH-amino acids, which is inconsistent with the number of the protein molecules. From the Coomassie Blue staining of the bands it seems that the yields of the 31 and 30 kDa proteins are equal. A probable explanation is that under the present experimental conditions a large percentage of the N-terminal glutamine residue of the 30 kDa protein was chemically converted into a pyroglutamate residue [29] that resists Edman degradation and therefore could not be sequenced. Taken together, the data from the Edman degradation and the results with the $\alpha 6^{\text{RKKR/A1}}$ and $\alpha 6^{\text{RKKR/A2}}$ mutants strongly suggest that cleavage occurs after Lys⁸⁸⁴.

Uncleaved $\alpha 6\beta 1$ integrins are not activated by PMA

We recently demonstrated that cleavage of the $\alpha 6\text{A}$ subunit is required for proper inside-out signal transduction by the $\alpha 6\beta 1$ integrin, but not for its ligand-binding capacity [17]. Uncleaved $\alpha 6\beta 1$ mediated adhesion to laminin-1 after activation by the stimulatory antibody TS2/16 [30,31] but did not respond to stimulation with the phorbol ester PMA, in contrast with wild-type cleaved $\alpha 6\beta 1$. To test whether there is the same relation between cleavage and activation of $\alpha 6\beta 1$ in the other mutant $\alpha 6\beta 1$ integrins, we examined the adhesion of the transfectants to laminin-1 after stimulation with TS2/16 or PMA. All transfectants bound to laminin-1 but only after stimulation with TS2/16 (Table 3). The proportions of bound cells of the different transfectants varied between 60% and 85%. Adhesion of all transfectants could be completely blocked by the anti- $\alpha 6$ mAb GoH3, which demonstrates that the adhesions were indeed mediated by the wild-type and mutant $\alpha 6\beta 1$ integrins.

Table 3 shows that the PMA-stimulated wild-type $\alpha 6\text{A}$ ($\alpha 6^{\text{RKKR}}$) transfectants adhered to laminin-1, and that the percentage of bound cells was lower than after stimulation with TS2/16, as previously reported [24]. The PMA-stimulated $\alpha 6^{\text{RKKR/A1}}$, $\alpha 6^{\text{RKKR/A2}}$, $\alpha 6^{\text{RKKR/B1}}$ and $\alpha 6^{\text{RKKR/C1}}$ transfectants adhered to laminin-1 and the percentages of bound cells were approximately similar, although slightly lower than that of $\alpha 6^{\text{RKKR}}$. As expected, the $\alpha 6^{\text{GKKR}}$, $\alpha 6^{\text{RKKG}}$ and $\alpha 6^{\text{RGGR}}$ transfectants did not bind to laminin-1 after treatment with PMA;

Table 3 Effect of TS2/16 and PMA stimulation of the transfectants on adhesion to laminin-1

Adhesion of unstimulated, TS2/16 (1:5 hybridoma supernatant) and PMA (10 ng/ml)-stimulated transfectants to laminin-1. Binding percentages are expressed as percentages of the total input per well (100%). Adhesion was measured in triplicate; results are means $\pm$ S.D. One representative experiment out of five is shown.

Cell type	Binding to laminin-1 (%)		
	Unstimulated	TS2/16	PMA
$\alpha 6^{\text{RKKR}}$	4 $\pm$ 1	83 $\pm$ 3	51 $\pm$ 2
$\alpha 6^{\text{GKKR}}$	2 $\pm$ 1	68 $\pm$ 2	4 $\pm$ 1
$\alpha 6^{\text{RKKG}}$	1 $\pm$ 1	80 $\pm$ 1	7 $\pm$ 1
$\alpha 6^{\text{RGGR}}$	1 $\pm$ 1	69 $\pm$ 3	6 $\pm$ 3
$\alpha 6^{\text{RKKR/A1}}$	4 $\pm$ 2	79 $\pm$ 3	43 $\pm$ 1
$\alpha 6^{\text{RKKR/A2}}$	5 $\pm$ 1	68 $\pm$ 1	29 $\pm$ 4
$\alpha 6^{\text{RKKR/B1}}$	8 $\pm$ 1	59 $\pm$ 4	35 $\pm$ 1
$\alpha 6^{\text{RKKR/C1}}$	10 $\pm$ 1	68 $\pm$ 1	38 $\pm$ 2
$\alpha 6^{\text{RGGR/B2C2}}$	1 $\pm$ 1	80 $\pm$ 1	7 $\pm$ 1

neither did the $\alpha 6^{\text{RGGR/B2C2}}$ transfectant. The results with these mutant $\alpha 6\text{A}$ transfectants are consistent with our previous findings: cleaved and uncleaved $\alpha 6\beta 1$ can bind to laminin-1, but only cleaved $\alpha 6\beta 1$ responds to PMA.

The RKKR sequence is required but is not sufficient for cleavage

To determine whether the RKKR sequence by itself allows cleavage of $\alpha 6\text{A}$ or whether surrounding amino acids are essential, we generated $\alpha 6\text{A}$ mutants with a shifted RKKR sequence. The RKKR sequence was shifted to the EK site in such a way that the arginine residue after which cleavage occurs is exactly in the position of Lys⁸⁸⁴ (Table 2). In the $\alpha 6^{\text{RK/A4}}$ mutant the KR sequence of the RKKR site was deleted and the ITEK sequence replaced by RKKR. In the $\alpha 6^{\text{RK/A4(E)}}$ mutant a glutamic acid residue was present after the RKKR sequence, which resembles more closely the RKKR cleavage site in wild-type $\alpha 6\text{A}$. The $\alpha 6^{\text{RK}}$ and $\alpha 6^{\text{RG/A4}}$ mutants were included as controls for cleavage. Because the two RK sequences in wild-type $\alpha 6\text{A}$ were not used for cleavage ($\alpha 6^{\text{RKKR/B1}}$ and $\alpha 6^{\text{RKKR/C1}}$ mutants), it was expected that the RK sequence in the $\alpha 6^{\text{RK}}$ mutant would not be cleaved either. The RG sequence was not a potential proteolytic cleavage site and thus the $\alpha 6^{\text{RG/A4}}$ mutant would not be cleaved at this site. In the experiments described below, all mutant $\alpha 6\text{A}$ cDNA species were transfected into K562 cells, their expression at the cell surface established and their cleavage subsequently investigated. As shown in Figure 5 the anti- $\alpha 6$ antibodies precipitated the 150 kDa $\alpha 6$ polypeptide chain and co-precipitated the $\beta 1$ subunit from the $\alpha 6^{\text{RK}}$ transfectant; thus the RK sequence is indeed not cleaved. The anti- $\alpha 6$ antibodies precipitated the 120 kDa heavy and the 30 kDa light chains and co-precipitated $\beta 1$ from the $\alpha 6^{\text{RK/A4}}$, $\alpha 6^{\text{RK/A4(E)}}$ and $\alpha 6^{\text{RG/A4}}$ transfectants (Figure 5). The 150 kDa $\alpha 6$ polypeptide chain was also precipitated from these transfectants (Figure 5B). Thus only a proportion of the $\alpha 6\text{A}$ subunits in each of these transfectants was cleaved at the RKKR site when it was moved to the EK site. Note that cleavage at the RKKR site in wild-type $\alpha 6\text{A}$ was complete, but cleavage at the EK site was also partial (Figure 3). Possibly the surrounding amino acids did not allow complete cleavage at this site. TS2/16 precipitated $\beta 1$ and co-precipitated $\alpha 5$ and $\alpha 6$ from all transfectants.

Next we investigated whether the RKKR sequence is used for cleavage of the $\alpha 6\text{A}$ subunit when shifted to positions other than

Figure 5 Identification of the cleavage in mutant $\alpha 6A$ subunits

(A) ^{125}I -labelled cell lysates of the $\alpha 6^{RKKR(D)}$, $\alpha 6^{RGGR(D)/A3}$, $\alpha 6^{RGGR(D)/B3}$, $\alpha 6^{RGGR(D)/C3}$, $\alpha 6^{RKKR}$, $\alpha 6^{RK}$, $\alpha 6^{RK/A4}$, $\alpha 6^{RK/A4(E)}$ and $\alpha 6^{RG/A4}$ transfectants were immunoprecipitated with GoH3 (anti- $\alpha 6$, lanes 1), K20 or TS2/16 (anti- $\beta 1$, lanes 2) or p $\alpha 6A$ (anti- $\alpha 6A$, lanes 3). Precipitates were analysed by SDS/PAGE (10% gel) under reducing conditions. (B) Shorter exposure of part of the autoradiogram shown in (A), revealing the $\alpha 6$ heavy chain (120 kDa) and the uncleaved $\alpha 6$ polypeptide chain (150 kDa). The 42 and 80 kDa bands represent non-specific products, which were not obtained in all experiments. The positions of molecular mass markers are shown at the left.

Figure 6 Identification of the cleavage in mutant $\alpha 6A$ subunits

(A) ^{125}I -labelled cell lysates of the $\alpha 6^{RKKR}$ and $\alpha 6^{RKKR/A5}$ transfectants were immunoprecipitated with GoH3 (anti- $\alpha 6$, lanes 1), TS2/16 (anti- $\beta 1$, lanes 2) or p $\alpha 6A$ (anti- $\alpha 6A$, lanes 3). Precipitates were analysed by SDS/PAGE (10% gel) under reducing conditions. (B) Shorter exposure of part of the autoradiogram shown in (A), revealing the $\alpha 6$ heavy chain (120 kDa) and the uncleaved $\alpha 6$ polypeptide chain (150 kDa). The 80 kDa band represents a non-specific product. The positions of molecular mass markers are shown at the left.

the EK site. In mutant $\alpha 6^{RGGR(D)/A3}$ the EKQI sequence was replaced by RKKR and the RKKR sequence mutated to RGGR (Table 2). The glutamic acid residue was changed to aspartic acid

to facilitate cloning (see the Materials and methods section). In this $\alpha 6A$ mutant the RKKR sequence was located two residues closer to the C-terminus of $\alpha 6A$ than in the $\alpha 6^{RK/A4}$, $\alpha 6^{RK/A4(E)}$ and $\alpha 6^{RG/A4}$ mutants (Table 2). The $\alpha 6^{RGGR(D)/B3}$ and $\alpha 6^{RGGR(D)/C3}$ mutants contained RKKR at the position of the first and second RK sites respectively (Table 2). Mutant $\alpha 6^{RKKR(D)}$ was generated as a control for the introduction of an aspartic acid instead of glutamic acid. In this experiment the anti- $\alpha 6$ antibodies precipitated the $\alpha 6$ heavy and light chains and co-precipitated the $\beta 1$ subunit from the $\alpha 6^{RKKR(D)}$ transfectants (Figure 5). Thus this mutant is cleaved at the RKKR and EK sites like wild-type $\alpha 6A$. The anti- $\alpha 6$ antibodies precipitated the 150 kDa $\alpha 6$ polypeptide chain and co-precipitated the $\beta 1$ subunit from the $\alpha 6^{RGGR(D)/A3}$, $\alpha 6^{RGGR(D)/B3}$ and $\alpha 6^{RGGR(D)/C3}$ transfectants (Figure 5). K20 precipitated $\beta 1$ and co-precipitated $\alpha 5$ and $\alpha 6$ from all transfectants. The results indicate that the mere presence of the RKKR sequence is not sufficient for cleavage. The RKKR sequence must also be present in its proper position and be surrounded by the appropriate amino acid residues. From these and the above results it can be concluded that both an intact RKKR sequence and its position within $\alpha 6A$ are essential for cleavage of the $\alpha 6A$ subunit. As expected all transfectants bound to laminin-1 but only after stimulation with TS2/16. One exception was the $\alpha 6^{RGGR(D)/B3}$ transfectant, which bound weakly to laminin-1 after stimulation with PMA (results not shown).

Amino acids surrounding the EK site are important for cleavage

The EK site is probably a monobasic cleavage site at which cleavage occurs after Lys⁸⁸⁴ (Figures 3 and 4). We observed that the glutamic residue at position -2 contributes to cleavage at the EK site because the $\alpha 6^{RKKR/A2}$ mutant, in which the glutamic acid residue was replaced by glycine, was not cleaved (Figure 3). To investigate whether the amino acid at position +1, glutamine, was also involved in cleavage we generated the $\alpha 6^{RKKR/A5}$ mutant, in which glutamine was replaced by glycine (Table 2). After transfection into K562 cells, the $\alpha 6^{RKKR/A5}$ subunit was immuno-

Figure 7 Identification of the cleavage in mutant $\alpha 6A$ subunits

(A) ^{125}I -labelled cell lysates of the $\alpha 6^{\text{RKKR}}$, $\alpha 6^{\text{KKR}}$ and $\alpha 6^{\text{KKR/A1}}$ transfectants were immunoprecipitated with GoH3 (anti- $\alpha 6$, lanes 1), TS2/16 (anti- $\beta 1$, lanes 2) or p $\alpha 6A$ (anti- $\alpha 6A$, lanes 3). Precipitates were analysed by SDS/PAGE (10% gel) under reducing conditions. (B) Shorter exposure of part of the autoradiogram shown in (A), revealing the $\alpha 6$ heavy chain (120 kDa) and the uncleaved $\alpha 6$ polypeptide chain (150 kDa). The 80 kDa band represents a non-specific product. The positions of molecular mass markers are shown at the left.

precipitated and whether it was cleaved was investigated. As shown in Figure 6, cleavage of $\alpha 6^{\text{RKKR/A5}}$ had occurred at the RKKR site but not at the EK site. We conclude from this and the above results that both Lys⁸⁸⁴ and the surrounding amino acid residues are essential for cleavage of the EK sequence. Like the $\alpha 6^{\text{RKKR/A1}}$ and $\alpha 6^{\text{RKKR/A2}}$ transfectants, the $\alpha 6^{\text{RKKR/A5}}$ transfectant bound to laminin-1 after stimulation with TS2/16 or PMA (Table 2).

$\alpha 6A$ subunits with an $\alpha 5$ -like cleavage site are not cleaved

The $\alpha 5$ subunit is also cleaved in K562 cells (Figures 3, 5, 6 and 7) and its cleavage site is the dibasic sequence KR¹ (Table 1). The KR sequence is no consensus sequence for furin, but it is a consensus site for other endoproteases of the constitutive secretory pathway, which require an arginine residue at position -1 and at least two basic residues at positions -2, -4 or -6 [11]. In the $\alpha 5$ subunit the basic residues arginine, lysine and histidine are present at positions -1, -2 and -6 respectively. The $\alpha 6$ subunit contains the RKKR sequence and the basic histidine residue at position -7 (Table 1). To investigate whether $\alpha 6A$ subunits with a similar cleavage site to that in $\alpha 5$, i.e. a histidine residue at position -6 and KR at positions -1 and -2, are cleaved in K562 cells, the $\alpha 6^{\text{KKR}}$ and $\alpha 6^{\text{KKR/A1}}$ mutants were generated (Table 2). After transfection of the mutant $\alpha 6A$ cDNA species into K562 cells, the cleavage of the mutant $\alpha 6A$ subunits was investigated. As shown in Figure 7 the anti- $\alpha 6$ antibodies precipitated the 150 kDa $\alpha 6$ polypeptide chain and co-precipitated the $\beta 1$ subunit from the $\alpha 6^{\text{KKR}}$ and $\alpha 6^{\text{KKR/A1}}$ transfectants. TS2/16 precipitated $\beta 1$ and co-precipitated $\alpha 5$ and $\alpha 6$ from these transfectants. The results demonstrate that the $\alpha 5$ -like cleavage site is not cleaved in the $\alpha 6A$ subunit. The

immunoprecipitations also show that the mutant $\alpha 6A$ subunits are associated with endogenous $\beta 1$.

DISCUSSION

In this study we show that the $\alpha 6$ integrin subunit is proteolytically cleaved into a heavy chain (120 kDa) and a light chain of either 31 or 30 kDa. Using site-directed mutagenesis we demonstrated that the RKKR and EK sites are used for cleavage, and microsequencing of the N-termini of the two light chains revealed that cleavage occurs after Arg⁸⁷⁹ and Lys⁸⁸⁴.

In K562 cells the transfected wild-type $\alpha 6A$ integrin subunit was processed in the same way as endogenous $\alpha 6$ in T24 carcinoma cells and platelets: all cell types express the heavy and the two light chains of $\alpha 6$. Thus K562 cells possess the enzymes responsible for proteolytic processing of the $\alpha 6$ subunit. The identity of the enzymes involved in the cleavage of $\alpha 6$ in K562 cells is not known. Recently it has been shown that $\alpha 6$ isolated from the furin-deficient human cell line LoVo is cleaved by recombinant furin [14]. Thus furin is most probably involved in the cleavage of $\alpha 6$ in K562 cells. This enzyme, a subtilisin-like serine protease, is a ubiquitously expressed transmembrane protein localized in the trans-Golgi network, where it is involved in processing precursor proteins in the constitutive secretory pathway [9,10,32]. The furin consensus sequence is RXR/KR¹, in which X can be any amino acid [13]. The RKKR sequence in $\alpha 6$ matches the recognition sequence of furin. Furthermore, the $\alpha 6^{\text{RKKR}}$ and $\alpha 6^{\text{GKKR}}$ mutants, in which the basic arginine residue immediately upstream of the cleavage site, at position -1 or position -4, was replaced by glycine, were not cleaved. This is consistent with the sequence requirements for furin, i.e. a basic arginine residue at position -1 and a basic residue, preferably arginine, at position -4 [33]. We also observed that the $\alpha 6$ subunit in which the lysine residues at positions -2 and -3 were replaced by glycines ($\alpha 6^{\text{RGGR}}$ and $\alpha 6^{\text{RGGR/B2C2}}$) was not cleaved. Furin, however, has been reported to recognize the RXXR sequence (X can be any amino acid) in several precursor proteins, although the cleavage efficiency at this site was sometimes low [33–35]. In spite of the fact that $\alpha 6$ subunits containing the RGGR sequence are not cleaved, but taking the other results into account, furin might still be the enzyme involved in processing $\alpha 6$ at the RKKR site. However, it cannot be excluded that other serine proteases, such as PACE4 and PC-6, which are also ubiquitously expressed and can recognize the RKKR sequence [35,36], are involved in cleavage of the RKKR site in $\alpha 6$.

We show that the sequence at the RKKR site is not itself sufficient for recognition by the endoprotease: its position in $\alpha 6$ is also important. Only $\alpha 6A$ subunits in which the RKKR sequence was located at sites that were cleaved in wild-type $\alpha 6A$, i.e. the RKKR and EK sites, were cleaved, whereas $\alpha 6A$ subunits with the RKKR sequence at other positions were not cleaved. Also $\alpha 6$ subunits with an $\alpha 5$ -like cleavage site were not cleaved, whereas the $\alpha 5$ subunit itself was cleaved in K562 cells. Thus the secondary structure of the $\alpha 6$ subunit, being different from that of $\alpha 5$, somehow prevented the recognition of the $\alpha 5$ -like cleavage site by endoproteases. That the secondary structure of a protein is important for endoproteolytic cleavage has been demonstrated for peptide hormone precursors, most of which are cleaved in α -helices, β -turns and ω -loops [37,38]. Processing at the RXK/RR site is prevented by the presence of an aliphatic residue at position +1 [39,40]. Thus the $\alpha 6^{\text{RGGR(D)/B3}}$ and $\alpha 6^{\text{RGGR(D)/C3}}$ mutants might not have been cleaved owing to the presence of a leucine or threonine residue at position +1 (see Table 2). At the EK site both glutamic acid and lysine are important for cleavage,

because mutation of either residue prevented cleavage. Similarly, we showed that the residue at position +1 (glutamine) is involved. It is unlikely that a subtilisin-like serine protease recognizes the EK site. Although a basic residue at position -6 is present in the primary structure of $\alpha 6$, consistent with the sequence requirements for cleavage by subtilisin-like serine proteases [11,12,39], this residue is not necessary for cleavage at the EK site. In the $\alpha 6^{\text{GKKR}}$ and $\alpha 6^{\text{RGGR}}$ mutants, in which an arginine residue is present at position -6, we observed no cleavage of $\alpha 6$ either at the EK or at the RKKR site. Moreover cleavage at the EK site seems to occur secondarily to cleavage at the RKKR site (see below). Hence no basic residues are available N-terminal to the EK site.

Cleavage at the EK site obviously depends on the presence of an intact RKKR site, the primary cleavage site in $\alpha 6$. Proteolytic cleavage at RKKR might generate a conformation that is required for recognition of the EK sequence. Recognition of the RKKR sequence does not require a previous cleavage in the $\alpha 6A$ subunit. When located at the proper position, either the RKKR or the EK site, the RKKR sequence is recognized and cleaved by endoproteases. Clearly the requirements for cleavage of the RKKR and EK sequences differ and thus different enzymes might be responsible for cleaving these sequences. In the cell types expressing wild-type $\alpha 6$ the RKKR sequence was cleaved in all $\alpha 6$ molecules, because the $\alpha 6A$ heavy chain migrated as a single band when $\alpha 6A$ precipitates were analysed by SDS/PAGE under reducing conditions. Cleavage at EK occurred in approximately half of the $\alpha 6$ molecules, as estimated from the intensities of the two $\alpha 6$ light chains precipitated from surface-labelled K562 transfectants, platelets and T24 cells. We assume that cleavage of the RKKR sequence precedes that at EK. Because we previously observed that proteolytic cleavage of the $\alpha 6$ precursor has the same kinetics as maturation of the N-linked glycans on $\alpha 6$ [19], and because furin is localized in the trans-Golgi network, cleavage at the RKKR site probably occurs in this compartment. Cleavage at the EK site might also occur during transport or at the plasma membrane by a membrane-bound protease.

The sequences encoding RKKR and EK are conserved in the human, murine and chick $\alpha 6$ cDNA species [21,22,41,42]. Consequently the $\alpha 6$ subunits of mouse and chicken are expected to be cleaved in the same way as human $\alpha 6$. The *Xenopus laevis* $\alpha 6$ cDNA encodes RHRR and ES sequences instead of RKKR and EK [43]. Its $\alpha 6$ subunit is predicted to be processed into one heavy and one light chain. The furin consensus sequence RXR/KR is present not only in $\alpha 6$ but also in the primary structures of the $\alpha 3$ [44], $\alpha 7$ [45], αIIb [46] and αE [5] subunits (Table 1). Cleavage by recombinant furin has been demonstrated for the $\alpha 3$ and $\alpha 6$ subunits [14]. It is therefore expected that all these α subunits are processed at the furin consensus sequence.

Cleavage of the $\alpha 6A$ subunit did not influence its $\beta 1$ -subunit association: both uncleaved $\alpha 6A$ subunits and those that were cleaved at one site associated with endogenous $\beta 1$, like wild-type $\alpha 6A$. We recently demonstrated that cleavage at the RKKR site in $\alpha 6A$ is not required for binding of the $\alpha 6A/\beta 1$ integrin to ligand, but it is necessary for inside-out signal transduction [17]. Indeed all mutant $\alpha 6A/\beta 1$ integrins are receptors for laminin-1 when activated by the anti- $\beta 1$ mAb TS2/16, but only the cleaved $\alpha 6A/\beta 1$ integrins can be activated by PMA to bind laminin-1. The results also show that multiple amino acid substitutions in the $\alpha 6A$ subunit are tolerated. They affect neither the association of the $\alpha 6A$ subunit with $\beta 1$ nor the ligand-binding capacity of the $\alpha 6A/\beta 1$ integrin. Apparently, cleavage at the EK site is not important for inside-out signal transduction by $\alpha 6A/\beta 1$ because the wild-type $\alpha 6A$ ($\alpha 6^{\text{RKKR}}$) and mutant $\alpha 6A$ with mutations in

the EK site ($\alpha 6^{\text{RKKR/A1}}$, $\alpha 6^{\text{RKKR/A2}}$, and $\alpha 6^{\text{RKKR/A5}}$) could be stimulated by PMA to bind laminin-1. At present the function, if any, of cleavage at the EK site is not clear. Among the cleaved α subunits, $\alpha 3$, $\alpha 6$, $\alpha 7$ and αIIb are processed into a heavy and two light chains; all others possess one light chain. The cleavage sites in the αIIb subunit have also been determined [7,8]. Further, it has been established that cleavage of αIIb does not influence the ligand binding of the $\alpha \text{IIb}/\beta 3$ integrin [16]. But there is no explanation for the existence of the two light chains of αIIb , except that the occurrence of the smaller light chain is variable, which may depend on whether or not outdated platelets (the αIIb source) had been used [16].

In conclusion, we have determined the two cleavage sites in the $\alpha 6$ subunit and the sequence requirements for cleavage at these sites. Furthermore we have confirmed that cleavage of the $\alpha 6$ subunit is required for inside-out signal transduction.

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