The two phenylalanines in the GFFKR motif of the integrin $\alpha 6A$ subunit are essential for heterodimerization

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The membrane-proximal domain of the integrin α subunit contains a conserved motif of five amino acid residues, GFFKR. We deleted this motif from the human $\alpha 6A$ subunit and found that in COS-7 cells this mutant cannot associate with the $\beta 1$ subunit and is retained in the endoplasmic reticulum. Point mutations in the GFFKR motif of the glycine residue or the two highly charged amino acids, or deletion of the lysine and arginine residues, had no effect on the ability of $\alpha 6$ to interact with $\beta 1$ and to be expressed at the cell surface. In contrast, by replacing either of the two phenylalanines with alanine, or by deletion of both of these residues, $\alpha 6$ was incapable of associating with $\beta 1$. The $\alpha 6$ point mutants that associated with $\beta 1$ were expressed in K562 cells and their responsiveness to integrin-activating factors was determined. None of these transfectants bound spontaneously to laminin-1, but binding could be induced by either PMA or the stimulating anti- $\beta 1$ antibody TS2/16 to the same extent as that

of the wild-type transfectant. The ability of these mutants to initiate focal-contact formation in CHO cells plated on laminin-1 substrates also appeared to be unaltered. Thus the behaviour of $\alpha 6$ mutants involving the glycine, lysine or arginine residues was indistinguishable from that of wild-type $\alpha 6$ both in inside-out and outside-in signalling. In contrast, deletion of the cytoplasmic domain of $\alpha 6$ C-terminal of the GFFKR motif resulted in a loss of responsiveness of $\alpha 6\beta 1$ to PMA stimulation and formation of focal contacts on laminin-1. However, this mutant was targeted to focal contacts formed by other integrins, even when they had not bound ligand. Together, these results suggest that the two phenylalanine residues of the GFFKR motif provide a site for interaction of the $\alpha 6A$ subunit with $\beta 1$, whereas the cytoplasmic domain C-terminal of this motif is involved in the regulation of bidirectional signalling via $\alpha 6A\beta 1$.

INTRODUCTION

Integrins are cell-surface receptors which consist of an α and a β subunit. Each subunit contains a large extracellular domain, a single membrane-spanning region and a short cytoplasmic tail. Integrins mediate cell–matrix and cell–cell interactions and, in addition, transduce signals from the extracellular environment to the cytosol [1,2]. After binding to ligand, integrins become concentrated in specific regions of close contact between the substrate and the basal side of the cells, called focal contacts [3]. These regions are anchoring sites for actin filaments and contain a large number of cytoskeletal and regulatory proteins. Several of these intracellular proteins appear to interact directly with the cytoplasmic domains of integrin subunits and probably participate in the signalling pathways [4,5]. The integrins not only transduce signals from outside the cell to the cytosol, but also in the opposite direction, since the affinity of various integrins for ligand appears to be regulated by signals from within the cell [6–8].

The molecular changes involved in the transduction of signals via integrins are still poorly defined. Several monoclonal antibodies (mAbs) against integrins of the $\beta 1$, $\beta 2$ or $\beta 3$ subfamilies recognize specific epitopes that become exposed on binding of the integrins to ligands or ligand-mimicking small peptides, or on activation of the integrins by stimulating mAbs, cations or integrin-activating factors [7,8]. These findings indicate that conformational changes occur in the extracellular region of the integrin on activation or ligand binding. Changes in conformation have also been demonstrated in biochemical experi-

ments [9,10], by NMR [11] and by resonance-energy-transfer measurements [12].

It is not clear how a conformational change in the extracellular domain can be induced by a conformational change in the cytoplasmic domain and vice versa. Briesewitz et al. [13] showed that a chimaeric $\alpha 1\beta 1$ integrin in which the cytoplasmic domains were swapped, although able to bind ligand, no longer mediated outside-in signalling. This suggests that in these chimaeric integrins the interactions between the cytoplasmic domains are disturbed. The membrane-proximal regions of both α and β subunits contain highly conserved amino acid sequences. All α subunits contain the motif GFFKR, whereas most β subunits contain the motif LLviXhDRRE (the less conserved residues are in lower case, X is not conserved) [6]. These conserved sequences were found to be essential for maintaining the $\alpha IIb\beta 3$ integrin in a state of low affinity. Deletion of these sequences resulted in an energy-independent constitutively active integrin, which suggests that the association between these regions needs to be changed in order to activate the integrin [14–16].

In contrast, $\alpha 2$, $\alpha 4$ and $\alpha 6$ subunits truncated N-terminal of the GFFKR motif did not reach the cell surface [17–19], and the levels of similar mutants of $\alpha 5$ [20] and αL [21] at the cell surface were very low. This suggests that the GFFKR motif is important for subunit association of integrins and their transport to the plasma membrane.

In a previous study, we showed that an $\alpha 6$ subunit that was truncated N-terminal of the GFFKR motif was not expressed at the cell surface, but was retained in the endoplasmic reticulum (ER). When the cytoplasmic domain of the $\alpha 6$ subunit was

Abbreviations used: endo Hf, endoglycosidase Hf; ER, endoplasmic reticulum; FBS, foetal bovine serum; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium.

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truncated C-terminal of this motif, $\alpha 6\beta 1$ did appear at the cell surface, but bidirectional signalling was impaired [17]. To study the function of the GFFKR motif in more detail, we have made a number of $\alpha 6A$ mutants with point mutations or small deletions in the GFFKR motif and have studied the capacity of these mutants to associate with $\beta 1$ and to transduce signals. We show that mutation of either one of the phenylalanines prevents subunit association and transport to the membrane, whereas mutation of the other three residues has no effect on either heterodimerization or signalling via integrins.

MATERIALS AND METHODS

Cells and antibodies

The $\alpha 6A$ transfectant of the human erythroleukaemic cell line K562 has been described previously [22]. Transfectants were grown in RPMI 1640 medium containing 10% foetal bovine serum (FBS), 100 IU/ml penicillin, 100 μ g/ml streptomycin (Gibco-BRL, Paisley, Scotland, U.K.) and 1 mg/ml Geneticin (G418 sulphate; Gibco-BRL). COS-7 (a simian virus 40-transformed monkey kidney fibroblast-like cell line) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin and streptomycin. CHO (a Chinese hamster ovary cell line) cells were grown in this medium, to which non-essential amino acids (Gibco-BRL) had been added.

The following mAbs were used: rat mAb GoH3 and mouse mAb J8H, against extracellular epitopes of $\alpha 6$ [23,24]; mouse mAb 1A10, against the cytoplasmic domain of $\alpha 6A$ [23,25]. The hybridoma producing the mouse anti- $\beta 1$ activating mAb TS2/16 [26] was purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Finally, the mouse anti- $\alpha 5$ mAb Sam-1 [27] was a gift from Dr. C. Figdor (University of Nijmegen, The Netherlands). Vinculin was detected by a mouse mAb purchased from Sigma Biosciences (St. Louis, MO, U.S.A.).

Construction of $\alpha 6$ mutants

Point mutations and deletions were introduced into the cDNA encoding the cytoplasmic domain of $\alpha 6A$ using synthetic oligonucleotides and overlapping PCR, as described previously [23,28]. The resulting fragment encompassing the mutation was digested with *Xba*I and *Bam*HI and exchanged with an identical fragment of pUC18- $\alpha 6A$ cDNA. Mutant clones were identified by sequence analysis. Correct mutant $\alpha 6$ cDNA fragments were isolated after digestion with *Hind*III and cloned into the *Hind*III site of the pRc/CMV expression vector (Invitrogen, San Diego, CA, U.S.A.).

Stable transfection in K562 cells and flow cytometry

The method of transfection and subsequent selection of expressing cells by flow cytometry has been described previously [22]. K562 cells (5×10^6) were suspended in 250 μ l of RPMI 1640 medium and mixed with 10 μ g of expression vector DNA. Electroporation was performed in a Gene Pulser Apparatus (Bio-Rad, Richmond, CA, U.S.A.) at 230 V and 960 μ F using a 0.4 cm cuvette. Cells were resuspended in RPMI 1640 medium containing 10% FBS and penicillin/streptomycin. After 2 days, 1 mg/ml Geneticin was added to the medium. Cells expressing $\alpha 6$ at high levels were selected after 2 weeks by sorting with a FACScan (Becton Dickinson, Mountain View, CA, U.S.A.), using the mAb J8H.

Transient transfection in COS-7 cells

A total of 1×10^6 cells were plated in 100 mm $\times$ 20 mm tissue-culture dishes (Falcon, Plymouth, Devon, U.K.). After 24 h, the cells were washed twice with PBS and then incubated for 30 min at 37 °C with 0.5 ml of PBS containing 50 μ g of DEAE-dextran and 5 μ g of $\alpha 6$ cDNA. After the addition of 6.5 ml of complete medium containing 80 μ M chloroquine to the cells, they were incubated for 2.5 h at 37 °C. The DNA-DEAE-dextran/chloroquine solution was aspirated and 2 ml of 10% DMSO in complete medium was added gently and left on the cells for 2.5 min. Then the DMSO was aspirated and 10 ml of complete medium was added to the cells. After 24 h, the cells were collected and transferred to six-well plates for pulse-chase experiments. Typically, cells were utilized for these experiments at 48–72 h after transfection.

Transient transfection in CHO cells

A total of 3×10^5 cells were plated per well in a six-well plate (Falcon). After 24 h, the cells were washed once with DMEM without supplements and once with Optimem 1 (Gibco-BRL). $\alpha 6$ cDNA (2 μ g) was mixed with 12 μ g of lipofectamine (Gibco-BRL) in 200 μ l of Optimem and incubated for 45 min at room temperature to allow the formation of DNA-liposome complexes. These complexes were added to the washed cells in a total volume of 1 ml of Optimem and incubated for 5 h at 37 °C. Then 1 ml of DMEM containing 20% FBS was added to the cells without removal of the transfection mixture. Next day, the medium was aspirated and the cells were then grown further in their usual medium. Cells were utilized for immunofluorescence 48 h after the start of the transfection.

Radiolabelling of transfected cells

K562 transfectants were surface-labelled with 125 I using the lactoperoxidase/ H_2O_2 method as described previously [24]. Labelled cells were washed three times with PBS and then incubated for 30 min at 4 °C with lysis buffer (1% Nonidet P40, 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 4 mM EDTA, 1 mM PMSF, 10 μ g/ml soyabean trypsin inhibitor and 10 μ g/ml leupeptin).

Transfected COS-7 cells were washed twice with PBS and incubated with DMEM without methionine and cysteine (ICN Biomedicals, Costa Mesa, CA, U.S.A.) for 1 h at 37 °C. Cells were pulsed with 50 μ Ci/ml [35 S]methionine/cysteine (Amersham International, Amersham, Bucks., U.K.), for 30 min at 37 °C and chased in the presence of unlabelled methionine/cysteine for 0, 1, 5 and 20 h. Cells were lysed as described above and lysates were stored at 4 °C.

Immunoprecipitation and enzyme digestion

Cell lysates were clarified by centrifugation in an Eppendorf microcentrifuge for 10 min at 16000 *g* (14000 rev./min) and used for immunoprecipitation. To reduce non-specific binding of proteins to the beads, cell lysates of metabolically labelled cells were precleared with Protein A-Sepharose CL-4B (Pharmacia LKB, Biotechnology Inc., Uppsala, Sweden) for 1.5 h or overnight at 4 °C. Integrins were precipitated from the lysates for 1 h at 4 °C with specific mouse or rat mAbs coupled to Protein A-Sepharose beads via rabbit anti-mouse immunoglobulin or rabbit anti-rat immunoglobulin antibodies. The beads were then washed three times with cold lysis buffer and twice with cold

PBS. After the beads had been heated for 5 min in SDS sample buffer at 95 °C, precipitated proteins were analysed by SDS/PAGE. For reduction, 5% 2-mercaptoethanol was added to the sample buffer.

Endoglycosidase Hf (endo Hf) treatment was performed directly on the immunocomplexes coupled to the beads. Proteins were denatured at 100 °C for 10 min in 0.5% SDS. Then 10 $\times$ reaction buffer (500 mM sodium citrate, pH 5.5) and 1 μ l of endo Hf (1 $\times 10^6$ units/ml) (New England Biolabs Inc.) were added. The total volume for the reaction was 26 μ l. Proteins were digested for 1 h at 37 °C and analysed by SDS/PAGE as described above.

Cell binding assays

Microtitre plates (96-well; Greiner GmbH, Frickenhausen, Germany) were coated with 20 μ g/ml laminin-1 (Collaborative Biomedical Products, Bedford, MA, U.S.A.) for 16 h at 4 °C. Coated plates were washed twice with PBS, and remaining binding sites on the plastic surface were blocked with PBS/BSA (0.35%) for 2 h at room temperature. Transfected K562 cells were washed twice in PBS and radiolabelled with ^{51}Cr as described previously [22]. After labelling, cells were washed three times in Iscove's medium (Gibco-BRL) containing 0.35% BSA and incubated with the anti- $\beta 1$ activating antibody TS2/16 (5 μ g/ml), with various concentrations of PMA (Sigma Chemical Co.) dissolved in DMSO or with DMSO only, for 10 min at 37 °C. Then 0.1 ml aliquots of the cells at a final concentration of 1 $\times 10^6$ /ml were added to individual substrate-coated wells and incubated for 45 min at 37 °C. Non-adherent cells were removed by inverting the microtitre plates on to tissue paper and absorption of the medium. After the plates had been washed five times in Iscove's/BSA, adherent cells were lysed in 2% (w/v) SDS, and bound radioactivity was determined by γ -spectrometry.

Immunofluorescence

Coverslips were coated with 20 μ g/ml mouse laminin-1 or human plasma fibronectin (Sigma) for 16 h at 4 °C. Transfected CHO cells were treated with 25 μ g/ml cycloheximide (Merck, Darmstadt, Germany) for 2 h before trypsin treatment. The trypsin-treated cells were washed twice with PBS and plated on to the coated coverslips in serum-free medium containing cycloheximide and incubated for 3 h at 37 °C. PMA (5 ng/ml) was added to the medium of cells plated on laminin-1-coated coverslips. Cells were fixed in 4% paraformaldehyde in PBS for 5 min and permeabilized with 0.2% Nonidet P40 in PBS for 5 min at room temperature. Non-specific staining was blocked by incubating the coverslips with 1% BSA in PBS for 30 min at 37 °C or overnight at 4 °C. After incubations with primary antibodies for 30 min at 37 °C, the cells were washed three times with PBS and incubated for 30 min with FITC-conjugated goat anti-mouse antibody (Zymed Laboratories, San Francisco, CA, U.S.A.). Cells were washed again with PBS and then incubated for 30 min with rhodamine-conjugated phalloidin (Sigma). Coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.), and viewed under a Bio-Rad MRC-600 confocal laser-scanning microscope (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

RESULTS

Expression of $\alpha 6$ A mutants in K562 cells

To examine the contribution of the GFFKR motif in the $\alpha 6$ A cytoplasmic domain to the expression of $\alpha 6$ A at the cell surface,

we deleted the sequence encoding this motif from the $\alpha 6$ A cDNA. In addition, each of the five residues was targeted by site-directed mutagenesis. G1017, F1018 or F1019 was substituted with an alanine residue, and the positively charged K1020 or R1021 with a non-polar leucine residue or a negatively charged aspartic acid residue (Table 1). K562 cells were transfected with the wild-type or mutant $\alpha 6$ A cDNAs and their expression was evaluated by FACS analysis. Surface expression of $\alpha 6$ was detected on cells transfected with $\alpha 6$ A carrying the G1017A mutation or either of the mutations of K1020 or R1021. After several rounds of cell sorting, the FACS profiles of $\alpha 6$ on the wild-type and mutant $\alpha 6$ A transfectants were similar (Figure 1), which suggests that there are no obvious differences in the stability of the wild-type and various mutant $\alpha 6\beta 1$ heterodimers. In agreement with the results of the FACS analysis, immunoprecipitation from lysates of ^{125}I -labelled transfectants showed that these mutant $\alpha 6$ A subunits (M_r 150 000) were present at the cell surface in association with $\beta 1$ (M_r 115 000) (Figure 2A). The mutations did not affect the cleavage of the $\alpha 6$ subunit into a heavy (M_r 120 000) and a light (M_r 30 000 or 31 000) chain (Figure 2B).

In contrast, $\alpha 6\text{A}(\Delta\text{GFFKR})$, $\alpha 6\text{A}(\text{F1018A})$ and $\alpha 6\text{A}(\text{F1019A})$ were not detectable at the cell surface by FACS analysis (Table 1). The importance of the phenylalanines for the cell-surface expression of $\alpha 6$ A was further confirmed with several deletion mutants. $\alpha 6$ A in which both K1020 and R1021 (ΔKR) were deleted was expressed at the cell surface, whereas two mutants from which both phenylalanine residues were deleted (ΔGFF and ΔFFK) were not expressed. Thus the presence of both phenylalanines is essential for membrane expression.

Synthesis and processing of $\alpha 6$ A mutants in COS-7 cells

To study whether the lack of expression of the $\alpha 6$ A subunits in which either one or both phenylalanines were replaced by alanines is due to the inability of the $\alpha 6$ A subunit to associate with $\beta 1$, pulse-chase analysis of the biosynthesis of the various mutant $\alpha 6$ A subunits was performed in transiently transfected COS-7 cells. Intracellular transport of the $\alpha 6$ A subunits was followed by determining the maturation of the N-linked oligosaccharides with endo Hf treatment. Precursor $\alpha 6$ A subunits that have not left the ER are completely sensitive to digestion with this enzyme, whereas mature $\alpha 6$ A, present in the Golgi complex and at the cell surface, is only partially sensitive, because of the conversion of most of the high-mannose N-linked oligosaccharides into complex-type glycans [24].

As indicated in Figure 3(A), wild-type human $\alpha 6$ A was synthesized in COS-7 cells, and the mature form, which migrated slightly more slowly than precursor $\alpha 6$ A and was less sensitive to endo Hf treatment, was first detected after 5 h. After 20 h of chase, most of the $\alpha 6$ A subunits had been processed to the mature form. The production of fully processed $\alpha 6$ A subunits was closely related to co-precipitation of $\beta 1$. These findings are consistent with the assumption that $\alpha 6$ subunits cannot be transported to the Golgi complex, unless associated with $\beta 1$.

Like that of human wild-type $\alpha 6$ A, the endo Hf-sensitive precursor of $\alpha 6\text{A}(\Delta\text{GFFKR})$ was synthesized in COS-7 cells. However, only very few of these precursor subunits were processed to mature proteins and no $\beta 1$ could be co-precipitated with them. Evidently, this mutant $\alpha 6$ A subunit cannot stably associate with $\beta 1$ and is retained in the ER (Figure 3B). The observation that a few precursor subunits escaped retention in the ER and were processed to mature proteins can probably be explained by the production of large quantities of transiently expressed proteins. The escaped $\alpha 6$ A subunits are possibly

Table 1 List of mutant $\alpha 6$ A subunits and their expression in K562 cells

(TM) means transmembrane region; the position of the original GFFKR motif is indicated in bold; the positions of the mutated or deleted amino acids are underlined; + means cell-surface expression on transfected K562 cells; – means no cell-surface expression on transfected K562 cells.

Mutant	Sequence	Expression
Wild-type $\alpha 6$ A	(TM) W KCG GFFKR NKKDHY	+
$\alpha 6$ A(Δ 1022)	(TM) W KCG GFFKR	+
$\alpha 6$ A(Δ GFFKR)	(TM) W KC _____NKKDHY	–
$\alpha 6$ A(G1017A)	(TM) W KCA FFKR NKKDHY	+
$\alpha 6$ A(F1018A)	(TM) W KCG A FKRNKKDHY	–
$\alpha 6$ A(F1019A)	(TM) W KCG F AKRNKKDHY	–
$\alpha 6$ A(K1020D)	(TM) W KCG FFDR NKKDHY	+
$\alpha 6$ A(K1020L)	(TM) W KCG FFLR NKKDHY	+
$\alpha 6$ A(R1021D)	(TM) W KCG FFKD NKKDHY	+
$\alpha 6$ A(R1021L)	(TM) W KCG FFKL NKKDHY	+
$\alpha 6$ A(Δ KR)	(TM) W KCG FF _____NKKDHY	+
$\alpha 6$ A(Δ GFF)	(TM) W KC _____ KR NKKDHY	–
$\alpha 6$ A(Δ FFK)	(TM) W KCG _____ RN KKDHY	–

Figure 1 Surface expression of wild-type or mutant $\alpha 6$ A subunits in transfected K562 cells

K562 cells were transfected with $\alpha 6$ A, $\alpha 6$ A(G1017A), $\alpha 6$ A(K1020D), $\alpha 6$ A(K1020L), $\alpha 6$ A(R1021D), $\alpha 6$ A(R1021L) or $\alpha 6$ A(Δ KR) cDNA. Cells were incubated with the $\alpha 6$ -specific mAb J8H (black) or PBS (white), then with an FITC-labelled goat anti-mouse secondary immunoglobulin, and analysed by flow cytometry.

expressed at the cell surface as single subunits, but are rapidly degraded, since they could not be detected after 20 h of chase.

The same analysis of $\alpha 6$ A carrying the various point mutations in the GFFKR motif showed that the rate of synthesis and processing of $\alpha 6$ A(G1017A), $\alpha 6$ A(K1020L) and $\alpha 6$ A(R1021L) were similar to those of wild-type $\alpha 6$ A, and that co-precipitation of $\beta 1$ coincided with the appearance of mature mutant $\alpha 6$ A subunits (Figure 3A). In contrast, no $\beta 1$ could be shown to associate with the $\alpha 6$ A(F1018A) and $\alpha 6$ A(F1019A) subunits. A fraction of these latter two mutant subunits also escaped retention in the ER, probably by the same mechanism as described above for $\alpha 6$ A(Δ GFFKR). This was also seen when $\alpha 6$ A(Δ GFF) and $\alpha 6$ A(Δ FFK) were expressed in COS-7 cells, but $\alpha 6$ A(Δ KR) was again synthesized and processed like wild-type $\alpha 6$ A (Figure 3B).

Thus these experiments show that both phenylalanines in the GFFKR motif of the $\alpha 6$ A integrin subunit are essential for a stable association with $\beta 1$, which is followed by expression at the cell surface.

Adherence mediated by $\alpha 6$ A mutant integrins on K562 cells

Next, the binding to laminin of the various $\alpha 6\beta 1$ -expressing K562 cells, which contain $\alpha 6$ A with a mutation in the GFFKR

Figure 2 Immunoprecipitation analysis of surface-labelled transfected K562 cells

$\alpha 6$ A-, $\alpha 6$ A(G1017A)-, $\alpha 6$ A(K1020D)-, $\alpha 6$ A(K1020L)-, $\alpha 6$ A(Δ KR)-, $\alpha 6$ A(R1021D)- or $\alpha 6$ A(R1021L)-transfected cells were labelled with 125 I. Cell lysates were immunoprecipitated with the anti- $\beta 1$ mAb TS2/16 (lanes 1), the anti- $\alpha 6$ mAb GoH3 (lanes 2) or the polyclonal anti- $\alpha 6$ A serum $\alpha 6$ A (lanes 3). Precipitates were analysed by SDS/PAGE on 5% gels under non-reducing conditions (A) or on 10% gels under reducing conditions (B).

motif, was studied. K562 cells expressing wild-type $\alpha 6$ A adhered poorly to laminin-1, but adhesion was induced by the anti- $\beta 1$ activating mAb TS2/16 or by PMA [22]. All transfectants expressing mutant $\alpha 6$ A showed weak spontaneous binding to the laminin-1 substrate, but were stimulated to bind to laminin-1 by incubation with TS2/16 to a similar extent to that of the wild-type $\alpha 6$ A transfectants (Figure 4). Binding to laminin-1 was also induced by PMA, although the $\alpha 6$ A(R1021D), $\alpha 6$ A(R1021L), $\alpha 6$ A(K1020L) and $\alpha 6$ A(Δ KR) transfectants seemed to be less sensitive to PMA activation than the other two mutant and wild-type $\alpha 6$ A transfectants. Independent transfections, however, revealed that this is an effect of clonal variation rather than of the mutation (not shown). Thus mutations of the glycine, lysine or arginine within the GFFKR domain do not affect inside-out signalling.

Localization into focal contacts

The effect of mutations of the glycine, lysine or arginine residue within the GFFKR motif on outside-in signalling was studied by determining the ability of the various mutant $\alpha 6$ subunits to induce the formation of focal contacts on laminin-1 in transiently

Figure 3 Biosynthesis of wild-type and mutant $\alpha 6/\beta 1$ complexes on transient expression in COS-7 cells

Cells were transfected with constructs encoding wild-type $\alpha 6$, $\alpha 6$ (G1017A), $\alpha 6$ (F1018A), $\alpha 6$ (F1019A), $\alpha 6$ (K1020L) or $\alpha 6$ (R1021L) (**A**) or $\alpha 6$ (Δ GFFKR), $\alpha 6$ (Δ KR), $\alpha 6$ (Δ GFF) or $\alpha 6$ (Δ FFK) (**B**). Transfectants were pulsed with [35 S]methionine/cysteine for 30 min and chased in the presence of unlabelled methionine/cysteine for the indicated periods of time. Cell lysates were immunoprecipitated with an antibody specific for human $\alpha 6$ (J8H). Half of each immunoprecipitated sample was subjected to endo Hf digestion (1 h; 37 °C) at each time point during the chase period (lanes 2, 4, 6 and 8). Precipitates were analysed by SDS/PAGE on a 5% gel under non-reducing conditions. The positions of precursor $\alpha 6$ (pre- $\alpha 6$) and mature $\alpha 6$ and $\beta 1$ are indicated on the left. * denotes endo Hf-treated forms of the $\alpha 6$ subunits, the positions of which are indicated on the right.

Figure 4 Adhesive properties of K562 cells transfected with wild-type or mutant $\alpha 6A$ cDNA

The cells were labelled with ^{51}Cr and incubated with various concentrations of PMA for 10 min. Stimulated cells were added to wells coated with laminin-1 (murine Engelbreth-Holm-Swarm-tumour laminin) and incubated for 45 min at 37 °C. Non-adherent cells were removed by washing with medium and adhesion was quantified as described in the Materials and methods section. (A) Wild-type $\alpha 6A$ - or $\alpha 6A(\text{G1017A})$ -transfected cells; (B) $\alpha 6A(\text{K1020D})$ -, $\alpha 6A(\text{K1020L})$ - or $\alpha 6A(\Delta\text{KR})$ -transfected cells; (C) $\alpha 6A(\text{R1021D})$ - or $\alpha 6A(\text{R1021L})$ -transfected cells. (D) Adhesion to laminin-1 of unstimulated transfectants (hatched bars) or transfectants stimulated with 5 $\mu\text{g/ml}$ anti- $\beta 1$ mAb TS2/16 (black bars). Binding percentages are expressed as percentage of the total input per well (100%). The data shown are means $\pm$ S.D. from a representative experiment carried out in triplicate.

transfected CHO cells. The mutant $\alpha 6(\Delta 1022)$ subunit (Table 1) was included in this study as a control for ligand-independent focal-contact localization.

CHO cells bound, but spread poorly on laminin-1, even after expression of wild-type human $\alpha 6A$. When PMA was added to the medium of $\alpha 6A$ -transfected CHO cells, which were plated on laminin-1, spreading was enhanced, whereas mock-transfected cells still spread poorly. Therefore PMA stimulation has been

used in all experiments on laminin-1 described below. First, we studied the formation of focal contacts in CHO cells plated on laminin-1 by staining the cells with anti-vinculin antibodies. The distribution of vinculin was diffuse in the few mock-transfected cells that had spread on laminin-1 (Figure 5A). Expression of human wild-type $\alpha 6A$ in CHO cells induced the localization of vinculin into focal contacts (Figure 5C). Double-staining with phalloidin showed that these focal contacts were located as thin

streaks at the ends of, and sometimes along, the stress fibres (not shown). The distribution of vinculin remained diffuse in cells expressing truncated $\alpha 6(\Delta 1022)$ (Figure 5B), which, like the mock-transfected cells, spread only poorly. Ligand binding of $\alpha 6(\Delta 1022)\beta 1$ cannot be stimulated by PMA and formation of focal contacts cannot be induced by this integrin, since both inside-out and outside-in signalling via this mutant integrin are impaired [17]. All transfectants expressing $\alpha 6A$ with a mutation of the glycine, lysine or arginine residue of the GFFKR motif spread well on laminin-1 after PMA stimulation and vinculin was localized in focal contacts as in the wild-type $\alpha 6A$ -transfected cells (not shown).

The human $\alpha 6$ subunit was localized in the focal contact sites on laminin-1 in cells expressing the wild-type $\alpha 6A$ subunit (Figure 5E), $\alpha 6A(G1017A)$ (Figure 5F), $\alpha 6A(K1020L)$ (Figure 5G) or $\alpha 6A(R1021L)$ (Figure 5H). Double staining with phalloidin showed that $\alpha 6$, like vinculin, was localized in thin streaks along and at the ends of stress fibres. Transfection of cells with $\alpha 6A(\Delta KR)$, $\alpha 6A(K1020D)$ or $\alpha 6A(R1021D)$ cDNA also resulted in the formation of $\alpha 6\beta 1$ heterodimers that are able to initiate focal-contact formation on laminin-1 (not shown). In contrast, $\alpha 6(\Delta 1022)$ was not localized in focal contacts. Sections perpendicular to the substrate showed that $\alpha 6(\Delta 1022)$ was equally distributed over the cell surface (Figure 5D, inset), whereas wild-type $\alpha 6A$ (Figure 5E, inset) and all other mutants tested (not shown) were localized only at the basal side of the cells.

CHO cells plated on fibronectin spread fast and produced very pronounced focal contacts at the ends of stress fibres, as detected by vinculin staining (Figure 6A). A similar difference between vinculin-staining patterns in cells plated on laminin and fibronectin has been described previously for the human ovarian carcinoma cell line OVCAR-4 [29]. CHO cells transfected with the different $\alpha 6$ mutants showed a similar distribution of vinculin (not shown). Although fibronectin is not a ligand for $\alpha 6\beta 1$, $\alpha 6(\Delta 1022)$ was localized at the basal side of the cells, in focal contacts (Figure 6B). In contrast, wild-type $\alpha 6A$ and all other mutants were diffusely distributed over the cell surface (Figure 6C–F).

Thus mutations in the GFFKR motif that do not affect surface expression of $\alpha 6A$ do not interfere with the formation of $\alpha 6\beta 1$ -containing focal contacts on a laminin substrate either, and neither do they induce ligand-independent focal-contact localization.

DISCUSSION

In a previous study, we showed that the cytoplasmic domain of the integrin $\alpha 6$ subunit C-terminal of the GFFKR motif is not required for expression of $\alpha 6\beta 1$ at the cell surface, but that association of $\alpha 6$ with the $\beta 1$ subunit is impaired when the cytoplasmic domain of $\alpha 6$ is truncated immediately C-terminal of the transmembrane domain [17]. Here, we have demonstrated that the two phenylalanine residues present in the GFFKR motif are required for association of the $\alpha 6A$ subunit with $\beta 1$ and for membrane expression, whereas the other three amino acids are irrelevant. Mutation of these other three residues also did not affect bidirectional signalling by $\alpha 6\beta 1$.

When the cDNA encoding $\alpha 6A$ with a deletion of the GFFKR motif was transfected into COS-7 cells, no $\alpha 6\beta 1$ was found on the cell surface. Pulse-chase analysis of ^{35}S -labelled cells revealed that mutant $\alpha 6A$ was synthesized, but that it was not transported to the Golgi apparatus to undergo modification of its attached oligosaccharides, and that it did not associate with $\beta 1$. Various studies have provided evidence that single integrin subunits do

Figure 5 Immunofluorescence of CHO cells transfected with wild-type or mutant human $\alpha 6A$ and plated on laminin-1

Mock-transfected CHO cells (A) and CHO cells transfected with $\alpha 6(\Delta 1022)$ (B, D), wild-type $\alpha 6A$ (C, E), $\alpha 6A(G1017A)$ (F), $\alpha 6A(K1020L)$ (G) or $\alpha 6A(R1021L)$ (H) cDNA were plated on laminin-1 (20 $\mu g/ml$)-coated coverslips in serum-free medium in the presence of 5 ng/ml PMA and 25 $\mu g/ml$ cycloheximide and allowed to spread for 3 h. Cells were examined by immunofluorescence using anti-vinculin (A–C) or anti-(human $\alpha 6$) antibodies (D–H). In (D) and (E) sections through the cells perpendicular to the substrate are shown. Bar, 10 μm .

not leave the ER because of retention signals present in the extracellular domain of α subunits and in the cytoplasmic or transmembrane domain of β subunits [21,30]. The retention signals are inactivated when heterodimers are formed, which are then transported to the Golgi apparatus, where their oligosaccharide side chains are further modified.

Our results confirm those of many other studies showing that the GFFKR motif is required for subunit association and membrane expression of $\beta 1$, $\beta 2$ and $\beta 3$ integrins. Briesewitz et al.

Figure 6 Immunofluorescence of CHO cells transfected with wild-type or mutant human $\alpha 6A$ and plated on fibronectin

Mock-transfected CHO cells (A) and CHO cells transfected with $\alpha 6(\Delta 1022)$ (B), wild-type $\alpha 6A$ (C), $\alpha 6A(G1017A)$ (D), $\alpha 6A(K1020L)$ (E) or $\alpha 6A(R1021L)$ (F) cDNA were plated on fibronectin (20 $\mu g/ml$)-coated coverslips in serum-free medium in the presence of 25 $\mu g/ml$ cycloheximide and allowed to spread for 3 h. Cells were examined by immunofluorescence using anti-vinculin (A) or anti-human $\alpha 6$ antibodies (B–F). In (B) and (C) sections through the cells perpendicular to the substrate are shown. Bar, 10 μm .

[13] showed that chimaeric $\alpha 1$ and $\beta 1$ subunits in which the cytoplasmic domains were exchanged only associated with each other and not with wild-type $\alpha 1$ or $\beta 1$. The presence of the GFFKR region of $\alpha 1$ was sufficient for this association, and the cytoplasmic domain of $\alpha 1$ C-terminal of this region and the $\beta 1$ transmembrane domain were not necessary for stable subunit association. These data suggested that the cytoplasmic domains are required for heterodimer formation and that GFFKR associates with sequences in the cytoplasmic domain of the β subunit. However, the role of the individual amino acids in the GFFKR motif was not studied. Surface levels on CHO cells of mutant $\alpha 5\beta 1$ which lacked the $\alpha 5$ cytoplasmic domain including the GFFKR motif were low compared with that of wild-type $\alpha 5\beta 1$ [20]. In the case of αL , various truncated mutants were generated, one of which lacked the cytoplasmic domain including the GFFKR motif. This mutant formed heterodimers with $\beta 2$ in double-transfected COS-7 cells, but, in spite of this association, the complexes were retained in the ER and rapidly degraded, which indicated that in this case GFFKR is essential for the stability of the integrin [21]. With $\alpha IIb\beta 3$, results similar to those for $\alpha 6\beta 1$ described in this paper were obtained. When the entire GFFKR motif was deleted or replaced by five alanine residues, the surface levels on transfected lymphocytes were much lower than those of wild-type $\alpha IIb\beta 3$ because of impaired intracellular assembly of heterodimers [31]. In contrast, the $\alpha 6$ subunit of which the cytoplasmic domain, including the GFFKR motif, was deleted, did form heterodimers with the $\beta 4$ subunit. However, surface levels of the mutant $\alpha 6\beta 4$ integrin were low, because of

an increased turnover of the complex [17]. Thus, for most integrins, the GFFKR motif appears to be essential for subunit association. Probably, an interaction occurs between this motif and a relatively well-conserved membrane-proximal region, LLviXhDRRE (the less conserved residues are in lower case, X is not conserved), present in all β subunits except $\beta 4$ and $\beta 8$ [6], but the exact nature of this interaction is not known. Here, we show that the phenylalanines are essential for the assembly and membrane expression of $\alpha 6\beta 1$. This suggests that interaction of $\alpha 6$ with $\beta 1$ is determined by hydrophobic bonds between the non-polar residues of the conserved membrane-proximal regions in these subunits.

In contrast, Hughes et al. [32] suggested the presence of a salt bridge between the positively charged arginine residue in the αIIb subunit and the negatively charged aspartic acid residue in the conserved membrane-proximal domain of the $\beta 3$ subunit. Indeed, substitution of this aspartic acid with arginine resulted in a constitutively active receptor, but an integrin made up of this mutant $\beta 3$ with a mutant αIIb in which the arginine was replaced by aspartic acid, a 'charge-reversal' mutant dimer, was inactive. It was concluded that the interaction between these residues maintains the low-affinity state of the integrin, and that disruption leads to activation. Furthermore αIIb or $\beta 3$ subunits which contain activating mutations in the membrane-proximal domain were recruited to focal contacts formed on fibronectin by endogenous integrins in CHO cells, whereas co-expression of the α and β charge-reversal mutants did not result in ligand-independent localization in focal contacts.

Although the experiments described in this paper do not exclude the presence of such a salt bridge, the replacement of the arginine in $\alpha 6A$ by leucine or aspartic acid did not result in a constitutively active receptor in transfected K562 cells. It is unlikely that this difference is due to the cell line used, since, also in K562 cells, a mutant of αIIb lacking the GFFKR motif was constitutively active when co-expressed with $\beta 3$ [14]. Furthermore $\alpha 6A(R1021L)$ and $\alpha 6A(R1021D)$ expressed in CHO cells were normally localized in focal contacts on laminin, but not on fibronectin, indicating that these mutations did not impair the outside-in signalling via $\alpha 6\beta 1$ either.

We have previously shown that the cytoplasmic domain C-terminal of the GFFKR motif of the $\alpha 6$ subunit ($\Delta 1022$) is essential for inside-out signalling by $\alpha 6\beta 1$ [17]. Here, we show that $\alpha 6(\Delta 1022)\beta 1$ cannot induce the formation of focal contacts on laminin, but that this mutant integrin is targeted to focal contacts formed by other integrins. This indicates that outside-in signalling is also impaired, as has also been shown for similar truncated mutants of $\alpha 1$, $\alpha 2$ and αIIb [33–35]. Although addition of four to seven amino acid residues C-terminal of the GFFKR motif of the $\alpha 2$ or $\alpha 4$ subunit was sufficient to restore signalling events [19,34], the adhesive strength and specificity of the response of an integrin induced by ligand binding depends on the sequence of the cytoplasmic domain of the α subunit [36,37].

The phenylalanines may constitute an important site for regulation of integrin function. Since they are required for the interaction between α and β subunits, binding of regulatory proteins to this region may alter the relationship between the two subunits and thus change the conformation of the integrin. Alternatively, they may be important for regulation of the adhesiveness of cells by modulating the level of expression at the cell surface. When they are masked, heterodimers cannot be formed and the α and β chains are retained in the ER as monomeric subunits.

In conclusion, the cytoplasmic domain of $\alpha 6A$ is involved in the association with $\beta 1$ and in the regulation of bidirectional signalling via $\alpha 6\beta 1$. The experiments described in this paper show that the two phenylalanines within the GFFKR motif of the $\alpha 6A$ subunit are crucial for integrin-subunit association and therefore for transport to the plasma membrane. It is possible that they are, together with (sequences in) the cytoplasmic domain C-terminal of the GFFKR motif, essential for integrin signalling.

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