Down-regulation of $\alpha v/\beta 3$ integrin via misrouting to lysosomes by overexpression of a $\beta 3$ Lamp1 fusion protein

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We present a general strategy for the dominant negative reduction in the levels of type-I membrane-bound heterodimeric proteins within the secretory pathway through fusion of the soluble ectodomain of one of the partners to the transmembrane-cytosolic tail of the lysosomal protein Lamp1. Thus, in human embryonic kidney (HEK)-293 cells, overexpression of an integrin $\beta 3$ Lamp1 chimera resulted in a drastic reduction of its endogenous partner, the integrin αv subunit. The mechanism involves the formation in the endoplasmic reticulum of a $\alpha v/\beta 3$ Lamp1 complex that is subsequently sorted towards a lysosomal/endosomal degradation pathway. The specificity of this approach is afforded by the invariance in the levels of the

endogenous integrins $\alpha 5$ and $\beta 1$ as compared with control cells. Conversely overexpression of integrin $\beta 3$ in HEK-293 cells led to an increased level of $\alpha v/\beta 3$ at the cell surface. Functionally $\beta 3$ Lamp1 and $\beta 3$ overexpressors exhibit decreased and increased adhesion to vitronectin, respectively, as well as diminished cellular aggregation. The application of this technology should enable the analysis of the functional importance of homodimers or heterodimers in the cell types of choice and the identification of novel partner proteins by proteomic approaches.

Key words: cellular mis-sorting, dominant negative, lysosomal degradation.

INTRODUCTION

With the advent of the genome project, sequences of a growing number of gene products of unknown or unsuspected function are being identified. Of the various tools devised to define the biological activity of novel proteins, suppression of gene expression is one of the most powerful. Transcriptional suppression may be obtained by: (i) homologous recombination resulting in the production of homozygote null cells and/or animals in the gene of interest or, (ii) oligonucleotides directing triple-helix formation at *cis*-acting elements of gene promoters and inhibiting gene transcription (reviewed in [1–4]). Some of the approaches used in translational suppression involve: (i) DNA- and RNA-based antisense molecules, (ii) RNA enzymes (ribozymes), as well as (iii) RNA interference (RNAi) (reviewed in [5–9]). At the post-translational level, the methods generally involve overexpression of dominant negative forms of proteins, truncated or mutated, resulting in the disruption of the activity of the wild-type protein (reviewed in [10–12]).

An alternative approach, not requiring extensive structural or functional knowledge of the targeted protein, was recently reported [13,14]. This post-translational strategy results in the reduction in the levels of homo/heterodimeric proteins within the secretory pathway through fusion of one of the partners to a lysosomal targeting signal. Thus cellular expression of chimeras, consisting of either the zymogen of a lysosomal hydrolase (e.g. cathepsin B [14,15]) or lysosomal targeting domains (e.g. Lamp1 [13]) fused to the N- or C-terminus of the soluble sequences, respectively, results in the lysosomal degradation of their partner(s). Fusion of the lysosomal protease cathepsin B to: (i) the cytosolic transcription factor MyoD resulted in the knockout of this homodimeric protein [13], or to (ii) the peptide 7B2 [14],

resulted in the elimination of its partner the proprotein convertase PC2. Fusing the ectodomain of the CD4 receptor to the lysosomal targeting signal of Lamp1 results in rerouting and degradation *in trans* of its ligand, the HIV gp160 viral surface glycoprotein [13].

Lamp1 is a type I transmembrane glycoprotein that is localized primarily in lysosomes and late endosomes. Newly synthesized molecules are mostly transported from the *trans*-Golgi network (TGN) directly to endosomes and then to lysosomes. A minor pathway involves transport via the plasma membrane [16]. The 11-amino-acid cytoplasmic tail of Lamp1 contains a tyrosine-based motif Y-X-X-I that has been shown to mediate sorting in the *trans*-Golgi network and rapid internalization at the plasma membrane. The Y-X-X-I sorting motif is sufficient to confer proper intracellular targeting and the spacing of this motif relative to the membrane is critical for proper lysosomal targeting [16].

Integrins are heterodimeric cell surface receptors implicated in cell–cell and cell–matrix interactions [17]. More than 15 α and 10 β different subunits are known, resulting in the formation of more than 25 functional $\alpha\beta$ heterodimers. Both α (120–180 kDa) and β (90–180 kDa) subunits are type I transmembrane proteins containing a generally short cytoplasmic tail that regulates their interaction with the cytoskeleton and signalling molecules. Heterodimerization occurs in the endoplasmic reticulum (ER) and the complex is transported to the cell surface via the constitutive secretory pathway. Association of α and β subunits, which determines the binding specificity of the receptor, is not random and occurs between specific members. For example, while $\beta 3$ exclusively associates with αv and αIIB , $\beta 1$ dimerizes with $\alpha 1$ to $\alpha 11$ or αv subunits.

In this study, we present an extension of the above dominant negative post-translational method to integrin heterodimeric

Abbreviations used: BFA, brefeldin A; CB, cathepsin B; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FACS, fluorescence activated cell sorting; FCS, fetal calf serum; G418, geneticin; HEK, human embryonic kidney; mAb, monoclonal antibody; PE, phycoerythrin; RNAi, RNA interference; TGN, *trans*-Golgi network; TM-CT, transmembrane-cytosolic tail.

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proteins. As a model oligomeric protein we chose the integrin $\alpha v\beta 3$, overexpressed a $\beta 3$ Lamp1 chimera in human embryonic kidney (HEK)-293 cells, and studied the fate of the associated αv chain. The results demonstrate the general feasibility of this post-translational approach towards the silencing of heterodimeric protein expression.

EXPERIMENTAL

Cell culture

HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Emerainville, France) supplemented with 10% fetal calf serum (FCS; BioWhittaker). For each experiment, cells were detached by treatment of subconfluent cell monolayers with 0.53 mM EDTA, 0.05% trypsin.

Transient and stable transfectants

The $\beta 3$ Lamp1 protein was obtained by fusion of the N-terminal human integrin $\beta 3$ to the transmembrane-cytosolic tail (TM-CT) of human Lamp1, a lysosomal protein (Figure 1). Wild-type $\beta 3$ integrin-subunit and the chimera $\beta 3$ Lamp1, both cloned into the pIRES2-EGFP vector (Clontech Laboratories, Erembodegem, Belgium) or empty vector were transfected into HEK-293 cells using EffecteneTM reagent (Qiagen, Courtaboeuf, France). Typically, cells grown to 30–40% confluence in 35-mm dishes, were transfected with 0.5–1 μ g of DNA at a 1:10 DNA:Effectene ratio according to the manufacturer's instructions. Transfection efficiency, measured by fluorescence activated cell sorting (FACS) analysis of enhanced green fluorescent protein (EGFP) expression, was better than 50%. After 18 h the medium was replaced with DMEM with 10% FCS, in the case of transient transfections, or the same medium containing 0.8 μ g/ml geneticin (G418) for the isolation of stable transfectants. After selection for resistance to G418, stable transfectants were sorted by FACS for high EGFP expression.

Lamp1 cytosolic tail polyclonal antibody

The peptide CGGRKRSHAGYQTI representing the 12 C-terminal residues of Lamp-1, plus an N-terminal cysteine for coupling, and a glycine spacer residue, was prepared by solid-phase synthesis with Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry. An ovalbumin-peptide conjugate was prepared with the bifunctional reagent, *N*-hydroxysuccinimidylbromoacetate and used for rabbit immunization. Conjugation, immunization and characterization of the resulting antiserum was carried out

Figure 1 Construction of the $\beta 3$ Lamp1 chimera

Schematic representation of integrin $\beta 3$, the lysosome associated membrane protein Lamp1 and a $\beta 3$ Lamp1 chimera consisting of the signal peptide (SP) and the ectodomain of $\beta 3$ fused to the transmembrane (TM) and cytoplasmic (CT) segments of Lamp1.

as described previously [18]. Affinity-purified immunoglobulin was prepared using a Sulfolink column (Pierce Rockford, IL, U.S.A.) substituted with the above peptide. The C-terminal Lamp-1 sequence is completely conserved between human, bovine, chicken, hamster, mouse, and rat.

Metabolic cell labelling, pulse-chase experiments and immunoprecipitation

After cotransfection of HEK-293 cells 48 h earlier, and plating on collagen type-I (Upstate Biotechnology) with recombinant pIRES2-EGFP bicistronic vectors encoding for αv and $\beta 3$ Lamp1 or αv and $\beta 3$ as control, cells were washed and pre-incubated for 2 h at 37 °C in methionine- and cysteine-deficient DMEM. Cells were then pulse-labelled for 30 min at 37 °C in the same medium containing 150 μ Ci $\cdot$ ml⁻¹ of Tran³⁵S-labelTM (Amersham Biosciences, Orsay, France). After washing, cells were chased for 5 h and 10 h in culture medium containing 10 mM methionine, 2 mM cysteine and 10% FCS in the presence or absence of either 10 μ g/ml of the fungal metabolite brefeldin A (BFA) or 20 mM NH₄Cl. After the pulse or the chase periods, cells were scraped into a lysis buffer consisting of 50 mM Tris/HCl, pH 7.8, 150 mM NaCl, 2 mM EDTA, and 1% (v/v) Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, plus a mixture of proteinase inhibitors ALI/POP (1 mM PMSF, 500 units/ml aprotinin, 1 μ g/ml leupeptin, 1 μ M pepstatin, 1 mM iodoacetamide and 1 mM ortho-phenanthroline) and clarified by centrifugation for 10 min at 15000 g. Three equal parts of the supernatant were immunoprecipitated during 18 h at 4 °C with either 5 μ g of anti αv - (AMF7; Beckman Coulter, Cassina De' Pecchi, Italy) or $\beta 3$ - (SZ21; Beckman Coulter) or $\beta 1$ -antibody (K20; Beckman Coulter). Immunoprecipitates were then selected by rollover shaking with protein G-agarose beads for 2 h at 4 °C. After washing, immunoprecipitates were eluted in non-reducing SDS/PAGE sample buffer and analysed by electrophoresis on 7% polyacrylamide gels. Gels were then fixed in methanol/acetic acid/water (40:10:50, v/v/v), impregnated with Amplify (Amersham), dried and exposed to Kodak X-O-Mat AR films at -80 °C. Quantification of band intensity in the scanned gels was performed with the NIH Image 1.62 software (<http://rsb.info.nih.gov/nih-image/about.html>).

Flow-cytometry analysis of cell surface integrin expression

Detached cells were resuspended and incubated for 1 h at 22 °C, in DMEM containing 0.1% BSA and 10 μ g/ml of the following primary antibodies against integrin subunits: 69-6-5 (anti- $\alpha 3$ prepared in our laboratory; [19]) and AMF7 (anti- αv), K20 (anti- $\beta 1$), SZ21 (anti- $\beta 3$), Sam1 (anti- $\alpha 5$; Beckman Coulter), G0H3 (anti- $\alpha 6$; Beckman Coulter). After washing, cells were incubated with phycoerythrin (PE)-labelled goat anti-mouse IgG for 2 h at 4 °C. Cells were washed and analysed using a FACSCAN apparatus (Becton Dickinson, Mountain View, CA, U.S.A.). Controls that included either isotype-matched antibodies or no primary antibody, and a PE secondary antibody yielded a mean fluorescence intensity below 0.7 arbitrary units of fluorescence in 99% of gated cells (results not shown).

Analysis of αv content

Analysis of αv content was performed either after immunoprecipitation with αv antibodies, as described above, or in membrane extracts. In the latter case, cells detached with EDTA were washed twice in a 0.05 M Tris/HCl, pH 7.2, 0.35 M sucrose buffer and permeated with the same solution containing 0.05% digitonin plus the ALI/POP mixture of proteinase inhibitors.

Samples were clarified by centrifugation for 10 min at 2000 *g*, the pellet was washed with the Tris/sucrose buffer and membrane proteins were solubilized for 15 min at 4 °C with the same solution containing 1% *N*-octylglucoside plus ALI/POP. Samples were clarified by centrifugation for 10 min at 15000 *g*. Protein content in the supernatant was measured by the Bradford method. Expression of α_v was analysed after electrophoresis by SDS/PAGE (7% gels) under reducing conditions and electro-transfer onto nitrocellulose (Hybond C+; Amersham). After saturation for 30 min at 22 °C with 50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 0.1% Tween 20, 5% non-fat dry milk, the nitrocellulose membrane was incubated with primary monoclonal antibody (mAb) 1960 (anti- α_v ; Chemicon International, Temecula, CA, U.S.A.) overnight at 4 °C. Bound antibodies were detected by a horseradish peroxidase-conjugated secondary antibody and revealed with a chemiluminescence system (Pierce, Rockford, IL, U.S.A.).

Adhesion assays

Adhesion substrates were prepared by coating flat-bottom 96-well microtitre plates overnight at 4 °C with 50 μ l of vitronectin (prepared in our laboratory) or laminin (Sigma) at various concentrations. Coated wells were then blocked with 1% BSA in PBS for 30 min. Cells were detached and resuspended as a single cell suspension after washing once with DMEM containing 0.1% BSA (adhesion buffer). Cells (30000 cells in 50 μ l) were added to each well and allowed to adhere to the substrata for 1 h at 37 °C in a cell culture incubator. Unattached cells were removed by gently washing twice with adhesion buffer and attached cells were fixed with 1% glutaraldehyde in PBS. After staining by 0.1% crystal violet in water and washing, cells were lysed with 1% SDS. The absorbance was measured at 600 nm with a microplate reader.

Cell-aggregation assays

For slow adhesion-independent assay, 100 mg of bacto-agar were dissolved in 15 ml of Ringer saline solution (6 g/l NaCl, 0.075 g/l KCl, 0.1 g/l CaCl₂, and 0.1 g/l NaHCO₃). After heating, flat-bottom 96-well microtitre plates were coated with 100 μ l of this solution and incubated for 1 h at 4 °C. Cells were detached and resuspended as a single cell suspension. Cells (2×10^4 cells/100 μ l DMEM, 10% FCS) were added to each well and allowed to aggregate for 48 h at 37 °C in a cell culture incubator. Aggregation was observed by microscopy (4 $\times$).

For adhesion- and time-dependent assay, 24-mm glass slides were incubated in H₂SO₄, washed and coated with 3-aminopropyl-triethoxysilane (Sigma). After washing, the slide surface was divided into four equal sectors with a hydrophobic track-leaving pen and incubated for 20 min in 10 mM borate, pH 8.5, 0.25% glutaraldehyde. After washing with PBS, sectors were coated overnight at 4 °C with adhesion substrates: collagen type-I (Sigma), vitronectin, fibronectin (Chemicon) or BSA (20 μ g/ml). Then all sectors were blocked with PBS/BSA (0.1% w/v), and the slide was transferred to an incubation chamber which was filled with a single suspension of cells in DMEM, 10 mM Hepes, 10% FCS. After sealing, the chamber was transferred to the thermostatic motorized stage of a computerized microscope (DMIRBE; Leica Microsystems GmbH, Mannheim, Germany) enabling time-lapse video microscopy (10 $\times$) by taking pictures of the same fields every 15 min for 14 h. In a single frame, the number of cells, which could be individualized, was counted at the beginning and end of the experiment. Three different fields, in sectors coated by the same adhesion substratum, were analysed.

RESULTS

β 3Lamp1 associates with α_v and the complex is degraded in a post ER/Golgi acidic compartment

We explored the possibility of reducing the levels of an integrin when one of the partners of the heterodimer is expressed as an N-terminal protein fused to the TM-CT of Lamp1 (Figure 1). We selected the integrin β 3 subunit as a decoy partner for α_v since they form a unique pair in most cells, normally devoid of the alternate β 3 partner α IIb that is expressed mainly in platelets [20]. In addition, our choice to construct a β 3, rather than an α v chimera, was inspired by the fact that although the $\alpha_v\beta$ 3 complex is favoured, α_v binds four other β subunits including β 1, β 5, β 6 and β 8 [21]. Since the distance of the C-terminus of Lamp1 to the TM seems critical for lysosomal/endosomal targeting efficiency [16], we opted to keep both the TM and CT of Lamp1, which were fused to the signal peptide-ectodomain of β 3 (Figure 1). The latter contains all the structural determinants needed for efficient ER translocation and α_v association [22].

We selected *de novo* biosynthesis as the method of choice to test the temporal association of β 3Lamp1 with α_v . We transiently transfected HEK-293 cells with our constructs, since these cells can be transfected with high efficiency and endogenously express relatively low levels of α_v and β 3 (pulse-labelling, Western-blot, and FACS analyses; results not shown). Therefore we cotransfected HEK-293 cells with vectors coding for α_v and either β 3 or β 3Lamp1. Post-transfection (48 h), the cells were pulse-labelled for 30 min with a Translabel mixture of [³⁵S]methionine and

Figure 2 α_v Associates with β 3Lamp1 and the complex is degraded in a TGN or a post-TGN compartment

HEK-293 cells were pulse labelled, 36 h after cotransfection with vectors coding for α_v and β 3 integrin subunits or α_v and β 3Lamp1, with [³⁵S]Methionine and [³⁵S]Cysteine for 30 min and chased for 0, 5 and 10 h in the absence (0) or presence of 10 μ g/ml of brefeldin A (BFA). Lysates were immunoprecipitated with an (A) anti- α_v or (B) anti- β 1 mAb. Immunoprecipitates selected by affinity for protein-G beads were analysed by non-reducing SDS/PAGE (7% gel) and fluorography. Arrows in (A, left-hand panel) point to ER- and Golgi-associated forms of α_v after α_v/β 3 transfections and arrowheads in (A, right-hand panel) point to fast migrating degradation products after α_v/β 3Lamp1 transfections.

Figure 3 Degradation of the $\alpha v/\beta 3$ Lamp1 complex is attenuated by treatment with NH_4Cl

HEK-293 cells, 36 h after cotransfection with vectors encoding either the integrin subunits αv and $\beta 3$ ($\alpha v + \beta 3$), or αv and $\beta 3$ Lamp1 ($\alpha v + \beta 3$ Lamp1), or pIRES2-EGFP (Empty Vector), were pulsed with Tran^{35}S -labelTM (Amersham) for 30 min and chased for 2 h or 10 h in absence (0) or presence (+) of 20 mM NH_4Cl . Equal volumes of cell lysates were immunoprecipitated (IPP) with (A) anti- $\beta 3$ mAb ($\beta 3$) or (B) polyclonal antibody to the Lamp1 cytoplasmic tail (Lamp1). Immunoprecipitates were analysed by non-reducing SDS/PAGE (7% gel) and fluorography.

Figure 4 Dominant negative effect of $\beta 3$ Lamp1 on the endogenous level of integrin αv

Membrane extracts of stable high expressor HEK-293 cell pools of pIRES2-EGFP (pIRES), $\beta 3$ Lamp1 and $\beta 3$ were prepared by N-octylglucoside solubilization of cells after permeation with digitonin. Western-blot analysis, following separation by reducing SDS/PAGE (7% gel) was performed with an anti- αv mAb 1960.

[^{35}S]cysteine and chased for 2, 5 and 10 h (Figure 2). Cell lysates were immunoprecipitated with either αv (Figure 2A), $\beta 3$ (results not shown) or $\beta 1$ (Figure 2B) specific mAb and immuno-

precipitates were analysed by SDS/PAGE followed by fluorography. At all times αv and $\beta 3$ were found to co-immunoprecipitate in the $\alpha v/\beta 3$ cotransfectant. Following a 5 h chase, we noted an accumulation of the slowly migrating Golgi-associated form of αv (Figure 2A) that is dominant at the 10 h chase point. At this latter chase time, we also noticed a slight decrease in the level of the $\alpha v/\beta 3$ complex, possibly due to its cellular turnover. Indeed no change in the level of the complex was detected at any chase time in the presence of the fungal metabolite BFA that causes the fusion of the *cis*-, medial- and *trans*-Golgi compartments (but not the TGN) with the ER [23]. This suggested that the observed slight decrease in the level of $\alpha v/\beta 3$ took place in either the TGN or a post-TGN compartment(s). In the case of the $\alpha v/\beta 3$ Lamp1 transfectant, we also noted that αv and $\beta 3$ Lamp1 co-immunoprecipitated. In contrast to the $\alpha v/\beta 3$ transfectant, two immunoreactive αv forms could be distinguished at all times. We interpret these as ER- (lower band) and Golgi- (upper band) associated forms [24]. Within the resolution of the SDS/PAGE system (8% gel) used, such a separation was more evident for the αv subunit exhibiting 13 N-glycosylation sites as opposed to only 5 such sites in $\beta 3$. While the levels of the ER-form of αv generally diminished over the chase periods for both $\alpha v/\beta 3$ and $\alpha v/\beta 3$ Lamp1 complexes, only for the latter do we observe a drastic diminution at the 10 h chase point (> 90%). Concomitantly, lower-molecular-mass cleavage products (open arrows, Figure 2A) were detected at the 5 h and 10 h chase periods. Here again, BFA treatment abolished these effects at all chase times (Figure 2A), suggesting that degradation takes place in either the TGN or a post-TGN compartment(s).

These results were confirmed by immunoprecipitations with a mAb to the $\beta 3$ subunit ectodomain (Figure 3A) or a polyclonal antibody against the Lamp1 cytoplasmic tail (Figure 3B). Levels of endogenous $\alpha v/\beta 3$ heterodimers were undetectable (Figure 3A, 'Empty Vector'). In cells transfected with cDNAs coding for αv and $\beta 3$, after 2 h of chase, high levels of synthesized heterodimers were found with anti- $\beta 3$ mAb, which remained unchanged over the 10 h chase period (Figure 3A, ' $\alpha v + \beta 3$ '). These species were not detected by anti-Lamp1 Ab as expected (Figure 3B, ' $\alpha v + \beta 3$ '). In αv and $\beta 3$ -Lamp1 transfectants, high levels of heterodimers were found at the 2 h chase point with both anti- $\beta 3$ and -Lamp1 Abs and 90% of produced complexes disappeared by 10 h of chase (Figures 3A and 3B, ' $\alpha v + \beta 3$ Lamp1'). It was also found that cell treatment with 20 mM NH_4Cl , known to neutralize acidic compartments [25,26], almost completely prevented the degradation of the $\alpha v/\beta 3$ Lamp1 heterodimer at the 10 h chase period (Figures 3A and 3B, ' $\alpha v + \beta 3$ Lamp1', ' NH_4Cl '). These data strongly suggest that degradation occurs in an acidic compartment.

In order to assess whether the observed degradation is selective to $\alpha v/\beta 3$ Lamp1, we sought to verify the fate of endogenous $\beta 1$ -containing integrins, which should remain unaffected by this process. Indeed in the absence of BFA, when the same cell lysates were immunoprecipitated with a $\beta 1$ -specific antibody, no detectable degradation of $\alpha v/\beta 1$ complexes was observed at all chase times (Figure 2B). Altogether the data suggest that αv associates with $\beta 3$ Lamp1, and that the complex is sorted to an acidic post-TGN compartment, possibly the lysosome, where degradation takes place, and that this process is limited to the above integrin pair.

Endogenous αv levels are significantly reduced in HEK-293 cells stably transfected with $\beta 3$ Lamp1

To explore the dominant negative effects of $\beta 3$ Lamp1 on endogenous αv , stable transfectants in HEK-293 cells of

Figure 5 Suppression of α_v cell surface expression in $\beta 3$ Lamp1 overexpressors

HEK-293 cell suspensions of pIRES2-EGFP, $\beta 3$ Lamp1 and $\beta 3$ stable pools were successively incubated with anti- α_v or isotype-matched control mAbs and a phycoerythrin (PE)-conjugated anti-mouse antibody. FACS analysis of cell surface α_v (left-hand panels). Note that the mean fluorescence intensity of 1.6 in 57.2% of $\beta 3$ Lamp1 cells (central panel) is close to background (0.7, see Materials and methods). FACS analysis of cell surface α_v (y-axis) as a function of EGFP expression (x-axis) (right-hand panels). The percentage and mean fluorescence intensity of cells fall into four categories: cells double negative for α_v and EGFP expression (R4), single positive for α_v and negative for EGFP (R2), double positive for both α_v and EGFP (R3) and cells expressing EGFP but no α_v (R5). Note that, in $\beta 3$ Lamp1 transfectants (central panel), cell surface expression of α_v is lost in high EGFP expressors (R5).

Figure 6 Underexpression of αv subunit in $\beta 3\text{-Lamp1}$ transfectants is associated with low adhesion to vitronectin while $\alpha 2\beta 1$ -dependent adhesion to collagen type-1 remains unaffected

Single cell suspensions of pIRES (open circles), $\beta 3\text{Lamp1}$ (open squares) and $\beta 3$ (filled squares) HEK-293 cell lines were seeded in 96-well culture dishes (3×10^4 cells/well) previously coated with increasing vitronectin (A) or collagen type-1 (B) concentrations. The levels of the attached cells were estimated by crystal violet coloration (600 nm). The means $\pm$ S.D. of quadruplicate determinations are shown.

$\beta 3\text{Lamp1}$ cDNA inserted in the bicistronic vector pIRES2-EGFP were obtained. These were compared to similar transfectants obtained with either an empty pIRES2-EGFP vector or one expressing the wild-type $\beta 3$ integrin subunit. To this end, after selection for neomycin resistance, the cells were sorted by flow cytometry for high EGFP fluorescence, and consequently high integrin transgene expression. The level of total endogenous αv in these high expression pools was first analysed directly by Western blotting with a αv mAb of total cell lysates (membrane

extracts) (Figure 4). Quantitative analysis of the data (obtained in duplicate) revealed that total αv levels are reduced by approx. 4-fold in the $\beta 3\text{Lamp1}$ pool and increased by approx. 2-fold in the $\beta 3$ pool, as compared with the levels of αv in the control pIRES2-EGFP pool.

FACS analysis was used to estimate the cell-surface expression of αv in the above transfectants (Figure 5). In agreement with the Western-blot results (Figure 4), the overall mean cell-surface immunoreactivity of αv was also reduced by approx. 3-fold in the $\beta 3\text{Lamp1}$ pool as compared to the control pIRES2-EGFP pool (Figure 5, left-hand panels). Within the $\beta 3\text{Lamp1}$ pool, only 43 % of the cells still expressed significant levels of αv at the cell surface at a mean level of approx. 71 % (15/21) of control, while the rest of the pool showed virtually no αv at the cell surface. In $\beta 3$ -overexpressing cells, we noted a 3-fold (63/21) increase in cell surface αv , a value that is significantly higher than the one deduced from Western-blot analysis (approx. 2-fold). This suggests the presence of an intracellular pool of αv that is recruited to the cell surface by overexpressed $\beta 3$. Analysis of αv cell-surface expression, as a function of EGFP levels within the various pools, revealed heterogeneous cell populations (Figure 5, right-hand panels). All the pools revealed the presence of αv -positive but EGFP-negative populations (left-hand side upper quadrant R2). This observed heterogeneity was also recapitulated following reselection for high EGFP expression, suggesting that it is related to a stochastic cellular drift. In control cells, the level of cell surface αv remains constant, independent of EGFP expression. In contrast, αv is either drastically reduced or increased as a function of EGFP expression in the $\beta 3\text{Lamp1}$ and $\beta 3$ pools, respectively. In the $\beta 3\text{Lamp1}$ pool, the levels of αv gradually diminish, reaching undetectable levels once a critical threshold level of EGFP has been achieved. This strongly suggests a competition of $\beta 3\text{Lamp1}$ with endogenous β partners.

Similar FACS analysis of the $\beta 3$ subunit immunoreactivity showed low expression in pIRES2-EGFP control cells and in $\beta 3\text{Lamp1}$ transfectants, but a 60-fold increase in the $\beta 3$ pool (results not shown). This argues for a minor contribution of endogenous $\beta 3$ to the cell surface $\alpha v\beta$ complexes, probably due to its limiting level in HEK-293 cells. Finally, FACS analysis demonstrated constant levels of $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$ and $\beta 1$ immunoreactivities in all pools, indicating that overexpression of either EGFP, $\beta 3$ or $\beta 3\text{Lamp1}$ does not alter the levels of these integrins.

Functional consequences of stable $\beta 3\text{Lamp1}$ expression in HEK-293 cells

Altogether the above data demonstrated that overexpression of $\beta 3\text{Lamp1}$ led to a very significant reduction in the level of endogenous cell surface αv integrin and that this effect was most prominent in high expressors (Figure 5). Conversely, cell surface $\alpha v\beta 3$ integrin level was highly stimulated in $\beta 3$ overexpressors. In order to assess the functional consequences of these observations, we compared the adhesion of these transfectants on vitronectin, a $\alpha v\beta 3$ ligand [27]. As compared to control cells, the ability to bind vitronectin was enhanced or reduced for $\beta 3$ or $\beta 3\text{Lamp1}$ transfectants, respectively (Figure 6A). These results are consistent with the observed cell surface levels of $\alpha v\beta 3$ in these transfectants (Figure 5). Further proof of the specificity of these observations was obtained by the analysis of cellular attachment to collagen type-1, a specific ligand for $\alpha 2\beta 1$ integrin, whereby all transfectants equally attached this substrate (Figure 6B). Similar results were obtained with fibronectin and laminin, specific ligands for $\alpha 5\beta 1$ and $\alpha 6\beta 1/\beta 4$ integrins, respectively (results not shown) [17,28,29]. In a similar vein, time-lapse video microscopy revealed that haptotactic migration on vitronectin

Figure 7 Increased cell aggregation of β 3Lamp1 stable transfectants

In all cases pools of β 3Lamp1 (β 3Lamp) and β 3 stable HEK-293 transfectants were observed by microscopy and the experiment repeated in triplicate or quadruplicate. **(A)** Adhesion-independent aggregation assay. Single cell suspensions of pIRES, β 3Lamp1 (β 3Lamp) and β 3 HEK-293 cell lines, obtained as described in Figure 4, were seeded (2×10^4 cells/100 μ l DMEM, 10% FCS) in flat-bottom 96-well microtitre plates coated with bacto-agar. After incubating for 48 h at 37 °C in a cell culture incubator, cultures were observed by microscopy (4 $\times$ magnification; scale bars, 500 μ m). **(B)** Adhesion- and time-dependent assay. Single cell suspensions of β 3Lamp1 (β 3Lamp) and β 3 (β 3) HEK-293 stable transfectant cell lines were seeded in an incubation chamber, the bottom glass slide of which was divided into four sectors coated with either BSA, vitronectin, fibronectin or collagen type-I. The same three fields in each of the sectors were examined at 10 $\times$ magnification (scale bars, 20 μ m) every 15 min for 14 h, using a computerized Leica microscope equipped with a thermostatic motorized stage and digital camera. Pictures of β 3Lamp1 and β 3 cells on vitronectin substrate of the same field taken at the beginning (0 h) and the end of the experiment (14 h). **(C)** Quantification of cell compaction in adhesion-dependent aggregation assays. In each of the examined fields, the number of cells whose contours could be determined with certainty was determined at the time of plating and 14 h later. Changes (%) in cell number were calculated [(number of cells at the end/number of cells at the beginning) - 1] $\times$ 100. The values shown correspond to the mean $\pm$ S.D. of three independent determinations. All values in pIRES cells differed significantly ($P < 0.01$, Student's *t*-test) from values in either β 3Lamp1 or β 3 cells, except on collagen (Coll) where significance only applied for pIRES and β 3Lamp cells. Vn, vitronectin; Fn, fibronectin; Coll, collagen.

substrates of β 3Lamp1 cells was 25 % and 50 % lower than the migration of pIRES2-EGFP and β 3 transfectants, respectively, while all cells showed the same high α 2 β 1-mediated migration on collagen type-I (results not shown).

Adhesion receptors that connect cells to each other, and to the surrounding extracellular matrix, play a crucial role in the control of tissue structure and of morphogenesis. It was recently demonstrated that the expression of β 1 in cells null for this integrin induced the disruption of intercellular adherens junctions and cell scattering [30]. In that context, morphological examination of our cells revealed visible changes correlating with the level of cell-surface α v β 3. Thus, β 3Lamp1 transfectants exhibit a much more compact architecture than β 3 overexpressors. In order to quantify this phenomenon, we used adhesion-independent and adhesion-dependent aggregation assays. In the former assay, using cells grown on an agarose substrate (Figure 7A), it is quite evident that β 3 overexpressors associate into

numerous small-sized aggregates (right-hand panel), while β 3Lamp1 cells are highly aggregated forming a massive ring of compacted cells (left-hand panel), with the pIRES2-EGFP control exhibiting an intermediate phenotype (central panel). We next extended these observations by measuring the time-dependent aggregation of our cells, seeded on glass coated with different adhesion substrates, namely vitronectin, fibronectin, collagen type-I and BSA. Selected fields of cells seeded on these substrates were video imaged every 15 min for 14 h to measure cell motility, the fate of cell-cell contacts and to verify the absence of cell detachment. Figure 7(B) shows β 3Lamp1 and β 3 cells seeded on vitronectin at the beginning of the time-lapse experiment (0 h, left-hand panels) and the same fields 14 h later (right-hand panels). Comparison of cell morphology at time zero (0 h) showed that β 3Lamp1 cells were round while most β 3 cells were well spread on vitronectin substrates. It should be emphasized that experiments started generally 45 min after cell

seeding, a time necessary for selecting the fields to be recorded, saving their X/Y parameters and taking fluorescence emission pictures to evaluate GFP (and therefore transgene expression) by individual cells. Thus, consistent with the results in Figure 6, the low αv expressor $\beta 3$ Lamp1 cells did not spread on vitronectin to which they attached poorly, while high αv expressor $\beta 3$ cells attached and spread well on this substrate. Analysis of images taken subsequently showed that the spreading defect of GFP-expressors in the $\beta 3$ Lamp1 pool (low αv and αv null cells, Figure 5) was not rescued by the interaction, for example, of these cells with their endogenously *de novo* synthesized cellular matrix. It was noticed, however, that $\beta 3$ Lamp1 expressors formed aggregates by coalescing with cells they had contacted at the start of the experiment and remained attached to. Such behaviour was much less frequently observed with $\beta 3$ overexpressors where cell-cell contacts were generally transient. Thus, after 14 h of incubation, $\beta 3$ Lamp1 cells appeared as aggregates made of packed GFP and non-GFP expressors, in contrast to many $\beta 3$ cells that remained isolated (Figure 7B, 14 h ' $\beta 3$ Lamp1' and ' $\beta 3$ ', respectively).

Quantification of the extent of aggregation was achieved by counting the number of cells whose contours could be determined with certainty at the time of plating and 14 h later. Thus the number of cells at the end of the analysis period was expected to decrease if aggregation occurred, and conversely, to increase for non-aggregating cells, due to cellular proliferation, as this was a dynamic assay lasting 14 h. As shown in Figure 7(C) (open bars), we consistently observed aggregation of $\beta 3$ Lamp1 cells on vitronectin while an increase in cell number was evident in the $\beta 3$ overexpressors and control pIRES cells displayed an intermediate phenotype. One hypothesis to explain these data would be that after ligation to vitronectin, cell-cell adhesion is negatively regulated by αv integrin-mediated signalling that is likely to be low for $\beta 3$ Lamp1 and high for $\beta 3$ cells. Surprisingly, however, this negative regulation was observed whatever matrix protein the cells were grown on (Figure 7C), suggesting that it did not require αv integrin ligation by the extracellular matrix. Indeed, it was observed with the poor adhesion substrate BSA (hatched bars), where highest aggregation was observed for $\beta 3$ Lamp1 cells, but where $\beta 3$ cells also remained dispersed. A similar pattern was found after seeding on the highly adhesive type-I collagen whereby all pools attached, spread and migrated equally well (Figure 6 and results not shown), consistent with them expressing similar levels of $\alpha 2\beta 1$ collagen receptors. In contrast to $\alpha 2\beta 1$ -mediated adhesion, aggregation of $\beta 3$ Lamp1 and pIRES cells was lowest on fibronectin suggesting that, in HEK-293 cells, ligation of the major $\alpha 5\beta 1$ integrin to fibronectin [31] counterbalances cellular aggregation to some extent. Thus, to summarize these findings, $\alpha v\beta 3$ overexpression in HEK-293 cells stimulated cell scattering, a process that did not seemingly require ligation of the integrin by the extracellular matrix vitronectin. Conversely, underexpression of these integrins in $\beta 3$ Lamp1 transfectants stimulated aggregation. This process was attenuated by $\alpha 5\beta 1$ integrin signalling but remained unaffected by engagement of $\alpha 2\beta 1$ integrins. Overall, the data suggest that cell-cell adhesion is negatively regulated by $\alpha v\beta 3$ integrins, similar to $\beta 1$ integrins [30].

DISCUSSION

Many proteins form either homodimers or heterodimers as well as oligomers with multiple subunits. These include receptors, hormones, enzyme-inhibitor complexes, protein-chaperone multimers, ion channels and transcription factors. A previous report [15] showed that expression of cathepsin B (CB) fused to one of

the subunits of a cytosolic complex such as β -galactosidase (tetramer) or MyoD (dimer) results in a reduction in the levels of the corresponding wild-type protein. This effect was intriguing, especially in view of the fact that both tested proteins are located in the cytosol/nucleus whereas CB transits through the secretory pathway to lysosomes. How these two molecules meet in the cell is still unclear. However, when applied to the cytosolic transcription factor MyoD, the method failed to reduce the level of its E12 and E47 partners [15], suggesting that only homo-multimerized complexes are affected.

In a recent report [14], we extended this CB-fusion strategy towards the proprotein convertase PC2/7B2 complex formed in the ER and concentrating in secretory granules. The data showed that CB-7B2 chimeras effectively rerouted PC2 towards the lysosomal degradation pathway. However, when we applied this strategy to integrins using a CB full-length $\beta 3$ chimera, endogenous αv levels were unaffected (results not shown). A likely explanation for the failure of this dominant negative approach is that heterodimerization of αv and CB full-length $\beta 3$ was impaired due to the N-terminal CB extension (results not shown). Indeed we also found that addition of an N-terminal 8-amino-acid FLAG antigen to $\beta 3$ significantly reduced its heterodimerization with αv (results not shown). These results can be rationalized in view of the critical importance of the N-terminal 6 amino acids of $\beta 3$ whose exposure is uniquely regulated by both bivalent cations and ligand [32], in agreement with the recently solved crystal structure of $\alpha v\beta 3$ [22]. Therefore we opted for a $\beta 3$ C-terminal modification strategy in which the TM-CT of $\beta 3$ was replaced by that of Lamp1, containing all the necessary endosomal/lysosomal sorting information [16]. A further advantage gained by this approach was the elimination of the cytosolic tail of $\beta 3$ integrin known to contain cytoskeletal binding sites [33], which may prevent their entry into endosomes.

The data obtained so far show that the $\beta 3$ Lamp1 chimera can heterodimerize with αv -integrin subunit and the resulting complex is routed towards acidic degradation compartments with a time-course consistent with a post-Golgi destination (Figures 2 and 3). Noteworthy is the dominant cell-sorting effect of the cytosolic tail of Lamp1 as compared with that of integrin α -chains [34]. The specificity of this misrouting strategy is evident from the unaffected synthesis of $\alpha \beta 1$ integrins in cells overexpressing $\beta 3$ Lamp1 (Figure 2). This approach led to an approx. 4-fold decrease in the level of cell surface αv (Figure 5) and a corresponding decrease in cell attachment to vitronectin (Figure 6). The overall effect is expected to be an underestimate of what can be achieved, since high expressors showed undetectable levels of cell surface αv (Figure 5). Finally, the fact that we observed a similar approx. 4-fold decrease in total and cell surface αv is in accord with the early complex formation of $\alpha v\beta 3$ Lamp1 and subsequent destruction of this complex in acidic compartments.

A further insight into the biosynthesis of $\alpha v\beta 3$ integrins was achieved from the observation that overexpression of $\beta 3$ in HEK-293 cells led to a significant increase in cell surface $\alpha v\beta 3$ (Figure 5), suggesting the existence of a latent pool of free αv that can be recruited and routed to the plasma membrane, provided enough β partners are synthesized. This is similar to the reported excess synthesis of $\beta 1$ in most cells, which remains trapped in the ER as an available pool of uncomplexed $\beta 1$ [35].

As expected, the limitation of the proposed misrouting strategy is the requirement for a high level of stable expression of the Lamp1 chimera in order to effectively suppress the partner protein. Notwithstanding this requirement, this method, unlike conventional gene knockout, is readily applicable to any cell type of choice without species limitation, including human

cells. The use of vectors allowing regulated expression, e.g. under tetracycline control, will permit the analysis of the function of a particular protein by switching off its expression in a tightly controlled and titratable way. This should permit unique, quantitative studies on the effect of complete or partial abrogation of a protein complex or a family of proteins. In addition this approach may be used to identify unknown interactors using appropriate proteomic-based characterization tools. All cases studied to date, including integrins $\alpha v\beta 3$ (this study), PC2/7B2 [14] and gp160/CD4 [13], involve high affinity binding partners, which initially interact in the ER. Further studies are needed to define the relative importance of these two criteria for the success of the method. In that respect, the available data suggest that lysosomal targeting domains seem to dominate over those controlling secretion from the constitutive or regulated pathways. Future studies will undoubtedly shed more light on the scope of applications of this technology to multiple systems, and address whether overexpressing chimeric dominant negative forms in transgenic mice or nematodes will bring it to the realm of whole animals, leading to the future design of novel gene therapies.

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