Integrin α^4 cysteines 278 and 717 modulate VLA-4 ligand binding and also contribute to $\alpha^{4/180}$ formation

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Here we describe experiments in which we mutated four of the six integrin α^4 subunit cysteine residues that are not present in most other integrin α subunits that lack an I domain. In four different types of ligand binding assay we found that optimal integrin $\alpha^4\beta_1$ (VLA-4) binding to vascular cell adhesion molecule 1 (VCAM-1) and/or to CS1 peptide required the presence of both α^4 Cys²⁷⁸ and Cys⁷¹⁷. In addition, optimal ligand binding required divalent cations and reduced cysteines, as evidenced by EDTA and *N*-ethylmaleimide inhibition results. In a control experiment, an α^4 mutation that completely eliminated the α^4 80/70 proteolytic

cleavage site had no effect on ligand binding. Notably, although Cys²⁷⁸ and Cys⁷¹⁷ mutations markedly altered ligand binding, they had no adverse effect on cell adhesion. Thus, compared with cell adhesion, ligand binding is a distinct and apparently more stringent test of VLA-4 integrin–ligand interactions. In addition, we have established that the formation of the previously described $\alpha^{4/180}$ [Parker, Pujades, Brenner and Hemler (1993) *J. Biol. Chem.* **268**, 7028–7035] also requires Cys²⁷⁸ and Cys⁷¹⁷, divalent cations and reduced cysteines. Thus $\alpha^{4/180}$ appears to be more functionally relevant than $\alpha^{4/150}$.

INTRODUCTION

The integrins $\alpha^4\beta_1$ (VLA-4) and $\alpha^4\beta_7$ are highly expressed on several types of leucocytes. The $\alpha^4\beta_1$ heterodimer mediates cell adhesion to vascular cell adhesion molecule 1 (VCAM-1) and to the CS1 region within alternately spliced forms of fibronectin (reviewed in [1–4]). The $\alpha^4\beta_7$ integrin heterodimer mediates adhesion to the gut homing molecule MadCAM [5] as well as to fibronectin and VCAM-1 [6,7]. An abundance of recent evidence has established that α^4 integrins play critical roles in leucocyte recruitment to inflammatory sites, homing of leucocytes and mobilization of progenitor cells (reviewed in [4]). The α^4 subunit is also highly expressed on several non-lymphoid tissues in the developing embryo [8,9]. Accordingly, in the absence of a functioning α^4 gene, mice show defects in placental and cardiac development, leading to embryonic lethality [10]. In addition, VLA-4 expression inhibited metastasis in a mouse melanoma model system [11].

The α^4 subunit does not resemble either of the two main structural groups of integrin α subunits because it contains neither an I domain (an inserted segment of 180–200 residues) nor a disulphide-linked post-translationally cleaved C-terminal fragment [1,12]. Instead, α^4 contains a site near the middle of the molecule that is variably cleaved to yield non-disulphide-linked fragments of 80 and 70 kDa [13,14]. Although the site of proteolytic cleavage has been identified and eliminated by site-directed mutagenesis, this had no apparent effect on cell adhesion [14]. Importantly, the 80 and 70 kDa cleavage fragments of α^4 are not disulphide-linked to each other, although each contains an odd number of extracellular cysteines [15]. Thus at least two α^4 cysteines must be present in a reduced state, in contrast with some other integrin α subunits (e.g. α^{11b}), in which all of the cysteines may be disulphide linked [16].

Another unusual feature of the α^4 subunit, not seen for other

integrin α subunits, is the appearance in SDS gels of a puzzling structural form, called $\alpha^{4/180}$. The $\alpha^{4/180}$ structure migrates more slowly than the commonly observed $\alpha^{4/150}$ band in non-reduced SDS gels, but disappears on reduction. This $\alpha^{4/180}$ structure has been noted in several reports [1,13,14,17,18], but despite extensive preliminary characterization [19], neither the biochemical basis for its appearance nor its functional significance have yet been established.

Because the $\alpha^{4/180}$ structure disappeared on reduction [19], we hypothesized that cysteines might be important for its formation, and here we have confirmed this in an *N*-ethylmaleimide (NEM) inhibition experiment. Thus we focused attention on the subset of α^4 cysteines that do not correspond to the 18 cysteines present in the integrin α^{11b} subunit, for which disulphide linkages have been mapped [16]. With this rationale, α^4 cysteines 278, 717, 767 and 828 were mutated, and the effects on ligand binding, cell adhesion and $\alpha^{4/180}$ formation were determined. Within each α^4 cysteine mutant, a mutation of the α^4 cleavage site [14] was also introduced, because $\alpha^{4/180}$ formation is much more easily studied in the absence of α^4 cleavage into 80 and 70 kDa fragments. Importantly, the mutation that eliminated proteolytic cleavage of α^4 (R558L) itself had no impact on ligand binding.

MATERIALS AND METHODS

Antibodies, cells and integrin ligand proteins

Monoclonal antibodies used were as follows: mouse anti-VLA-4, B-5G10 [13], HP2/1 and HP1/7 [20]; mouse anti- β_1 , A-1A5 [21] and TS2/16 [22]; rat anti- β_1 , 9EG7 [23] and monoclonal antibody (mAb) 13 [24]; and the negative control antibodies P3 [25] and J-2A2 [26]. Function-blocking antibodies to VLA-4 (A4-PUJ1) and VLA-5 (A5-PUJ2) were generated by injection of α^4 -

Abbreviations used: CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MEM, minimal essential medium; NEM, *N*-ethylmaleimide; TBS, Tris-buffered saline; VCAM-1, vascular cell adhesion molecule 1; VLA-4, integrin $\alpha^4\beta_1$.

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transfected K562 cells into RBF/DnJ mice according to standard procedures [27].

Purified VCAM-mouse C kappa fusion protein (VCAM1-k), and a rat monoclonal antibody to mouse C kappa were obtained from Dr. D. Dottavio, Sandoz Co., East Hanover, NJ, U.S.A. Recombinant soluble VCAM-1 (rsVCAM-1) [28] was obtained from Dr. Roy Lobb, Biogen Co. Purified CS1 peptide (GPEILDVPST), derived from the major VLA-4 binding site of fibronectin, was synthesized at the Dana-Farber molecular biology core facility. CS1 peptide conjugated to BSA (CS1-BSA) was a gift from Dr. Tadashi Shimo-Oka (Iwaki Glass Co., Tokyo, Japan). The human cell line K-562 was maintained in RPMI 1640 medium and Chinese hamster ovary (CHO) cells were maintained in minimal essential medium (MEM) with ribonucleosides and deoxyribonucleosides (MEM α +). Both were supplemented with 10% (w/v) fetal calf serum and antibiotics. After transfection, K562 cells were maintained in RPMI 1640 medium with 10% fetal calf serum, whereas CHO cells were grown in MEM α - medium (without ribonucleosides and deoxyribonucleosides) supplemented with 10% dialysed fetal calf serum. G418 (GIBCO BRL Co.) (2.0 mg/ml) was also included for both transfected cell types.

Site-directed mutagenesis and transfections

Site-directed mutagenesis of full-length α^4 cDNA in Bluescript [15] was carried out with the Bio-Rad Muta-Gene kit. Oligonucleotides representing α^4 bp 963–981 (for Cys²⁷⁸ to Ser), 2281–2300 (for Cys⁷¹⁷ to Leu), 2429–2450 (for Cys⁷⁶⁷ to Ser) and 2613–2634 (for Cys⁸²⁸ to Gly) were used as primers containing the appropriate mismatches. The Arg⁵⁵⁸ to Leu (R558L) and Lys⁵⁵⁷ to Gln (K557Q) single mutations that abolished α^4 cleavage, and another mutation (K574N/E575A) that did not, have been previously described [14]. Double mutants containing one of the Cys mutations plus the R558L mutation were prepared sequentially, by using the α^4 cDNA with the R558L mutation as a template for making the second mutations. Base substitutions at the sites of mutation caused gain or loss of restriction enzyme sites, facilitating identification of mutations that were then confirmed by DNA sequencing. Mutated inserts were excised and ligated into the pFNeo expression vector in *Sall*–*Xba*I sites as previously described [29].

For transfection, 1×10^7 K562 or CHO cells were electroporated with 20 μ g of pFNeo vector containing mutant or wild-type α^4 , together with 200 μ g of salmon sperm carrier DNA. Then, 48 h later, cells were placed in media containing 2.0 mg/ml of G418 (GIBCO, Grand Island, NY, U.S.A.). For CHO cell transfections, 1 μ g of the dhfr⁺ p901 vector [30] was co-transfected with α^4 cDNA, and cells were kept in MEM α + medium for 2 days, then switched to MEM α - medium for 2 additional days, and finally selected in MEM α - medium containing 2.0 mg/ml of G418 (GIBCO). G418-resistant cells were analysed by flow cytometry for VLA-4 surface expression using a FACScan (Becton Dickinson), as previously described [29], and enriched for VLA-4-positive cells by immunomagnetic bead selection (Dynal Co.) with the anti- α^4 mAb B-5G10.

Cell labelling and immunoprecipitation

Cells were surface-labelled with Na¹²⁵I (Du Pont–New England Nuclear) by using lactoperoxidase, and then lysed as previously described [19]. For metabolic ³⁵S labelling, cells were preincubated for 30 min in methionine- and cysteine-free RPMI

1640 containing 10% dialysed fetal calf serum and then pulsed with 1 mCi of EXPRE³⁵S³⁵S protein labelling mix (DuPont–New England Nuclear) containing 77% [³⁵S]methionine and 18% [³⁵S]cysteine. Immunoprecipitation was performed as described previously [19] and samples were eluted from Protein-A–Sephacrose beads by boiling in 1 $\times$ sample buffer containing 10% glycerol, 0.1 M Tris/HCl, pH 6.8, 0.01% Bromophenol Blue and 0.05% SDS. Eluted samples were analysed by SDS/PAGE (7% gels) and non-reducing conditions.

Whole-cell VCAM-1 and CS1 binding assays

Cells were washed with 5 mM EDTA in TBS (10 mM Tris/HCl, pH 7.4, 150 mM NaCl), and then incubated with saturating amounts of VCAM1-k (approx. 1.5 μ M) in the presence or absence of EDTA or different divalent cations for 30 min at 4 °C. Unbound VCAM1-k was removed by two washes (while retaining the same divalent cation concentrations), and then bound VCAM1-k was revealed with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse kappa affinity-purified antibody (Caltag, San Francisco, CA, U.S.A.), and flow cytometry was performed with a FACScan machine (Becton Dickinson Co., Mountainview, CA, U.S.A.).

To assess CS1 peptide binding, cells (1.5×10^5) were washed in PBS and incubated with soluble CS1 peptide (0–2 mM) in the presence of 0.1 mM MnCl₂ for 15 min at 37 °C in 5% (w/v) BSA/PBS and 0.02% sodium azide to avoid receptor internalization. Then cells were further incubated for 30 min at 4 °C with approx. 2 μ g/ml rat mAb 9EG7 (an antibody that detects ligand-occupied β_1 integrins) and washed twice with washing buffer (2% BSA/PBS and sodium azide). Finally, to detect bound 9EG7 mAb, cells were incubated for 30 min at 4 °C with FITC-conjugated goat anti-rat IgG (Sigma, St. Louis, MO, U.S.A.), and flow cytometry was performed.

Cell-free VCAM-1 binding assays

Monoclonal rat anti-mouse kappa chain antibody was coupled to CNBr-activated Sepharose beads (Pharmacia LKB Biotechnology Inc.) at 0.5 mg/ml packed beads. Coupled beads were then equilibrated in TBS and incubated at 4 °C for 24 h with VCAM1-k to yield approx. 1 μ g of bound VCAM1-k per 25 μ l aliquot of beads used in each experiment. Transfected K562 and CHO cells were labelled with Na¹²⁵I as described above, washed in 2 mM EDTA, and then lysed in octyl-thioglucopyranoside (Calbiochem), 0.1 M n-octyl-glucopyranoside (Sigma) in TBS, pH 7.4, in the presence of iodoacetamide (8 mM) and protease inhibitors (0.15 mg/ml PMSF and 5 μ g/ml aprotinin) for 1 h at 4 °C. To remove irrelevant background binding material, lysates (supplemented with various amounts of divalent cations) were preincubated with 20 μ l of CNBr-activated Sepharose beads (non-coupled). In some experiments (involving later addition of mAb TS2/16) K562 cell lysates were also depleted of VLA-5 proteins by pretreatment with mAb A5-PUJ2, followed by goat anti-mouse antibody coupled to agarose beads (Sigma Immunochemicals).

Pretreated and clarified supernatants were then incubated with 20 μ l of Sepharose beads, containing bound VCAM1-k, for at least 18 h at 4 °C in the presence of various amounts of divalent cations, and/or in the presence of the anti- β_1 stimulatory antibody TS2/16 [31]. The bound complexes were washed five times with 0.025 M octyl-thioglucopyranoside, 0.025 M n-octyl-glucopyranoside in TBS at pH 7.4 (with cation levels retained

throughout). Bound integrin proteins were eluted by heating in SDS sample buffer, and then analysed by SDS/PAGE under non-reducing conditions.

Cell adhesion assays

Cell adhesion was carried out as previously described [32,33]. Briefly, cells labelled with fluorescent dye BCECF-AM were added to 96-well dishes (5×10^4 cells per well) previously coated with CS1-BSA or VCAM-1. After incubation for 30 min at 37 °C and removal of unbound cells, adherent cells were quantified with a fluorescence analyser (CytoFluor 2300,

Millipore Co.). Results are reported as the means of triplicate points $\pm$ S.D.

RESULTS

Structural consequences of mutating α^4 cysteines

Within the α^4 sequence, 18 of the 24 cysteines exactly align with cysteines in α^{11b} , for which disulphide bond pairing has previously been assigned [16]. These putative pairs are indicated in the α^4 schematic diagram shown in Figure 1. Although not found in α^{11b} , the third and fourth cysteines in α^4 are closely spaced and assumed to pair. Remaining cysteines, for which disulphide pairing is not assigned, include those at positions 278 (in the 80 kDa fragment) and 717, 767 and 828 (in the 70 kDa fragment). To assess the structural and functional relevance of these cysteines, they were each eliminated by single-point mutations, termed C278S, C717L, C767S and C828G, and these mutant forms of α^4 were expressed in K562 cells. Mutation of Cys²⁷⁸, Cys⁷¹⁷ and Cys⁸²⁸ yielded VLA-4 α^4 and β_1 protein bands immunoprecipitated by anti- α^4 (Figure 2A, lanes f, i and l) or anti- β_1 antibodies (lanes e, h and k), that were essentially no different from wild-type α^4 (lanes b and c), except that the 70 kDa α^4 fragment from the Cys⁸²⁸ mutant had a slightly larger apparent size (lanes k and l). Small variations in the amount of α^5 co-precipitated along with α^4 by the anti- β_1 antibody (lanes b, e, h and k) are not relevant to the analysis of α^4 .

As previously observed [14], most α^4 in K562 cells appeared in the cleaved form (80 and 70 kDa fragments), and the previously described $\alpha^{4/180}$ and $\alpha^{4/150}$ forms [19] were not visible. However, ³⁵S pulse labelling yielded $\alpha^{4/180}$ precursor bands in addition to $\alpha^{4/150}$ precursor bands, for both wild-type α^4 and the Cys⁸²⁸ mutant (Figure 2B, lanes b and e). In contrast, mutation of Cys²⁷⁸ or Cys⁷¹⁷ completely eliminated the $\alpha^{4/180}$ but not the $\alpha^{4/150}$ precursor bands (lanes c and d). In this pulse-labelling experiment, the α^4 80 and 70 kDa cleavage fragments were not yet obvious.

Figure 1 Schematic diagram of the α^4 subunit and mutations

The α^4 protein is shown with putative disulphide linkages extrapolated from α^{11b} assignments [16]. Specific amino acid numbers are indicated for those cysteines for which pairing cannot be predicted. The α^4 cleavage site between the 80 (left) and 70 kDa (right) fragments is indicated by the arrowhead. The transmembrane region (near the carboxy terminus) and the three EF-hand-like sites (within the 80 kDa fragment) are also indicated, as are potential N-linked glycosylation sites.

Figure 2 Immunoprecipitation of mutant forms of α^4

(A) K562 cells expressing wild-type or singly mutated α^4 were labelled with ¹²⁵I and then lysates immunoprecipitated with control mAb P3 (lanes a, d, g and j), anti- β_1 mAb A-1A5 (lanes b, e, h and k) or anti- α^4 mAb B-5G10 (lanes c, f, i and l). (B) K562 cells were labelled with ³⁵S for 1 h and immunoprecipitated with control mAb P3 (lane a) or anti- α^4 mAb B-5G10 (lanes b–e). The Cys⁷⁶⁷ single mutant is not shown in (A) or (B) because we could not obtain good expression levels. (C) K562 cells expressing α^4 mutated singly (lanes a, b and g) or doubly (lanes c–f and h–k) were labelled with ¹²⁵I and immunoprecipitated with mAb P3 (lane a), anti- β_1 mAb A-1A5 (lanes b–f) or anti- α^4 mAb B-5G10 (lanes g–k).

Figure 3 Flow cytometry analysis of α^4 mutants expressed in K562 cells

The R558L single mutant and the four R558L/cysteine double mutants were analysed by using the control mAb P3, anti- α^4 mAbs HP1/7, HP2/1 and B-5G10 (recognizing three distinct α^4 epitopes [35]) and anti- β_1 mAb A-1A5.

Because α^4 cleavage prevented the appearance of the mature $\alpha^{4/180}$ and $\alpha^{4/150}$ forms in K562 cells (Figure 2A), each cysteine mutation was then remade within α^4 cDNA that also contained an Arg⁵⁵⁸ → Leu (R558L) mutation, previously shown to eliminate cleavage of α^4 into 80 and 70 kDa fragments [14]. Mature ¹²⁵I-labelled $\alpha^{4/180}$ could be readily seen in the R558L/C767S (Figure 2C, lanes e and j) and R558L/C828G (lanes f and k) double mutants, and R558L single mutant α^4 (lanes b and g). However, $\alpha^{4/180}$ was not observed in either the R558L/C278S or the R558L/C717L mutants, immunoprecipitated with either anti- β_1 (lanes c and d) or anti- α^4 (lanes h and i) antibodies. As expected, no α^4 cleavage fragments can be seen in Figure 2C.

Neither the R558L mutation alone nor the combination of R558L with C278S, C717L and C767S caused a major alteration of the overall α^4 structure, because nearly identical expression of three distinct α^4 epitopes was observed for each mutant (Figure 3). The R558L/C828G double mutant tended to show diminished overall expression of α^4 , consistent with lower stability, and thus was not analysed further. The same α^4 mutants were also expressed at comparable expression levels in CHO cells (results not shown).

Cysteines: Binding of VCAM-1 to intact cells

To analyse ligand binding directly, VCAM1-k protein was added to α^4 -transfected K562 cells in the presence of 1 mM Mn²⁺, and then bound VCAM1-k was quantified with FITC-anti-kappa, followed by flow cytometry. In a preliminary experiment, the apparent K_d for VCAM1-k binding in the presence of 1 mM Mn²⁺ was approx. 50 nM, and thereafter 1.5 μ M VCAM1-k was used to provide reasonably saturating conditions. The VCAM1-k bound moderately well to K562 cells expressing the R558L single mutation (Figure 4A), but this binding was markedly reduced (by approx. 50%) on cells expressing the R558L/C278S double mutant (Figure 4B), and binding was also reduced (by approx. 30%) for the R558L/C717L double mutant (Figure 4C). These differences in VCAM1-k binding could not be explained

Figure 4 Effect of C278S and C717L mutations on VCAM1-k binding

K562 cells expressing R558L (A,D), R558L/C278S (B), R558L/C717L (C) or no α^4 (E) were washed in 5 mM EDTA and then incubated with saturating amounts of VCAM1-k (1.5 μ M) for 30 min at 4 °C in the presence of 1 mM Mn²⁺ or 1 mM Mg²⁺ as indicated. In each experiment, background binding in the presence of 2 mM EDTA was also assessed (peaks marked with E). In (F), α^4 flow cytometry profiles for R558L (broken line), R558L/C278S (solid line), and R558L/C717L (dotted line) are shown.

simply by differences in α^4 expression levels, because expression of the R558L, R558L/C278S and R558L/C717L α^4 mutants were nearly overlapping (Figure 4F). Whereas binding of VCAM1-k to the R558L mutant was well supported by Mn²⁺ (Figure 4A), practically no binding was observed in the presence of either Mg²⁺ (Figure 4D) or Ca²⁺ (results not shown). In a control experiment, no VCAM1-k bound to untransfected K562 cells (Figure 4E).

A more detailed analysis over a wide range of Mn²⁺ concentrations (Figure 5A) revealed consistently decreased binding of VCAM1-k to the R558L/C278S and R558L/C717L mutants compared with the R558L and R558L/C767S mutants in the presence of a saturating amount of VCAM1-k (1.5 μ M). Comparable results were also obtained with α^4 -CHO transfectants (results not shown). In addition we found that the R558L/C278S and R558L/C717L mutants supported 50–60% less binding by a divalent VCAM-Ig protein (results not shown). In these experiments, performed in collaboration with Dr. R. Lobb and Dr. L. Burkly (Biogen, Inc.), VCAM-Ig was directly detected on the cell surface as recently described [34].

Considering that Cys²⁷⁸ and Cys⁷¹⁷ are important for ligand binding, and that at least two cysteines (possibly Cys²⁷⁸ and Cys⁷¹⁷) are present in the reduced state, it was expected that NEM might inhibit ligand binding. Indeed, the binding of VCAM1-k to K562 cells expressing VLA-4 (with no mutated

Figure 5 Ligand binding to α^4 mutants

(A) K562 cells bearing α^4 with the indicated mutations were incubated with VCAM1-k (approx. $1.5 \mu M$) for 30 min at $4^\circ C$ in the presence of increasing amounts of $MnCl_2$, and then VCAM1-k binding was determined by flow cytometry as described in the Materials and methods section. Background signals, obtained in the presence of 5 mM EDTA, were subtracted from each point. MFI units (y-axis) correspond to mean fluorescence intensity values. (B) K562 transfectants were incubated with increasing amounts of CS1 peptide for 15 min at $37^\circ C$ in the presence of 0.1 mM Mn^{2+} , and then ligand binding was determined indirectly by measuring induction of the 9EG7 epitope, as described in the Materials and methods section. Results are expressed as the fraction of β_1 expressing the 9EG7 epitope relative to total β_1 subunit (measured with mAb 13). Both 9EG7 and mAb 13 binding were detected by using FITC-conjugated rabbit anti-rat second antibody.

cysteines) was diminished by approx. 50% when the cells were preincubated with 1 mM NEM (results not shown).

Functional role of α^4 cysteines: binding of CS1 peptide to intact cells

To analyse further the functional role of α^4 cysteines, VLA-4-dependent binding of CS1 peptide was studied. Preliminary experiments established that CS1 peptide binding, in the presence of 0.1 mM Mn^{2+} , yielded a dose-dependent induction of the mAb 9EG7 epitope on the β_1 subunit [34a]. As indicated in Figure 5B, CS1 peptide titrated up to 1 mM caused substantial 9EG7 epitope induction for the R558L mutant. In comparison, epitope induction was markedly reduced for the R558L/C278S and R558L/C717L mutants, but only slightly reduced for the R558L/C767S mutant. In a control experiment, no CS1-dependent induction of the 9EG7 epitope was detected on K562 cells lacking α^4 expression (results not shown), confirming that we were studying a VLA-4-dependent effect. Furthermore the 9EG7 epitope was induced equally well for all of the mutants on titration with Mn^{2+} alone. Thus there is no inherent deficiency in the 9EG7 epitope itself caused by the mutations.

Figure 6 VCAM-1 binding to α^4 cleavage mutants

K562 cells, bearing α^4 with the indicated cleavage site mutations, were incubated with increasing amounts of VCAM1-k for 30 min at $4^\circ C$, in the presence of 2 mM Mn^{2+} . Binding of VCAM1-k was then determined by flow cytometry as described in the Materials and methods section.

Figure 7 Comparison of α^4 mutant binding to VCAM1-k-Sepharose beads

Octylglucoside lysates from transfected K562 cells were incubated with VCAM1-k-Sepharose beads (approx. $25 \mu l$, containing approx. $1 \mu g$ of VCAM1-k) in the presence of the indicated divalent cations (at 1 mM) for 18 h at $4^\circ C$. Bound integrin complexes were eluted and analysed by SDS/PAGE. Despite washing in 2 mM EDTA before cell lysis, intracellular divalent cation concentrations were probably sufficient to allow the background binding seen even in the absence of added divalent cations.

Effect of α^4 cleavage on ligand binding

We previously described two mutations (R558L and K557Q) that abolished α^4 cleavage into 80 and 70 kDa fragments, and another mutation (K574N/E575A) that did not [14]. Thus, to assess further the specificity of the effects of our cysteine mutations on α^4 -dependent ligand binding, this other panel of α^4 mutants was analysed for VCAM1-k binding (Figure 6). As indicated, comparable VCAM1-k binding was obtained for wild-type α^4 , for mutants that lost the α^4 cleavage site (R558L and K557Q), and for another mutant that retained cleavage (K574N/E575A). Thus proteolytic cleavage of α^4 subunit into 80 and 70 kDa fragments seems to have no influence on ligand binding.

Binding of solubilized mutant VLA-4 to VCAM-1-Sepharose beads

To analyse further the ligand binding properties of mutant VLA-4, ^{125}I -labelled cells were solubilized in octyl glucoside, and the soluble VLA-4 was tested for binding to VCAM1-k-Sepharose beads. As shown in Figure 7, the R558L and R558L/C767S

Figure 8 Divalent cation-dependent binding of R558L and R558L/C717L mutants to VCAM1-k-Sepharose beads

Lysates from ¹²⁵I-labelled K562 cells were incubated with VCAM1-k beads, in the presence of the indicated levels of Mn²⁺ (A), Ca²⁺ (B) or Mg²⁺ (C). For this experiment the anti- β_1 stimulatory antibody TS2/16 was included to enhance binding. Bound material was analysed by SDS/PAGE as described in the Materials and methods section, and the resulting autoradiograms (results not shown) were scanned on an Ultrascan XL enhanced laser densitometer (Pharmacia Biotech Inc.). For each point, the area corresponding to total α^4 ($\alpha^{4/180} + \alpha^{4/150}$) was normalized relative to the area corresponding to total α^4 obtained when no divalent cations were added.

mutants showed strong binding to VCAM1-k-Sepharose beads, particularly when 1 mM Mn²⁺ or 1 mM Ca²⁺ was present. For these two mutants, binding was not markedly elevated in the presence of 1 mM Mg²⁺, and still did not increase even if 10 mM Mg²⁺ was added (results not shown). In marked contrast, the R558L/C278S and R558L/C717L mutants showed only marginal binding to the VCAM1-k beads, regardless of which divalent cation was present. In CHO cells, similar results were obtained, with moderate Mn²⁺-dependent binding by the R558L mutant, and substantially lower binding by the R558L/C278S and R558L/C717L mutants (results not shown). Thus the same mutants that were unable to form $\alpha^{4/180}$, and caused diminished VCAM-1 binding to intact cells, also showed diminished ligand binding properties in a cell-free assay. In a control experiment no binding was observed if lysates of untransfected K562 cells were used. Notably in Figure 7, increased binding generally correlated with more α^4 present in the $\alpha^{4/180}$ form.

Divalent-cation-dependent binding was investigated in more detail (Figure 8), using two representative mutants (R558L and R558L/C717L) and multiple concentrations of each divalent cation. As indicated, the R558L mutant again showed increased Mn²⁺-dependent VLA-4 binding (Figure 8A), a smaller amount of Ca²⁺-dependent binding (Figure 8B) and little Mg²⁺-dependent binding (Figure 8C). In contrast, the R558L/C717L mutant showed a minimal increase in binding, regardless of which divalent cation was added.

Effects of cysteine mutations on cell adhesion

To analyse further the possible functional roles of α^4 cysteines, extensive cell adhesion assays were performed, with both CS1 peptide and VCAM-1. As indicated in Figure 9A, for neither ligand was there any consistent difference in cell adhesion between mutants capable of forming $\alpha^{4/180}$ (R558L and R558L/C767S), and mutants in which $\alpha^{4/180}$ formation had been abolished (R558L/C278S and R558L/C717L). The overall level of cell adhesion was amplified in the presence of the stimulatory anti- β_1 mAb TS2/16 (Figure 9B), but there were still no consistent differences between the two sets of mutants. For each α^4 transfectant, adhesion was VLA-4-specific, as confirmed by the minimal adhesion obtained for mock-transfected K562 cells. Sensitivity to EDTA inhibition was also essentially invariant between the different mutants (Figure 9C). Finally, adhesion for each mutant was tested over a wide range of Mn²⁺, Ca²⁺ and Mg²⁺ concentrations, but again no consistent differences emerged. This point is illustrated in representative adhesion experiments performed in the presence of 2 mM Mg²⁺ (Figure 9D). Furthermore the thiol-reactive agents dithiothreitol and NEM (at 1–2 mM) had no consistent impact on cell adhesion mediated by wild-type α^4 or any of the mutants (results not shown). Together these results indicate that free α^4 thiols do not play a perceptible role during cell adhesion.

Further parallels between ligand binding and $\alpha^{4/180}$ formation

As indicated above, Cys²⁷⁸, Cys⁷¹⁷, reduced cysteines and divalent cations were all required for optimal ligand binding, whereas Cys²⁷⁸ and Cys⁷¹⁷ (but not Cys⁷⁶⁷ or Cys⁸²⁸) were also required for the formation of $\alpha^{4/180}$. Here we show that $\alpha^{4/180}$ formation also requires reduced cysteines and divalent cations. Pretreatment of an immunoprecipitated α^4 sample with the thiol derivatizing agent NEM, before boiling in SDS, completely abolished the appearance of $\alpha^{4/180}$ in the R558L mutant (Figure 10A, lanes a and b). Although the $\alpha^{4/180}$ band disappeared, and the total $\alpha^{4/150}$ was increased, the β_1 level was unchanged. In contrast, the R558L/C717L mutant was already incapable of forming $\alpha^{4/180}$ and thus NEM treatment had no effect (lanes c and d). In a control experiment, NEM treatment also did not alter the sizes of immunoprecipitated VLA-5 bands, although the quantity obtained was diminished for unknown reasons (lanes e and f).

Pretreatment of immunoprecipitated α^4 (the R558L mutant) with 1 mM EDTA for 10 min at 37 °C almost completely prevented the appearance of $\alpha^{4/180}$ (Figure 10B, compare lanes a and b). Notably, for effective inhibition of $\alpha^{4/180}$, EDTA did not need to be present when the sample was heated in SDS. If divalent cations were added back after EDTA treatment, the ability to form $\alpha^{4/180}$ was not restored, indicating that the EDTA had an irreversible effect on the isolated integrin. In addition, heating in the presence of a higher SDS concentration (2% instead of 0.05%, lanes a and c) prevented the appearance of $\alpha^{4/180}$. Thus $\alpha^{4/180}$ formation is favoured by less denaturing conditions.

Figure 9 Effects of α^4 cysteine mutations on cell adhesion

K562 cells transfected with mutated α^4 were tested for adhesion to surfaces coated with either CS1-BSA (left panels) or rsVCAM-1 (right panels). Adhesion was performed as described in the Materials and methods section, with RPMI media containing 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+} , either in the absence (**A**) or presence (**B**) of 1 μ g/ml TS2/16. (**C**) Cells were washed in TBS (without any divalent cations) and then adhesion was performed in the presence of increasing concentrations of EDTA. Results are presented as a fraction of the non-inhibited adhesion level. (**D**) Cells were washed in TBS and then adhesion to surfaces coated with 2.5 μ g/ml CS1-BSA or rsVCAM-1 was performed in the presence of 2 mM Mg^{2+} in TBS.

Figure 10 Formation of $\alpha^4/180$ requires reduced cysteines and divalent cations

(A) From ^{125}I -labelled K562 cells, bearing α^4 mutants R558L (lanes a and b) or R558L/C717L (lanes c–f), integrins were immunoprecipitated with either the anti- α^4 mAb A4-PUJ1 (lanes a–d) or the anti- α^5 mAb A5-PUJ2 (lanes e–f). While bound to Protein-A–Sepharose beads, precipitated material was treated (+) or untreated (–) with 2 mM NEM for 5 min at 25 °C. NEM was then removed by washing and the sample was analysed by SDS/PAGE under non-reducing conditions. (B) From ^{125}I -labelled K562 cells expressing the R558L mutation, the α^4 chain was immunoprecipitated with mAb A4-PUJ1. Before boiling in either 0.05% or 2.0% SDS, samples were treated with (+) or without (–) 0.5 mM EDTA for 10 min at 25 °C.

DISCUSSION

Effects of cysteine mutations on ligand binding

Mutation of Cys²⁷⁸ or Cys⁷¹⁷, to a greater extent than Cys⁷⁶⁷, caused a marked reduction in ligand binding by the integrin VLA-4. To our knowledge this is the first report to show the functional consequences of mutating integrin cysteine residues. Of the 24 cysteines present within the human α^4 sequence, 16 are conserved in nearly all integrin α chains, and 18 are conserved in all those integrin α chains that lack an I domain [12]. These more highly conserved cysteines were not mutated because they seem

unlikely to contribute to the specialized functional and structural features of α^4 and are more probably needed for maintaining structural features common to many integrins. The overall structure of the α^4 molecule was not obviously disrupted by mutation of either Cys²⁷⁸ or Cys⁷¹⁷, as indicated by the complete retention of previously defined epitopes A, B and C, including two epitopes (A and B) important for ligand binding [35,36].

Our ligand binding results were confirmed by using four different types of assay. First, cell-surface binding of soluble VCAM1-k was detected by flow cytometry with anti-Ig antibodies. A similar approach has been used successfully elsewhere [37]. Secondly, cell-surface binding of CS1 peptide was detected by using an antibody (9EG7) that recognizes a ligand-occupied conformation of β_1 integrins [34a]. The use of inducible integrin neoepitopes to detect ligand binding has been well established for other integrins [38]. Thirdly, we used a VCAM-1-bead assay to detect binding to detergent-solubilized VLA-4. We have previously used this approach to study VLA-2/collagen interactions [33]. Fourthly, we used a directly labelled, high-affinity, divalent VCAM1-Ig chimera for cell-surface ligand binding studies. As recently described elsewhere, this approach has been very effective [34].

In an important control experiment, two mutations (K557Q and R558L) that abolished α^4 cleavage had no effect on the binding of soluble VCAM to VLA-4. This result provides further evidence that the Cys²⁷⁸ and Cys⁷¹⁷ mutations are indeed exerting specific effects on α^4 ligand binding function. Also this finding is consistent with previous results showing that proteolytic cleavage of α^4 into 80 and 70 kDa fragments had no influence on VLA-4-mediated cell adhesion to either VCAM-1 or fibronectin [14].

Contrast between ligand binding and cell adhesion results

Whereas two different α^4 cleavage site mutations had no effect on either ligand binding or cell adhesion, the Cys²⁷⁸ and Cys⁷¹⁷ mutations each caused diminished ligand binding without affecting cell adhesion. To verify our cell adhesion assay results, we used two distinct cell types, multiple ligands, different divalent cations and a range of divalent cation and ligand doses. In no case did we obtain results suggesting that any of our cysteine mutations could influence cell adhesion. Furthermore, although the thiol-reactive agent NEM inhibited ligand binding to a similar extent to the Cys²⁷⁸ and Cys⁷¹⁷ mutations, it also did not affect cell adhesion. Together these results emphasize that ligand binding assays and static cell adhesion assays measure distinct parameters. In this regard it has been demonstrated previously that cell adhesion and ligand binding can be regulated independently [34,39,40]. It is well established that cell adhesion requires not only integrin ligand binding, but also an intact cytoskeleton [41] and metabolic energy [33] that are probably required for receptor redistribution and adhesion strengthening [42]. Thus, above a certain threshold of ligand binding, later steps in the complex series of adhesion events could become rate-limiting. These steps are likely to involve integrin clustering and/or adhesion strengthening.

Furthermore we must consider that integrins do not solely function to mediate cell adhesion. Others have obtained intriguing results that suggest that high-affinity sites exist for purposes other than adhesion [40]. In this regard, a host of studies have shown that soluble integrin ligands can stimulate or co-stimulate various leucocyte functions including proliferation and cytokine production [43]. Also it is notable that platelet $\alpha^{\text{IIb}}\beta_3$ binding to soluble fibrinogen is regulated much more stringently than platelet adhesion to insoluble fibrinogen [44].

$\alpha^{4/180}$ originates from an active conformation of α^4

Previously we described $\alpha^{4/180}$, a novel form of the integrin α^4 subunit with anomalous slower migration (compared with $\alpha^{4/150}$) in non-reducing SDS/gel electrophoresis [19]. Here we have evidence that $\alpha^{4/180}$ may be the more functionally relevant form of α^4 . The same four conditions (reactive thiol groups, Cys²⁷⁸, Cys⁷¹⁷ and divalent cations) required for optimal ligand binding activity are essential for the formation of $\alpha^{4/180}$.

We propose that divalent cations, perhaps together with key cysteine residues, help to stabilize an active form of α^4 . Then, on heating in SDS, the likely occurrence of thiol–disulphide interchanges could secure the active form of α^4 as $\alpha^{4/180}$. Consistent with this model, it is well established that divalent cations are required for integrin ligand binding and can markedly alter integrin conformation [45]. Also, results seen here and previously [19] suggest that $\alpha^{4/180}$ is less denatured than $\alpha^{4/150}$. For example, reducing agents, heat, elevated pH, EDTA and excess SDS all contribute to the conversion of $\alpha^{4/180}$ to $\alpha^{4/150}$. In addition, compared with $\alpha^{4/150}$, the $\alpha^{4/180}$ molecule is derived from a population of α^4 molecules that is more protease-resistant [19].

The occurrence of thiol–disulphide interchange has been observed for other proteins on heating in SDS [46]. Consistent with this idea, (i) the α^4 integrin contains at least two free thiols (see the Introduction section), (ii) thiols are required for $\alpha^{4/180}$ formation (as seen by NEM inhibition) and (iii) reducing agents convert $\alpha^{4/180}$ to $\alpha^{4/150}$ [19]. Obviously Cys²⁷⁸ and Cys⁷¹⁷ should play key roles in the proposed thiol–disulphide interchange, because the effects of NEM almost exactly paralleled the effects of mutating these cysteines. However, at this point we cannot definitely indicate whether Cys²⁷⁸ and/or Cys⁷¹⁷ would act as donors or recipients in such an interchange.

As previously mentioned [19], the shift of α^4 from 180 kDa to a lower apparent molecular mass (150 kDa) on reduction is highly atypical. On reduction, the unfolding of many proteins in fact leads to a larger hydrodynamic radius and increased apparent molecular mass. We now speculate that Cys⁷¹⁷ and Cys²⁷⁸, by participating in thiol–disulphide interchanges, contribute to long-range interactions that stabilize an asymmetrical shape, with a correspondingly larger hydrodynamic radius. This, together with the structural stabilization provided by divalent cations, is obviously essential for $\alpha^{4/180}$ formation. It is perhaps significant that Cys²⁷⁸ occurs in close proximity to the first putative divalent cation site in α^4 (Figure 1).

Mutation of two other cysteines (Cys⁷⁶⁷ and Cys⁸²⁸) did not prevent the appearance of $\alpha^{4/180}$, emphasizing that the structural contributions of Cys²⁷⁸ and Cys⁷¹⁷ seem to be highly specific. Also previous studies showed that mutations within the α^4 cytoplasmic domain [33,47] and within the putative EF-hand divalent cation sites [32] did not prevent the formation of $\alpha^{4/180}$, further pointing to the specialized properties of Cys²⁷⁸ and Cys⁷¹⁷.

Comparisons with other integrins

Although the studies reported here have exclusively involved $\alpha^4\beta_1$ (VLA-4), we expect that $\alpha^4\beta_7$ binding to its ligand MadCAM-1 [5] may be similarly facilitated by α^4 cysteines 278 and 717. Also the functional importance of Cys²⁷⁸ and Cys⁷¹⁷ in human α^4 is reinforced by the presence of comparable cysteines in the mouse α^4 sequence [48].

Among the 15 published human integrin α -chain sequences, only α^L has cysteines in positions comparable to both Cys²⁷⁸ and Cys⁷¹⁷ in α^4 . Thus it is notable that, under non-reducing conditions, we observed the migration of α^L at 210 kDa as well

as at 180 kDa. In contrast we saw no structures of higher apparent molecular mass for α^M and α^X or any other integrin α -chains (results not shown). This result provides further evidence that the appearance of $\alpha^{4/180}$ (and the analogous $\alpha^{L/210}$) is highly dependent on the presence of specific cysteine residues that are uniquely able to stabilize active α -chain conformations on heating in SDS. It is notable that α^L and α^4 are the two integrin α -chains most often found on lymphocytes, and both participate in binding to cell-surface ligands in the immunoglobulin superfamily (VCAM, MadCAM and ICAM). Thus we hypothesize that these two integrin α -chains may have similar specialized structural features uniquely suited for their related functional activities.

In conclusion, we have carried out genetic and biochemical manipulations of the integrin α^4 subunit that strongly implicate key cysteines as being required for optimal ligand binding function. The effect of these alterations on ligand binding but not cell adhesion reaffirms that these are distinct, independently regulatable events. Finally, the same biochemical manipulations that promote optimal ligand binding had a parallel effect on the appearance of $\alpha^{4/180}$. Thus it is now clear that $\alpha^{4/180}$, defined in the context of SDS/PAGE, represents a less denatured, and more functionally relevant, conformation than the more commonly observed $\alpha^{4/150}$.

We thank Elizabeth Murphy for assistance in the preparation of α^4 mutants, and Dr. D. Dottavio (Sandoz Co., East Hanover, NJ, U.S.A.) for VCAM1-k fusion protein used in ligand binding assays, Dr. Roy Lobb (Biogen Co., Cambridge, MA, U.S.A.) for recombinant soluble VCAM-1, and Dr. Tadashi Shimo-Oka (Iwaki Glass Co., Tokyo, Japan) for CS1–BSA. This work was supported by National Institutes of Health Grant GM38903, and by postdoctoral fellowships from the Spanish Ministerio de Educacion y Ciencia and the Lady Tata Trust (to C.P.).

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