A novel region of the $\alpha 4$ integrin subunit with a modulatory role in VLA-4-mediated cell adhesion to fibronectin

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The integrin VLA-4 ($\alpha 4\beta 1$) is a receptor for fibronectin and vascular cell-adhesion molecule 1 (VCAM-1). Four functionally different epitopes, designated A, B1, B2 and C, have previously been defined on the $\alpha 4$ subunit. Using K562 $\alpha 4$ mutant transfectants we found that $\alpha 4$ amino acids Tyr¹⁵¹, Gln¹⁵², Asp¹⁵³, Tyr¹⁵⁴ and Val¹⁵⁵ are important for the structure of the epitope B2. Mutations at $\alpha 4$ Gln¹⁵² substantially impaired the transfectant adhesion to a CS-1-containing fragment of fibronectin (FN-H89), whereas this adhesion was not affected on the other $\alpha 4$ mutant transfectants. None of the $\alpha 4$ mutations significantly altered the adhesion of the different $\alpha 4$ transfectants to VCAM-1. In addition, we have identified residues Gln¹⁵², Asp¹⁵³ and Tyr¹⁵⁴ as part of the $\alpha 4$ epitope B2 involved in homotypic cell aggregation. The decrease in adhesion to FN-H89 shown by Gln¹⁵² $\alpha 4$ mutant transfectants was the result of an inefficient

binding of FN-H89 by VLA-4 mutated at this residue. Also, mutant VLA-4 displayed an altered reactivity with HUTS-21, an anti- $\beta 1$ monoclonal antibody that reacts with functionally active VLA integrins. Adhesion to FN-H89 was not restored unless stimuli that increase the ligand-binding affinity of VLA heterodimers were added, suggesting that cell adhesion was affected in the initial phases. These results indicate that $\alpha 4$ Gln¹⁵² modulates cell adhesion to FN-H89 by playing important roles in the maintenance and/or the acquisition of an active state of VLA-4, an integrin that is normally expressed on the cell surface in a range of multiple activation states. The location of the $\alpha 4$ Gln¹⁵² residue on a loop of the upper surface of the proposed β -propeller structure suggests a close association with potential ligand-binding sites.

INTRODUCTION

The integrins are adhesion molecules involved in cell–cell and cell–extracellular matrix interactions, which influence a wide number of differentiation and migration processes [1]. VLA-4 ($\alpha 4\beta 1$) is a member of the $\beta 1$ subfamily of the integrins, mainly expressed on haemopoietic cells and also on several melanomas and osteosarcomas, that mediates cell adhesion to the connecting segment 1 (CS-1) region of fibronectin and to vascular cell-adhesion molecule 1 (VCAM-1) [2–6]. The activity of VLA-4, as well as other $\beta 1$ integrins, can be modulated rapidly by various stimuli, including bivalent cations [7] and certain anti-(integrin $\beta 1$ subunit) monoclonal antibodies (mAbs) [8,9]. Accordingly they can exist in different states of activation [7], and it has been shown that several anti- $\beta 1$ mAbs, such as HUTS-21 and 15/7, can detect functionally active forms of the VLA integrins [10–12].

VLA-4 is involved in leucocyte recruitment to sites of inflammation, and it has been implicated in several inflammatory pathologies [13–15]. In addition, VLA-4 participates in the adhesion of haemopoietic progenitors to bone marrow stroma [16–18] and it is required for the normal development of both T-cells and B-cells in the bone marrow [19].

Different epitopes in the $\alpha 4$ subunit, designated A, B and C, were originally defined according to the interference of a wide panel of anti- $\alpha 4$ mAbs with VLA-4-mediated cell adhesion functions [20]. The $\alpha 4$ epitope B included the main interaction sites with the CS-1-containing FN-40 fragment of fibronectin and with VCAM-1, and was subdivided into epitopes B1 and B2, because some of the antibodies that blocked these adhesions also triggered homotypic cell aggregation, which constitutes another functional property of VLA-4. The epitope A anti- $\alpha 4$ mAb partly blocked cell adhesion to FN-40 but not to VCAM-1, and were also able to induce homotypic aggregation [20]. We have

recently reported that mutations at $\alpha 4$ epitope A residues Arg⁸⁹Asp⁹⁰ affects both VLA-4-mediated cell adhesion to FN-40 and homotypic aggregation triggered by anti- $\alpha 4$ epitope A mAbs [21]. Electron microscopy studies on integrins revealed that a globular domain composed of the N-terminal portions of the α and β subunits contained the ligand-binding site [22]. A characteristic feature of integrin α subunit N-terminal domains is the presence of seven repeats of approx. 60 amino acid residues each [23]. It has been recently proposed that these repeats fold into a β -propeller domain, which is based on the presence of potential secondary structures of integrin α subunits similar to secondary structures found in several proteins with β -propeller domains [24]. According to this model, the upper surface of the β -propeller contains the α subunit ligand-binding sites.

In the present study we have mutated selected residues at the N-terminal portion of $\alpha 4$ and investigated the effect of the mutations on both VLA-4 structure and cell adhesion mediated by VLA-4. Our results identify modulatory regions in close association with potential ligand-binding sites.

MATERIALS AND METHODS

Cells, VLA-4 ligand proteins and antibodies

The human cell line K562 was cultured in RPMI 1640 medium supplemented with 10 % (v/v) fetal calf serum and antibiotics (complete medium). K562 $\alpha 4$ transfectants were maintained in the same medium containing 1 mg/ml of G418 (Gibco BRL). The recombinant FN-H89 fragment of fibronectin, which contains the CS-1 site, and recombinant soluble VCAM-1 (sVCAM-1) consisting of domains 1–4 were generated as previously described [21,25]. mAbs used in this study included P3X63 [26], TP1/36 (anti-CD43) [27], TS2/16, Lia 1/2.1 and HUTS-21 [anti-

Abbreviations used: BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; CS-1, connecting segment 1; mAb, monoclonal antibody; sVCAM-1, soluble vascular cell adhesion molecule 1; VCAM-1, vascular cell-adhesion molecule 1.

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Table 1 Functional epitope mapping of anti- $\alpha 4$ mAbs ALC1/4.1 and ALC1/5.1

Wild-type $\alpha 4$ transfectants (4M7) were preincubated at 37 °C for 20 min with the indicated anti- $\alpha 4$ mAbs, added to wells coated with FN-H89 (7.5 $\mu\text{g/ml}$) or sVCAM-1 (3 $\mu\text{g/ml}$) and the extent of adhesion was analysed as described in the Materials and methods section. For aggregation, 4M7 cells were incubated with the anti- $\alpha 4$ mAbs and the induction of aggregation was determined after 3 h by the method of Rothlein and Springer [35]. Symbols: + + +, more than 85% inhibition; + +, 65–85% inhibition; +, 30–65% inhibition; $\pm$, 10–25% inhibition; —, no inhibition; 1, less than 10% of cells aggregating; 4, more than 80% of cells aggregating.

mAb	Inhibition of cell adhesion to:		Induction of aggregation
	FN-H89	VCAM-1	
ALC1/4.1	+ + +	+ +	4
ALC1/5.1	+ + +	+ +	4
HP2/4 (B2)	+ + +	+ +	4
HP2/1 (B1)	+ + +	+ + +	1
HP1/1 (A)	+	$\pm$	4
B-5G10 (C)	—	—	1

(VLA- $\beta 1$) subunit] [11,28,29], SAM-1 (anti- $\alpha 5$) [30], and the following anti- $\alpha 4$: HP1/1, HP1/7, HP2/1, HP2/4 [31] and B-5G10 [32]. The ALC1/4.1 and ALC1/5.1 mAbs were generated by immunizing Balb/c mice with human monocytes and were identified as anti- $\alpha 4$ antibodies as they reacted with K562 cells expressing transfected wild-type $\alpha 4$ subunit (4M7 cells) [28], but not with parental VLA-4-negative K562 cells (results not shown). Functional characterization of mAbs ALC1/4.1 and ALC1/5.1 revealed that they fully inhibited the adhesion of 4M7 cells to FN-H89, partly inhibited the adhesion to sVCAM-1 and were able to trigger the homotypic cell aggregation of the same cells, similarly to the epitope B2 anti- $\alpha 4$ mAb HP2/4 (Table 1). These results indicated that ALC1/4.1 and ALC1/5.1 were epitope B2 mAbs, according to the functional designation assigned previously [20].

Site-directed mutagenesis and transfections

The $\alpha 4$ cDNA in Bluescript [23] was subjected to site-directed mutagenesis with the Bio-Rad Muta-Gene kit, as recommended. Oligonucleotides representing $\alpha 4$ bp 578–603 (for Y151Q), 580–611 (for Q152E/D153A), 585–613 (for D153A/Y154S), 586–615 (for Y154L/V155S), 592–618 (for V155S/K156Q), 596–624 (for K157N/F158I), 491–524 (for N123H/E124A) and 640–670 (for F172V/Y173N) were used as primers containing selected mismatches. Base substitutions at sites of mutations raised new endonuclease restriction sites, which facilitated the identification of the mutations introduced, that were later confirmed by DNA sequencing with a Perkin-Elmer automatic DNA sequencer (Model ABI Prism®). Mutated $\alpha 4$ inserts were then excised and ligated into the pFNeo expression vector [33,34] in *SalI*–*XbaI* sites. For transfection, 25 μg of pFNeo vector containing mutant $\alpha 4$ was electroporated into 10^7 K562 cells; after 48 h, 1 mg/ml of G418 was added to the media. G418-resistant cells were analysed by flow cytometry with a Coulter Epics XL, and enriched for VLA-4-positive cells by immunomagnetic bead selection (Dynal Co., Oslo, Norway), with the anti- $\alpha 4$ mAb B-5G10.

Cell adhesion and aggregation assays

For cell adhesion, $\alpha 4$ transfectants or K562 cells were labelled in complete medium with the fluorescent dye 2',7'-bis-(2-carboxy-

Figure 1 Structure predictions and mutagenesis of the integrin $\alpha 4$ subunit

Surface probability profile of the N-terminal 200 amino acid residues from the integrin $\alpha 4$ subunit (**A**). The index of surface probability for each $\alpha 4$ residue is plotted with the aid of the Peptidestructure program, following the method of Emini [55]. The indicated pair of residues possess the peak surface probability indices inside short regions. The amino acids subjected to mutagenesis and the designation of the $\alpha 4$ mutant transfectants are indicated (**B**). C denotes the cysteine residues.

ethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Leiden, The Netherlands), and then added in RPMI medium containing 0.4% BSA to 96-well dishes (Costar) (6×10^4 cells per well) coated with FN-H89 or sVCAM-1 (100 μl per well). After incubation for 20 min at 37 °C, unbound cells were removed by three washes with RPMI medium, and adhered cells were quantified with a fluorescence analyser (Cyto-Fluor 2300; Millipore Co.). Homotypic cell aggregation assays were performed essentially as described [36], and the degree of cell aggregation was measured at 3, 7 and 20 h in a semi-quantitative manner by the method of Rothlein and Springer [35].

RESULTS

Expression of mutated $\alpha 4$ subunits

Previous studies showed that most anti- $\alpha 4$ mAbs that inhibited VLA-4-mediated cell adhesion to fibronectin and VCAM-1 mapped in the first N-terminal 200 residues of the $\alpha 4$ subunit

Figure 2 Flow cytometry analysis of K562 $\alpha 4$ transfectants

K562 cells expressing transfected wild-type $\alpha 4$ subunit (4M7 cells) and the indicated $\alpha 4$ mutant cells were incubated at 4 °C for 40 min with saturating concentrations of different anti- $\alpha 4$, anti- $\beta 1$ or control mAbs. After washing, cells were incubated at 4 °C for 30 min with FITC-conjugated secondary antibody (Dako, Glostrup, Denmark), and finally analysed with a Coulter Epics XL flow cytometer.

[37,38]. Inside this portion of $\alpha 4$ there are several regions ranging from four to ten residues long that have high indices of hydrophilicity and surface probability (Figure 1A). Within these regions, peak indices correspond to residues 89 and 90, 101 and 102, 123 and 124, 153 and 154, and 172 and 173. We have previously reported that residues Arg⁸⁹Asp⁹⁰ are important in the structure of the $\alpha 4$ epitope A [21]. In the present study we mutated the $\alpha 4$ residues Asn¹²³Glu¹²⁴, Asp¹⁵³Tyr¹⁵⁴ and Phe¹⁷²Tyr¹⁷³, to investigate their role in the structure and function of VLA-4. The mutated full-length $\alpha 4$ cDNA species in the expression vector pFNeo were transfected into VLA-4-negative

K562 cells and the resulting geneticin-resistant transfectants were designated as indicated in Figure 1(B). Expression of VLA-4 by the transfectants was analysed by flow cytometry with anti- $\alpha 4$ mAbs that recognize different epitopes on the $\alpha 4$ subunit. VLA-4 expressed by 4M7 wild type, as well as by NE(123–124)HA and FY(172–173)VN mutant $\alpha 4$ transfectants, showed a similar reactivity with all anti- $\alpha 4$ mAbs (Figure 2A). However, VLA-4 on DY(153–154)AS transfectants exhibited a decreased reactivity with epitope B2 HP2/4, ALC1/4.1 and ALC1/5.1 anti- $\alpha 4$ mAbs compared with HP1/1, HP2/1 and B-5G10 (Figures 2A and 2B). In contrast, 4M7 (Figure 2B), NE(123–

Figure 3 Flow cytometry analysis of $\alpha 4$ transfectants expressing mutations at residues 151–158

The transfectants were incubated with the indicated anti- $\alpha 4$, anti- $\beta 1$ or control mAbs and processed as mentioned in the legend to Figure 2.

124)HA and FY(172–173)VN (results not shown), bound ALC1/4.1 and ALC1/5.1 at levels comparable to epitope A, B1 and C anti- $\alpha 4$ mAbs. We next generated additional $\alpha 4$ transfectants mutated at residues around Asp¹⁵³Tyr¹⁵⁴, in accordance with the scheme shown in Figure 1(B). Transfectants expressing VLA-4 mutated at $\alpha 4$ Tyr¹⁵¹ and Gln¹⁵²Asp¹⁵³ displayed a decrease in their reactivity with HP2/4 and a partial or total abolition of ALC1/4.1 and ALC1/5.1 binding, compared with epitopes A, B1 and C anti- $\alpha 4$ mAbs (Figure 3). Reactivity with ALC1/5.1 was still absent from transfectants mutated at $\alpha 4$ residues Tyr¹⁵⁴Val¹⁵⁵, whereas partial and total binding was obtained with ALC1/4.1 and HP2/4 respectively (Figure 3). Transfectants expressing mutations at $\alpha 4$ residues Val¹⁵⁵Lys¹⁵⁶ reacted poorly with ALC1/4.1, whereas all the anti- $\alpha 4$ mAbs reacted similarly with the $\alpha 4$ subunit mutated at Lys¹⁵⁷Phe¹⁵⁸ (Figure 3). These results showed that mutations at residues Tyr¹⁵¹, Gln¹⁵², Asp¹⁵³, Tyr¹⁵⁴ and Val¹⁵⁵ locally affected the reactivity of epitope B2 but not that of epitope A, B1 and C anti- $\alpha 4$ mAbs, indicating that the overall VLA-4 conformation was maintained.

Adhesion of $\alpha 4$ mutant transfectants to FN-H89 and to sVCAM-1

To investigate the effect of mutations at $\alpha 4$ residues 151–158 on cell adhesion to the FN-H89 fragment of fibronectin (which contains the CS-1 region) and to sVCAM-1, we incubated the different mutants as well as wild-type $\alpha 4$ transfectants with increasing coating concentrations of both ligands and measured

the extent of adhered cells. Adhesion of QD(152–153)EA transfectants to FN-H89 was substantially impaired compared with adhesion attained by wild-type 4M7 cells or other $\alpha 4$ mutant transfectants (Figure 4). The decrease in QD(152–153)EA cell adhesion to FN-H89 related to that of 4M7 cells ranged between 55 % and 75 %. Adhesion levels to increasing concentrations of sVCAM-1 were similar among the different mutant and wild-type $\alpha 4$ transfectants, although the extent of the adhesion of QD(152–153)EA transfectants to low sVCAM-1 concentrations was slightly lower than that of the remaining cells, but they achieved comparable adhesion at the higher concentrations (Figure 4). Transfectants expressing mutations at $\alpha 4$ residues Asn¹²³Glu¹²⁴ and Phe¹⁷²Tyr¹⁷³ adhered to FN-H89 and sVCAM-1 similarly to 4M7 cells (results not shown).

Expression of HUTS-21 epitope on mutant VLA-4

The anti- $\beta 1$ HUTS-21 mAb binds to an epitope on the $\beta 1$ subunit that is induced by Mn²⁺ ions and ligands of VLA heterodimers, and its expression correlates with functional activation and ligand-binding activity of the VLA proteins [11,12]. Thus the HUTS-21 mAb can be used as an indicator of the extent of ligand bound by a VLA heterodimer. To try to understand the mechanisms underlying the decrease in QD(152–153)EA cell adhesion to FN-H89, we first analysed by flow cytometry their expression of the HUTS-21 epitope in the absence or in the presence of Mn²⁺ ions, or after the addition of soluble FN-H89.

Figure 4 Adhesion of $\alpha 4$ transfectants to FN-H89 and sVCAM-1

Cells were labelled with BCECF-AM and added to 96-well microtitre plates coated with increasing concentrations of FN-H89 or sVCAM-1. After incubation for 20 min at 37 °C, plates were washed and adhesion was quantified using a fluorescence analyser. Each panel shows a representative result from at least five experiments and each point is the mean of triplicate determinations, with a standard deviation of less than 10%. K562 cells showed no adhesion at any concentration of FN-H89 or sVCAM-1 tested (results not shown).

Table 2 Expression of HUTS-21 epitope on $\alpha 4$ transfectants

Cells were incubated in PBS in the absence or in the presence of 0.5 mM MnCl_2 , or with 15 $\mu\text{g/ml}$ of FN-H89, at 37 °C for 15 min with saturating concentrations of the anti- $\beta 1$ HUTS-21 mAb. After being washed, cells were incubated at 4 °C with FITC-conjugated secondary antibody and analysed in a flow cytometer. The data are presented as the percentage of cells positive for HUTS-21 epitope expression, after subtracting negative control (P3) values, and correspond to a representative experiment of six. The expression of VLA-5 was similar between the three transfectants and did not change in the presence of Mn^{2+} ions.

Cells	Normalized expression (% of cells positive)		
	— Mn^{2+}	+ Mn^{2+}	+ FN-H89
4M7	30	71	48
QD(152–153)EA	16	44	28
DY(153–154)AS	35	70	46

Table 3 Effect of different stimuli on the adhesion of 4M7, QD(152–153)EA and DY(153–154)AS $\alpha 4$ transfectants to FN-H89

After being labelled with BCECF-AM, cells were preincubated for 15 min at 37 °C with 2 $\mu\text{g/ml}$ anti- $\beta 1$ TS2/16 mAb or control P3 mAb, or with 0.5 M MnCl_2 , and assayed for adhesion to FN-H89 (5 $\mu\text{g/ml}$). Adhesion was performed at 37 °C for 20 min and quantified in a fluorescence analyser. Values indicate cells bound/ mm^2 . Basal adhesions to wells without FN-H89 were less than 30 cells bound/ mm^2 . A representative result from five separate experiments is shown.

Cells	Stimulus ...	Adhesion (cells bound/ mm^2)		
		P3	TS2/16	Mn^{2+}
4M7		891	1197	1072
QD(152–153)EA		364	1074	758
DY(153–154)AS		766	1079	886

Whereas 100 % of the cells expressed the $\beta 1$ subunit, as detected with the TS2/16 mAb (results not shown), in the absence of Mn^{2+} ions the QD(152–153)EA transfectants displayed lower expression levels of the $\beta 1$ HUTS-21 epitope than 4M7 and DY(153–154)AS cells (Table 2). On addition of Mn^{2+} ions, the expression of this epitope on the QD(152–153)EA transfectants increased significantly, although it did not reach the levels of 4M7 and DY(153–154)AS. The binding of soluble FN-H89 induced similarly the HUTS-21 epitope expression on the three transfectants, but the level of expression of this epitope on QD(152–153)EA was also lower than that of 4M7 and DY(153–154)AS (Table 2). In addition, the specific binding of ^{125}I -FN-H89 to QD(152–153)EA transfectants was severely decreased compared with the binding to 4M7 cells (results not shown). These results indicated that there was a deficient binding of FN-H89 to VLA-4 on the QD(152–153)EA transfectants, which was correlated with a lower degree of functional activation of this integrin.

Effect of stimulating agents on QD(152–153)EA cell adhesion to FN-H89

To investigate whether the decrease in adhesion efficiency to FN-H89 shown by QD(152–153)EA transfectants could be reversed by different stimuli, we preincubated them, as well as 4M7 and DY(153–154)AS, with the anti- $\beta 1$ mAb TS2/16 or with Mn^{2+}

ions. Incubation of QD(152–153)EA cells with TS2/16 resulted in an increase in adhesion to FN-H89 to levels similar to those of 4M7 and DY(153–154)AS transfectants (Table 3). Addition of Mn^{2+} ions also increased the adhesion of QD(152–153)EA cells to FN-H89, although the degree of induction was lower than with mAb TS2/16 (Table 3). Thus the partly active conformation of VLA-4 on QD(152–153)EA cells was modified by TS2/16 or Mn^{2+} ions, which are known to increase the ligand-binding affinity of VLA integrins, and enabled these cells to adhere efficiently to FN-H89.

Homotypic aggregation of $\alpha 4$ mutant transfectants triggered by anti- $\alpha 4$ mAbs

To analyse the effect of the $\alpha 4$ mutations on homotypic cell aggregation, we incubated the transfectants with epitope B2 mAb HP2/4 or with the epitope A anti- $\alpha 4$ mAb HP1/7. After 3 h at 37 °C, the wild-type 4M7 and mutant Y151Q, QD(152–153)EA, DY(153–154)AS and VK(155–156)SQ $\alpha 4$ transfectants aggregated equally well in response to HP1/7 (Table 4). In the presence of HP2/4, the wild-type 4M7, VK(155–156)SQ and Y151Q transfectants exhibited large aggregates, whereas QD(152–153)EA and DY(153–154)AS failed to aggregate (Table 4). All $\alpha 4$ transfectants rapidly aggregated in the presence of the anti- $\beta 1$ Lial/2 and anti-CD43 mAb TP1/36, and failed to

Table 4 Homotypic aggregation of wild-type and mutant $\alpha 4$ transfectants induced by anti- $\alpha 4$ mAbs that recognize different epitopes on the $\alpha 4$ subunit

K562 or $\alpha 4$ -transfected K562 cells were incubated for 3 h with anti- $\beta 1$ mAb or with anti- $\alpha 4$ mAbs recognizing $\alpha 4$ epitope A (HP1/7), B1 (HP2/1), B2 (HP2/4) or C (B-5G10), and cell aggregation was scored visually: 0, no aggregation; 1, less than 10% of cells aggregating; 2, 10–50% of cells aggregating; 3, 50–80% of cells aggregating; 4, more than 80% of cells in small aggregates; 5, more than 90% of cells in large and compact aggregates. No aggregation and nearly 100% aggregation were determined with the control mAbs P3 and TP1/36 respectively.

Cells	Aggregation						
	P3	TP1/36 (CD43)	Lia1/2 ($\beta 1$)	$\alpha 4$			
				HP1/7	HP2/1	HP2/4	B-5G10
K562	0	5	0	0	0	0	0
4M7	0	5	5	5	2	5	0
QD(152–153)EA	0	5	5	5	2	2	0
DY(153–154)AS	0	5	5	4	2	1	0
VK(155–156)SQ	1	5	5	5	2	5	0
Y151Q	0	5	5	5	2	5	0

aggregate in the presence of control P3, or with the epitope C anti- $\alpha 4$ mAb B-5G10 (Table 4).

DISCUSSION

In the present study we found that $\alpha 4$ subunit residues Tyr¹⁵¹, Gln¹⁵², Asp¹⁵³, Tyr¹⁵⁴ and Val¹⁵⁵ form part of epitope B2 of $\alpha 4$ and are important for the structure of this epitope. Mutations at these residues affected the reactivity of VLA-4 expressed by K562 $\alpha 4$ transfectants with epitope B2 anti- $\alpha 4$ mAbs, but not with epitopes A, B1 and C anti- $\alpha 4$ mAbs, indicating that the mutations did not alter the overall conformation of VLA-4. Our results narrow to a few amino acid residues two previous reports that used $\alpha 4$ murine/human chimaeric constructs to map the $\alpha 4$ epitope B2 between residues 108 and 203 [37,38].

The epitope B2 of $\alpha 4$ was functionally defined as participating in VLA-4-mediated cell adhesion to fibronectin and VCAM-1 [20]. Here we report that transfectants expressing VLA-4 mutated at $\alpha 4$ Gln¹⁵²Asp¹⁵³ showed a substantial decrease in their adhesion to the CS-1-containing FN-H89 fragment of fibronectin. As transfectants expressing mutations at residues around Gln¹⁵² Asp¹⁵³, such as Y151Q, DY(153–154)AS and YV(154–155)LS, adhered to this fragment similarly to 4M7 wild-type $\alpha 4$ transfectants, this indicated that the main mutation affecting cell adhesion to FN-H89 corresponded to Gln¹⁵². The basis for the decrease in the adhesion arises from an inefficient binding of FN-H89 by VLA-4 mutated at $\alpha 4$ Gln¹⁵². This is probably due to an alteration in the state of activation of mutated VLA-4, which we have detected with HUTS-21, an anti- $\beta 1$ antibody that reacts mainly with functionally active VLA integrins [11,12]. This conclusion is supported by the fact that stimuli that increase the ligand-binding affinity of VLA heterodimers, such as TS2/16 and Mn²⁺ [39,40], augmented the adhesion of Gln¹⁵² $\alpha 4$ mutant transfectants to FN-H89 to levels similar to 4M7 cells. The results indicate that mutation at $\alpha 4$ Gln¹⁵² affects VLA-4 adhesion to FN-H89 in the initial phases, i.e. ligand binding, of the adhesion cascade, and that if no stimulus is added, the whole process of adhesion will be compromised. Therefore this residue modulates cell adhesion to FN-H89 by playing an important role in the maintenance and/or acquisition of an active state of VLA-4. The importance of Gln¹⁵² is emphasized by the fact that even if VLA-4 that had been mutated at $\alpha 4$ Tyr¹⁵¹, Asp¹⁵³, Tyr¹⁵⁴ and Val¹⁵⁵ showed a decreased reactivity with epitope B2 anti- $\alpha 4$ mAbs, the heterodimer retains a totally active conformation for adhesion to FN-H89.

Adhesion to sVCAM-1 was not significantly altered by the $\alpha 4$ mutations and we detected only a minimal decrease in the adhesion of $\alpha 4$ Gln¹⁵² mutant transfectants to low concentrations of sVCAM-1. The results suggest that this residue does not have a role in VLA-4 cell adhesion to VCAM-1, although the fact that VLA-4 displays a higher affinity for VCAM-1 than for CS-1 [7,32], might obscure a possible small implication of Gln¹⁵² in cell adhesion to VCAM-1.

According to the predicted β -propeller structure recently proposed by Springer for the seven sequence repeats of the $\alpha 4$ subunit [24], the ligand-binding site would be located on the upper surface of this structure. In this model Gln¹⁵² is situated on a loop connecting strand 4 of the W2 repeat with strand 1 of the W3 repeat. This loop is found on the upper surface of the β -propeller, indicating that it has the potential to participate in $\alpha 4$ ligand binding. A previous study showed that mutations at $\alpha 4$ Tyr¹⁸⁷ and Gly¹⁹⁰ severely affected VLA-4-mediated cell adhesion to both CS-1 and VCAM-1, as well as the binding of soluble VCAM-1 [41]. These residues are also located on the upper surface of the β -propeller structure on a loop connecting strands 2 and 3 of the W3 repeat. Both loops containing residues Gln¹⁵² and Tyr¹⁸⁷–Gly¹⁹⁰ are adjacent in the structure and have the ability to contact each other, as indicated by Springer [24]. The spatial closeness of Gln¹⁵² with Tyr¹⁸⁷ and Gly¹⁹⁰ is also suggested by results showing that the anti- $\alpha 4$ epitope B2 mAb HP2/4 binds distant regions in the $\alpha 4$ primary sequence covering approximately residues 100–200 [37,38], and other studies reported that mutations at $\alpha 4$ Gly¹⁹⁰ abolished binding of the anti- $\alpha 4$ epitope B2 mAb P4C2 [41]. This fact is probably the basis of anti- $\alpha 4$ epitope B2 mAb blocking of VLA-4-mediated cell adhesion to CS-1/fibronectin and VCAM-1 [20]. All these results indicate that an important interaction site for CS-1/fibronectin on the $\alpha 4$ subunit corresponds to residues Tyr¹⁸⁷ and Gly¹⁹⁰, and that Gln¹⁵² is probably associated with the ligand-binding site, having the potential to influence its conformation; it also indicates that, as suggested above, Gln¹⁵² is a good candidate for modulating VLA-4/fibronectin interactions. As VLA-4, as well as other integrins, is expressed on the cell surface in a range of multiple activation states [1,7], this modulation could have important roles in VLA-4-mediated cell adhesion in different processes including haemopoiesis [42], thymocyte maturation [43] and leucocyte migration [44–46]. The absence of significant effects of mutations at Gln¹⁵² on cell adhesion to VCAM-1 suggests that the modulation of this interaction could be different from VLA-4/fibronectin interaction. Accordingly, VLA-4 displays higher

affinity for VCAM-1 than for CS-1/fibronectin, and also the extent of stimulation of adhesion to VCAM-1 by different agents is usually lower than to CS-1/fibronectin [7].

Additional $\alpha 4$ residues that have been implicated in VLA-4-mediated cell adhesion include Cys²⁷⁸ and Cys⁷¹⁷, which modulate ligand binding, although their mutations do not alter cell adhesion [47]. Mutations at epitope A $\alpha 4$ residues Arg⁸⁹Asp⁹⁰ affect cell adhesion to CS-1/fibronectin [21], most probably due to post-ligand binding events (M. Muñoz, M. Guerrero and J. Teixido, unpublished work). In addition, the $\alpha 4$ putative bivalent-cation-binding sites and the cytoplasmic $\alpha 4$ tail modulate cell adhesion to VLA-4 ligands [48,49], and it has been suggested that this later region of $\alpha 4$ could be implicated in the regulation of the adhesion strengthening phase [50].

In the present study we have also identified residues Gln¹⁵², Asp¹⁵³ and Tyr¹⁵⁴ as part of the $\alpha 4$ epitope B2 involved in homotypic cell aggregation. VLA-4, as well as the cytoskeletal proteins F-actin and talin, redistribute to areas of cell contact on cell aggregation induced by anti- $\alpha 4$ mAbs [51], and it has been reported that the $\alpha 4$ integrin subunit can interact with $\alpha 4\beta 1$ and $\alpha 4\beta 7$ [52], suggesting that cells could use the $\alpha 4$ integrins to adhere in a homotypic manner. This type of adhesion mediated by VLA-4 could have a role in the initial stages of melanoma metastasis [53] and sarcoma invasion [54].

In summary, the results from the present study further expand the knowledge of mechanisms controlling VLA-4-mediated adhesion, and contribute to the understanding of cellular responses in several differentiation and migration processes.

We thank José Luis Alonso and Reyes Tejedor for their help in the generation of sVCAM-1; Dr. Martin J. Humphries for the gift of fibronectin recombinants; Dr. Carlos Cabañas, Dr. Martin E. Hemler, Dr. Carl G. Figdor and Dr. Angel Corbi for providing different antibodies; and Dr. Juan José Calvete and Dr. Mario García for help in the analyses of the $\alpha 4$ structure. This work was supported by grants from SmithKline Beecham-CDTI and PM95-0017 from the PGC-MEC (Spain).

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