Identification of putative ligand-binding sites of the integrin $\alpha 4\beta 1$ (VLA-4, CD49d/CD29)

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Integrin $\alpha 4\beta 1$ recognizes both fibronectin (CS-1 sequence) and vascular cell adhesion molecule-1 (VCAM-1). To localize the ligand-binding sites of $\alpha 4$, we located the epitopes for function-blocking anti- $\alpha 4$ monoclonal antibodies (mAbs), including those that recognize previously described (but not yet physically localized) functional epitopes (A, B1, B2 and C) using interspecies $\alpha 4$ chimeras expressed in mammalian cells. Epitopes B1 and B2 were associated with ligand binding, and epitopes A and B2 with homotypic cellular aggregation. mAbs P4C2 (epitope B2), 20E4 and PS/2 were mapped within residues 108–182; mAbs HP2/1 (epitope B1), SG/73 and R1-2 within residues 195–268; mAbs HP1/3 (epitope A) and P4G9 within residues 1–38; and B5G10 (epitope C) within residues 269–548. The data suggest that residues 108–268, which do not include bivalent-cation-binding

motifs, are related to VCAM-1 and CS-1 binding, and more N-terminal portions of $\alpha 4$ (residues 1–38 and 108–182) to homotypic aggregation. Since mAbs PS/2 and HP2/1 block $\alpha 4\beta 7$ binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1), the MAdCAM-1-binding site is close to, or overlapping with, VCAM-1- and CS-1-binding sites. The role of Asp-130 of $\beta 1$ in the binding to VCAM-1 and CS-1 peptide was examined. Chinese hamster ovary (CHO) cells expressing $\beta 1$ (D130A) (Asp-130 to Ala mutant of $\beta 1$) and $\alpha 4$ showed much less binding to both ligands than CHO cells expressing wild-type $\beta 1$ and $\alpha 4$ [a dominant negative effect of $\beta 1$ (D130A)], suggesting that Asp-130 of $\beta 1$ is critical for binding to both ligands and that the two ligands share common binding mechanisms.

INTRODUCTION

The integrins are a family of heterodimers of cell-surface receptors that are composed of at least eight β subunits and 15 distinct α subunits. They recognize multiple ligands and mediate cell–cell and cell–extracellular matrix interactions [1–6]. Integrin $\alpha 4\beta 1$ [very late antigen 4 (VLA-4), CD49d/CD29] recognizes both the IIICS region of fibronectin (CS-1 and CS-5 peptide sequences) [7–10] and vascular cell adhesion molecule-1 (VCAM-1) [11], which is expressed on activated endothelial cells [12,13] and constitutively expressed on bone-marrow stromal cells [14]. $\alpha 4\beta 1$ is possibly involved in the interaction of leucocytes with activated endothelial cells (e.g. atherosclerosis formation [15], experimental allergic encephalitis [16]) and in the interaction of lymphocyte precursor with bone-marrow stromal cells [14,17]. The $\alpha 4$ subunit is also involved in $\alpha 4\beta 7$ ($\alpha 4\beta p$), which has been implicated in lymphocyte adhesion to Peyer's patch high endothelial venules through interaction with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) [18]. MAdCAM-1 binds to $\alpha 4\beta 7$, but not to $\alpha 4\beta 1$ [19,20]. VCAM-1 binds to $\alpha 4\beta 1$, and only weakly to $\alpha 4\beta 7$ [19].

It has not been determined whether multiple ligands bind to distinct or identical binding sites of $\alpha 4\beta 1$. It has been reported that $\alpha 4\beta 1$ binding to VCAM-1 and fibronectin 40 kDa fragment (containing CS-1 sequence) is independently inhibitable [11,21] and that binding sites for different ligands are distinct, although located in the same region [21]. It has also been reported that CS-1 peptide and VCAM-1 compete for binding to $\alpha 4\beta 1$, and therefore they bind to identical or very close sites [22]. To resolve the controversy, more information about ligand-binding sites of

$\alpha 4\beta 1$ is required. In this report, we localized the putative ligand-binding sites within the $\alpha 4$ subunit by mapping function-blocking anti- $\alpha 4$ antibodies using interspecies chimeras. The data indicate that the putative binding sites for VCAM-1, CS-1 and MAdCAM-1 are mapped within a small region (residues 107–268) of $\alpha 4$. We also describe that a mutant of $\beta 1$, $\beta 1$ (D130A) [23] exerts a dominant negative effect on the binding of $\alpha 4\beta 1$ to both CS-1 and VCAM-1, suggesting Asp-130 residues of $\beta 1$ are essential to binding of $\alpha 4\beta 1$ to both ligands, and both ligands share common binding mechanisms.

MATERIALS AND METHODS

Materials

Anti-(human $\alpha 4$) monoclonal antibody (mAb), B5G10 [24], was obtained from M. E. Hemler (Dana-Farber Cancer Institute, Boston, MA, U.S.A.). mAbs HP1/3 and HP2/1 [25] were from F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain), P4G9 and P4C2 [7] from E. Wayner (University of Minnesota, Minneapolis, MN, U.S.A.), 20E4 (aggregation-inducing; B. McIntyre, unpublished work) from B. McIntyre (M.D. Anderson Cancer Center, Houston, TX, U.S.A.), SG/73 [26] from K. Miyake (Saga Medical School, Saga, Japan). Two anti-(mouse $\alpha 4$) mAbs PS/2 [27] and R1-2 [28] were purchased from the American Type Culture Collection. 7E2 (to hamster $\beta 1$), PB1 (to hamster $\alpha 5$) were obtained from R. L. Juliano (University of North Carolina, Chapel Hill, NC, U.S.A.) and A1A5 (to human $\beta 1$) from M. E. Hemler.

Abbreviations used: CHO, Chinese hamster ovary; FACS, fluorescence-activated cell sorter; mAbs, monoclonal antibodies; MAdCAM-1, mucosal addressin cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; VLA, very late antigen.

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Construction of wild-type, chimeric and mutant cDNAs

The *EcoRV* fragment of human $\alpha 4$ cDNA [29], containing the whole coding region, was subcloned into the *XbaI* site of pBJ-1 vector with SR α promoter [30] using *XbaI* linker. The *XbaI*-*NheI* fragment of wild-type $\beta 1$ or mutant $\beta 1$ (D130A) cDNA [23] was also subcloned into the pBJ-1 vector. cDNAs for m182/h $\alpha 4$ (N-terminal 182 amino acid residues are from mouse $\alpha 4$ and the rest from human $\alpha 4$), m194/h $\alpha 4$, m268/h $\alpha 4$ and m548/h $\alpha 4$ chimeras were prepared by fusing mouse $\alpha 4$ cDNA (pZ5/6) [31] and human $\alpha 4$ cDNA [29] using common *ApaI*, *AccI*, *HindIII* and *SpyI* restriction sites respectively. cDNAs for m38/h $\alpha 4$ and m107/h $\alpha 4$ were prepared by amplifying mouse cDNA fragments coding N-terminal 38 and 107 residues with artificial *SmaI* and *BamHI* sites at the 3' boundaries using synthetic oligonucleotides 5'-GTAAATCGCCCCGGGATTGACC-3' and 5'-CAAGTC-ACGATGGATCCATTTTCTCC-3' respectively, and fusing them with human $\alpha 4$ fragments coding the rest of the $\alpha 4$ molecule. Authenticity of the PCR products was confirmed by sequencing. All of the chimeric $\alpha 4$ cDNAs were subcloned into pBJ-1 expression vector except m268/h $\alpha 4$ cDNA that was subcloned into CDM8 vector [32].

Transfection and selection of the expressing cells

Chinese hamster ovary (CHO)-K1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal-calf serum at 37 °C in a 6% CO₂ incubator. A sample (10 μ g) of the wild-type or chimeric $\alpha 4$ cDNA construct was transfected into CHO-K1 cells (8×10^6 cells) with 1 μ g of pFneo DNA by electroporation. Transfected cells were maintained for 2 days in the above medium, and then transferred to Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal-calf serum and 700 μ g/ml G418 (Gibco). After 10–14 days, resulting colonies were harvested. Usually 30–50% of cells express $\alpha 4$ as detected by fluorescence-activated cell sorter (FACS) analysis. CHO cells expressing high levels of $\alpha 4$ were cloned by sorting with mAb B5G10 in FACStar (Becton-Dickinson) (designated $\alpha 4$ -CHO cells). To obtain CHO cells stably expressing $\alpha 4\beta 1$ and $\alpha 4\beta 1$ (D130A), $\beta 1$ or $\beta 1$ (D130A) cDNA constructs (10 μ g) were transfected into clonal $\alpha 4$ -CHO cells with pCD-hygromycin (1 μ g) by electroporation. The transfected cells were selected with 500 μ g/ml hygromycin for 10–14 days. CHO cells expressing high-level human $\beta 1$ or $\beta 1$ (D130A) were cloned by sorting as described above with anti-(human $\beta 1$) mAb A1A5 (designated $\alpha 4\beta 1$ - and $\alpha 4\beta 1$ (D130A)-CHO cells respectively).

Adhesion assay

Wells of 96-well Immulon-2 microtitre plates were coated first with 100 μ l of anti-(mouse IgG) κ chain (2 μ g/ml) (Caltag Laboratories, San Francisco, CA, U.S.A.) in PBS (10 mM phosphate, 0.15 M NaCl, pH 7.4) and incubated overnight at 4 °C and then with 100 μ l of recombinant VCAM-1/mouse C κ chain hybrid (2 μ g/ml) (a kind gift from D. Dottavio, Sandoz Pharmaceuticals, East Hanover, NJ, U.S.A.) in PBS, and incubated for 1 h at room temperature. Remaining protein-binding sites were blocked by incubating with 1% BSA for 1 h at room temperature. Cells (1×10^5 cells per well) in 100 μ l of Dulbecco's modified Eagle's medium unsupplemented with serum were added to the wells and incubated for 1 h at 37 °C. After gently rinsing the wells three times with PBS to remove unbound cells, bound cells were determined by assaying endogenous phosphatase activity [33].

Other methods

Immunoprecipitation of surface-labelled cell extract and FACS analysis were carried out as described [23,34].

RESULTS

Expression of wild-type and chimeric $\alpha 4$ on CHO cells

We localized the putative ligand-binding sites on the $\alpha 4$ subunit for VCAM-1, CS-1 and MAdCAM-1 by mapping function-blocking anti- $\alpha 4$ mAbs using interspecies chimeric $\alpha 4$ subunits. The rationale of the epitope mapping using interspecies chimeras is that, although human $\alpha 4$ and mouse $\alpha 4$ are more than 85% homologous [31], anti-(human $\alpha 4$) or anti-(mouse $\alpha 4$) mAbs recognize only human and mouse $\alpha 4$ respectively. We constructed chimeric $\alpha 4$ cDNAs between human and mouse $\alpha 4$, which contained various lengths of mouse $\alpha 4$ sequence on the N-terminal side (Figure 1), and these cDNAs were used to transfect CHO cells. Wild-type and chimeric $\alpha 4$ chains were expressed stably on CHO cells. Flow cytometric analysis showed that at least 30% of the G418-resistant population of transfected CHO cells express wild-type or chimeric $\alpha 4$ subunit (Table 1).

Immunoprecipitation of surface-labelled cell extract (Figure 2) showed protein bands that correspond to $\alpha 4$ subunit (140 kDa) and its fragments (80 kDa and 70 kDa) [24]. An associated band (110 kDa) is most probably endogenous hamster $\beta 1$ subunit. Ligand-binding functions of CHO cells expressing chimeric $\alpha 4$ subunits were examined. All the CHO cells expressing wild-type or chimeric $\alpha 4$ bound to VCAM-1, but parent CHO cells did not (Figure 3), suggesting that chimeric $\alpha 4$ subunits are functional and properly folded.

Localization of epitopes for function-blocking anti- $\alpha 4$ mAbs

The reactivities of anti- $\alpha 4$ mAbs towards the wild-type or chimeric $\alpha 4$ on CHO cells were tested by flow cytometry (Table 1). All the anti-(human $\alpha 4$) mAbs tested recognize human $\alpha 4$ but not mouse $\alpha 4$. The two anti-(mouse $\alpha 4$) mAbs tested (PS/2 and

Figure 1 Mouse/human $\alpha 4$ chimeras

Table 1 The reactivities of anti- $\alpha 4$ mAbs to mouse/human $\alpha 4$ chimeras detected by FACS

CHO cells stably expressing different $\alpha 4$ chimeras were stained by mAbs, followed by fluorescein isothiocyanate-labelled secondary anti-(mouse IgG) or anti-(rat IgG) antibodies and analysed using FACS. The numbers in the Table represent the percentage of positive cells. /+ represents positive reactivity. CHO cells expressing human wild-type (wt) $\alpha 4$ were cloned by sorting to obtain homogeneous cells.

Antibody	CHO cells	Human wt	m38/h	m107/h	m182/h	m194/h	m268/h	m548/h	Mouse wt
Mouse IgG	1.2	2.5	3.5	2.6	4.7	4.1	3.5	3.4	2.8
HP1/3 (A)	0.9	97.4/+	4.2	2.6	3.3	3.6	3.4	3.1	2.9
P4G9	1.4	97.3/+	3.4	1.9	2.7	2.6	2.7	2.7	1.8
P4C2 (B2)	1.5	94.9/+	40.4/+	56.9/+	2.5	2.8	2.5	2.7	2.6
20E4	1.1	95.7/+	31.2/+	42.6/+	6.7	4.6	4.4	4.6	2.7
HP2/1 (B1)	1.0	97.2/+	47.2/+	63.8/+	50.7/+	44.2/+	4.0	2.8	3.2
SG/73	1.5	96.0/+	34.0/+	47.5/+	47.7/+	43.2/+	2.9	2.9	2.9
B5G10 (C)	2.1	98.4/+	42.9/+	75.6/+	69.0/+	48.5/+	35.5/+	1.2	1.2
PS/2*	3.0	0.8	2.4	2.3	61.9/+	37.5/+	30.7/+	31.4/+	68.6/+
R1-2*	2.4	0.9	1.3	1.0	1.0	2.1	30.0/+	30.0/+	67.0/+

* Anti-(mouse $\alpha 4$) mAbs; A, B1, B2 or C, anti-(human $\alpha 4$) mAbs assigned to epitope A, B1, B2 or C respectively.

Figure 2 Immunoprecipitation of ^{125}I -surface-labelled CHO cells expressing wild-type or chimeric $\alpha 4$

CHO cells expressing wild-type or chimeric $\alpha 4$ were surface-labelled with ^{125}I using iodogen according to the manufacturer's instructions. Lysates were immunoprecipitated with mAbs specific for mouse or human $\alpha 4$ (PS/2 and SG/73, respectively) or rabbit polyclonal anti- $\alpha 4$ antibody that reacts with both human and mouse $\alpha 4$. Samples were analysed in a 7% gel under non-reducing conditions. The data show chimeric $\alpha 4$ subunits are expressed on CHO cells as heterodimers with endogenous hamster $\beta 1$ subunit. Rabbit preimmune serum does not precipitate $\alpha 4$ or $\beta 1$ (results not shown) from lysates of CHO cells used. Parent CHO cells do not express $\alpha 4$.

R1-2) recognized mouse $\alpha 4$ but not human $\alpha 4$. P4G9 [7] and HP1/3 recognize wild-type $\alpha 4$ but no chimeras, including m38/h $\alpha 4$, suggesting that P4G9 and HP1/3 recognize epitopes within

Figure 3 Adhesion of CHO cells expressing mouse/human $\alpha 4$ chimeras to VCAM-1

CHO cells expressing wild-type or chimeric $\alpha 4$ were incubated with VCAM-1 or BSA immobilized on plastic wells for 1 h at 37 °C. After rinsing the wells to remove unbound cells, the adherent cells were quantified by endogenous phosphatase assay. Transfected CHO cells, after G418 selection, were used without further enrichment of the $\alpha 4$ -positive population, except for human $\alpha 4$ -expressing CHO cells that are clonal. Data (% of bound cells) are expressed as means $\pm$ S.D. of triplicate experiments. Expression of $\alpha 4$ was determined by flow cytometry with mAbs B5G10 or PS/2 (only for mouse wild-type and m548/h $\alpha 4$). The data show wild-type or chimeric $\alpha 4$ have VCAM-1 binding function, which roughly correlates with the level of $\alpha 4$ -positive cells in the G418-resistant population.

residues 1–38 of $\alpha 4$. P4C2 [7] and 20E4 reacted only with m38/h $\alpha 4$ and m107/h $\alpha 4$, indicating that these mAbs recognize residues 108–182. SG/73 [26] and HP2/1 reacted with all the $\alpha 4$ chimeras except m268/h and m548/h, suggesting that SG/73 and HP2/1 recognize epitopes within residues 195–268. Anti-(mouse $\alpha 4$) mAb PS/2 recognized m182/h to m548/h $\alpha 4$, but not m38/h nor m107/h $\alpha 4$, suggesting that PS/2 recognizes epitopes within residues 108–182. Another anti-(mouse $\alpha 4$) mAb R1-2 recognizes mouse wild-type, m268/h and m548/h $\alpha 4$, but no other $\alpha 4$ chimeras, indicating that R1-2 recognizes residues 195–268. mAb B5G10 reacted with all the $\alpha 4$ chimeras except for m548/h $\alpha 4$, suggesting that B5G10 recognizes an epitope within residues 269–548 of $\alpha 4$. If we assume that function-blocking mAbs bind close to the ligand-binding sites, the present data suggest the putative ligand-binding sites may be located within or around residues 108–268 of $\alpha 4$.

Figure 4 Profiles of $\beta 1$ integrin expression on $\alpha 4\beta 1$ - and $\alpha 4\beta 1(D130A)$ -CHO cells

Clonal CHO cells stably expressing $\alpha 4\beta 1$ and $\alpha 4\beta 1(D130A)$ integrin were stained with mAbs specific to human $\alpha 4$ (B5G10), human $\beta 1$ (A1A5), hamster $\alpha 5$ (PB1) or hamster $\beta 1$ (7E2), followed by fluorescein isothiocyanate-labelled goat anti-(mouse IgG) and analysed using FACScan (Becton-Dickinson). Mean fluorescence intensities are 276, 216 and 4.1 for human $\beta 1$; 209, 209 and 4.4 for human $\alpha 4$; 184, 123 and 315 for hamster $\beta 1$; 107, 101 and 86 for hamster $\alpha 5$ [to $\alpha 4\beta 1$ -CHO, $\alpha 4\beta 1(D130A)$ -CHO and parent CHO cells respectively].

The effects of the D130A mutation of $\beta 1$ on binding of VCAM-1 and CS-1 to $\alpha 4\beta 1$

We previously reported that Asp-130 of $\beta 1$ is critical for ligand binding of $\alpha 5\beta 1$ [23]. To ask whether VCAM-1 and CS-1 binding to $\alpha 4\beta 1$ share the same binding mechanism, we examined the effect of the Asp-130 to Ala mutation (D130A) on the ligand-binding function of $\alpha 4\beta 1$. $\alpha 4\beta 1$ - or $\alpha 4\beta 1(D130A)$ -CHO cells were prepared by transfecting wild-type human $\beta 1$ or $\beta 1(D130A)$ cDNA into clonal $\alpha 4$ -CHO cells, followed by cloning to obtain cells expressing high-level $\beta 1$ or $\beta 1(D130A)$ as described in the Materials and methods section. Human $\alpha 4\beta 1$ and $\alpha 4\beta 1(D130A)$

expression in $\alpha 4\beta 1$ - and $\alpha 4\beta 1(D130A)$ -CHO cells are comparable and approx. 65% of $\beta 1$ is exogenous human $\beta 1$ in each case, as assessed by flow cytometric analysis after staining with specific mAbs (Figure 4). Immunoprecipitation with specific antibodies detected human $\alpha 4$ (140 kDa) and 80 kDa and 70 kDa fragments, and $\beta 1/\beta 1(D130A)$ (110 kDa) on the surface of $\alpha 4\beta 1$ - and $\alpha 4\beta 1(D130A)$ -CHO cells (Figure 5).

The binding of $\alpha 4\beta 1$ - and $\alpha 4\beta 1(D130A)$ -CHO cells to CS-1 peptide was examined (Figures 6a and 6b). Certain anti-(human $\beta 1$) mAbs, including 8A2, have been known to activate the ligand-binding function of $\beta 1$ integrins [35–38]. mAb 8A2 binds to wild-type $\beta 1$ and $\beta 1(D130A)$ mutant to the same extent, but

Figure 5 Immunoprecipitation of ^{125}I -surface-labelled $\alpha 4\beta 1$ - or $\alpha 4\beta 1(\text{D130A})$ -CHO cells

Samples were analysed in a 7% gel under non-reducing conditions. The $\alpha 4$ and $\beta 1$ subunits do not co-precipitate, but $\alpha 4$ and $\beta 1(\text{D130A})$ co-precipitated on immunoprecipitation under the conditions used. The association of $\alpha 4$ and $\beta 1$ has been reported to be weaker than that between other integrin α and β . The affinity of $\beta 1(\text{D130A})$ to $\alpha 4$ may be higher than that of $\beta 1$.

does not recognize (thus does not activate) hamster $\beta 1$ (results not shown). $\alpha 4\beta 1$ -CHO cells bind to CS-1 peptide-coated plastic only in the presence of activating anti-(human $\beta 1$) mAb 8A2 [36], suggesting the avidity to CS-1 is low without activation by mAb. In contrast, $\alpha 4\beta 1(\text{D130A})$ cells did not show any binding to CS-1 peptide-coated plastic even in the presence of mAb 8A2. $\alpha 4$ -CHO cells (expressing $\alpha 4$ /hamster $\beta 1$) show little or no binding to CS-1 peptide even in the presence of mAb 8A2 since mAb 8A2 activates only $\alpha 4$ /human $\beta 1$ but not $\alpha 4$ /hamster $\beta 1$ (results not shown). The results suggest that Asp-130 of $\beta 1$ is critical for binding of $\alpha 4\beta 1$ to CS-1 peptide.

The binding of $\alpha 4\beta 1$ - and $\alpha 4\beta 1(\text{D130A})$ -CHO cells to VCAM-1 was examined (Figure 6c). It has been reported that VCAM-1 has higher affinity towards $\alpha 4\beta 1$ than CS-1 peptide [39]. Consistently, mAb 8A2 did not significantly increase the adhesion of $\alpha 4\beta 1$ -, or $\alpha 4\beta 1(\text{D130A})$ -CHO cells to VCAM-1 (results not shown). Approx. 80% of added cells bind to VCAM-1 with $\alpha 4$ -CHO and $\alpha 4\beta 1$ -CHO cells at 0.25 $\mu\text{g}/\text{ml}$ coating concentration (Figure 6c) without activating mAb 8A2. $\alpha 4\beta 1(\text{D130A})$ -CHO cells showed significantly lower adhesion to VCAM-1 (only approx. 40% at the same VCAM-1 concentration) than $\alpha 4\beta 1$ -CHO cells. CHO cells show no detectable binding function to VCAM-1. Since human $\alpha 4$, human $\beta 1$ or $\beta 1(\text{D130A})$, hamster $\beta 1$ and hamster $\alpha 5$ levels on $\alpha 4\beta 1$ -CHO cells and $\alpha 4\beta 1(\text{D130A})$ -CHO cells are comparable (Figure 4), the $\alpha 4$ /human $\beta 1$ or $\beta 1(\text{D130A})$ level or $\alpha 4$ /hamster $\beta 1$ level on each cell line may be comparable. Therefore the difference of the VCAM-1 binding between $\alpha 4\beta 1$ -CHO and $\alpha 4\beta 1(\text{D130A})$ -CHO cells reflects the functional difference of $\alpha 4\beta 1$ and $\alpha 4\beta 1(\text{D130A})$ (the dominant negative effect of D130A mutation [23]). These data suggest that Asp-130 of $\beta 1$ is critical for the binding of $\alpha 4\beta 1$ to VCAM-1 as well.

DISCUSSION

In the present study we localized the epitopes for anti- $\alpha 4$ mAbs to identify the ligand-binding sites on the $\alpha 4$ subunit. The strategy we used to locate the ligand-binding sites is based on the assumption that function-blocking antibodies bind close to the ligand-binding sites. We recently located epitopes for function-blocking anti- $\alpha 2$ mAbs within a small region of the I domain of the $\alpha 2$ subunit [40] and identified the critical residues for ligand (collagen) binding within the epitope region by site-directed

Figure 6 Ligand-binding properties of $\alpha 4\beta 1$ - and $\alpha 4\beta 1(\text{D130A})$ -CHO cells

Wells of 96-well Immulon-2 microtitre plates were coated with CS-1 peptide (a and b) or recombinant VCAM-1/mouse C κ chain hybrid (c) as described in the Materials and methods section. Cells (1×10^5 cells per well) were added to the wells and incubated for 1 h at 37 $^{\circ}\text{C}$ in the presence (a) and absence (b and c) of mAb 8A2 and bound cells were determined after gently rinsing the wells. Data are shown as means $\pm$ S.D. of triplicate experiments. $\alpha 4$ - ($\circ$), $\alpha 4\beta 1$ - ($\square$), $\alpha 4\beta 1(\text{D130A})$ - ($\triangle$) and parent CHO cells ($\blacksquare$). mAb 8A2 was used as 1:500 dilution of ascites (a saturating concentration). In a separate experiment, only $1.6 \pm 0.7\%$ of CHO cells bound to VCAM-1, whereas 78 ± 3.2 and $81 \pm 3.6\%$ of $\alpha 4$ - and $\alpha 4\beta 1$ -CHO cells, respectively, bound at a 2 $\mu\text{g}/\text{ml}$ coating concentration, indicating CHO cells do not interact with VCAM-1.

mutagenesis [41]. This suggests that we will be able to locate the ligand-binding sites if we locate the epitopes for multiple function-blocking antibodies.

Some of the anti- $\alpha 4$ mAbs we mapped have been reported to recognize epitopes A, B1, B2 and C on the $\alpha 4$ subunit, which have been described based on the cross-competitive cell binding and protease-sensitivity assays [21], but not yet physically mapped. Epitopes B1 and B2 are related to cell attachment to

Figure 7 Localization of the putative ligand-binding sites on the $\alpha 4$ subunit

1, The fibrinogen γ -peptide cross-linking region of α IIb (residues 294–314 of α IIb). 2, the RGD peptide cross-linking region of α V (residues 139–349 of α V). *mAbs that block lymphocyte binding to purified MAdCAM-1 [19]. The mAbs HP1/3, P4C2, HP2/1 and B5G10 have been assigned to epitopes A, B2, B1 and C respectively [21]. The region spanning from residue 108 to 268 may be related to binding to ligands VCAM-1, CS-1 and MAdCAM-1 (the putative ligand-binding region). The epitopes A (residues 1–38) and B2 (residues 108–182) are related to $\alpha 4\beta 1$ -mediated homotypic cellular aggregation. Three regions shown with bold bar are bivalent-cation-binding motifs.

fibronectin and VCAM-1, but epitope A or C is not (or only slightly). The present mapping data suggest the putative VCAM-1- and CS-1-binding sites may be located within, or around, residues 108–268 of $\alpha 4$ (epitopes B1 and B2) (Figure 7). Also, HP2/1 and anti-(mouse $\alpha 4$) mAb PS/2 have been reported to block binding of mouse lymphocytes to purified MAdCAM-1 [19], and another anti-(mouse $\alpha 4$) mAb, R1-2, to block adhesion of TK-1 mouse lymphoma cells to frozen section of Peyer's patch [42]. Since PS/2 is mapped to residues 108–182 (B2) and HP2/1 and R1-2 to residues 195–268 (B1), MAdCAM-1-binding sites on the $\alpha 4$ subunit may also be close to, or overlapping with, VCAM-1- and CS-1-binding sites. The epitopes B1 and B2 that span residues 108 to 268 of $\alpha 4$ overlaps to the Arg-Gly-Asp (RGD) peptide cross-linking region of α V (residues 139–349 of α V) [43]. The fibrinogen γ -peptide cross-linking region of α IIb (residues 294–314 of α IIb, the cation-binding site of α IIb) [44] corresponds to the first bivalent-cation-binding site of $\alpha 4$ (residues 278–297 of $\alpha 4$). This region is not involved in the putative ligand-binding region of $\alpha 4$. Since the mutant within the corresponding cation-binding site of $\alpha 4$ (Asp-283 to Glu) still retains binding function to VCAM-1, invasin and CS-1 [45], the first bivalent-cation-binding site of $\alpha 4$ may not be critical for ligand binding. However, since the cation-binding site is very close to the putative ligand-binding site of $\alpha 4$, we can not exclude the possibility that the cation-binding domain of $\alpha 4$ is involved in ligand recognition and binding. Epitopes A and B2 have been reported to be related to $\alpha 4\beta 1$ -mediated homotypic cellular aggregation [21]. The present data suggest that the aggregation is related to a more N-terminal portion of the $\alpha 4$ subunit, suggesting that the aggregation is mediated by mechanisms distinct from that for VCAM-1 or CS-1 binding. Further studies to localize the critical residues for ligand binding around or within the putative ligand-binding sites of $\alpha 4$ will be needed to answer whether or not multiple ligands bind distinct binding sites of $\alpha 4$. The studies in this direction using site-directed mutagenesis or synthetic peptides are in progress.

Asp-130 is located in a region which is highly conserved among different integrin β subunits, and there is the EF hand-like structure around Asp-130. Arg-Gly-Asp (RGD) peptide

cross-links to the corresponding region of $\beta 3$ (109–171 of $\beta 3$) [46]. Also a thrombasthenic patient has a mutation in a corresponding residue Asp-119 (corresponding to Asp-130 of $\beta 1$) to Tyr of platelet α IIb $\beta 3$ (GPIIb-IIIa) $\beta 3$ subunit [47]. These findings underscore the importance of the region around Asp-130 of $\beta 1$ in receptor–ligand interactions. We previously established that a point mutation of Asp-130 to Ala in a highly conserved region of the $\beta 1$ subunit abrogates the RGD-dependent binding of $\alpha 5\beta 1$ to fibronectin and that the substitution also interferes with the recognition of invasin that lacks the RGD sequence. This suggests that Asp-130 is critical in multiple ligand– $\alpha 5\beta 1$ interactions [23]. Recently a point mutation of Asp-140 of $\beta 6$, which corresponds to Asp-130 of $\beta 1$ and Asp-119 of $\beta 3$, has been shown to abolish the binding of $\alpha v\beta 6$ to a ligand, fibronectin [48], suggesting the ubiquitous importance of the Asp residue in integrin–ligand interactions.

The Asp-130 to Ala substitution of $\beta 1$ does not result in a major structural change in the whole $\beta 1$ molecule (e.g. without disrupting gross structure or heterodimer assembly) [23]. The present paper shows that the mutation of Asp-130 of $\beta 1$ to Ala has a dominant negative effect on both VCAM-1 and CS-1– $\alpha 4\beta 1$ interactions, suggesting both ligands share a common ligand–receptor binding mechanism. While a chemical cross-linking study of ligand-derived peptide to $\alpha 4\beta 1$ has not been reported, the present findings suggest the possibility that CS-1 peptide or VCAM-1-derived peptides would be chemically cross-linked to the region around Asp-130 of $\beta 1$, if we assume that Asp-130 of $\beta 1$ is directly involved in the ligand binding. It remains to be established, however, whether Asp-130 is directly involved in the interactions with RGD or CS-1 sequences. One possibility could be that Asp-130 is electrostatically interacting with the arginine residue of the RGD sequence. However, it may be difficult to explain the interaction with the EILDVPST sequence in CS-1 peptide in the same way since the EILDVPST sequence has no positively charged residue.

Another possibility is that Asp-130 is indirectly involved in the VCAM-1 and CS-1 binding. In preliminary experiments with an activation-dependent anti- $\beta 1$ mAb, we found that the activation-dependent epitope is induced in wild-type $\beta 1/\alpha 5$ by an activating anti- $\beta 1$ mAb, but repressed by an inhibiting anti- $\beta 1$ mAb, while $\beta 1$ (D130A)/ $\alpha 5$ constitutively expresses the epitope and does not respond to the activating or inhibiting mAbs (W. Puzon, T. Yednock and Y. Takada, unpublished work). These findings suggest that $\alpha 5/\beta 1$ (D130A) mutant has a conformation frozen at an activated stage, although $\alpha 5/\beta 1$ (D130A) mutant is inactive, and that the D130A mutation may have an effect on the conformation of the $\beta 1$ subunit. Presumably D130A mutation of $\beta 1$ may have a similar effect on $\alpha 4\beta 1$ integrin. The study on the role of Asp-130 of $\beta 1$ in the regulation of integrin conformation is in progress.

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