Critical amino acid residues for ligand binding are clustered in a predicted β -turn of the third N-terminal repeat in the integrin $\alpha 4$ and $\alpha 5$ subunits

Atsushi Irie, Tetsuji Kamata,
Wilma Puzon-McLaughlin and
Yoshikazu Takada¹

Department of Vascular Biology, The Scripps Research Institute,
10666 North Torrey Pines Rd, La Jolla, CA 92037, USA

¹Corresponding author

Integrin $\alpha 4\beta 1$ is a receptor for vascular cell adhesion molecule (VCAM)-1 and fibronectin (CS-1). The $\alpha 4\beta 1$ -ligand interaction is involved in the pathogenesis of diseases and is, therefore, a therapeutic target. Here, we identified critical residues of $\alpha 4$ for ligand binding using alanine-scanning mutagenesis of the previously localized putative ligand binding sites (residues 108–268). Among 43 mutations tested, mutations of Tyr187, Trp188 and Gly190 significantly inhibited cell adhesion to both VCAM-1 and CS-1. This inhibition was not due to any gross structural changes of $\alpha 4\beta 1$. These critical residues are clustered in a predicted β -turn structure (residues 181–190) of the third N-terminal repeat in $\alpha 4$. The repeat does not contain divalent cation binding motifs. Notably, the mutations within the corresponding region of $\alpha 5$ significantly reduced fibronectin- $\alpha 5\beta 1$ interaction. These findings suggest that the predicted β -turn structure could be ubiquitously involved in ligand binding of non-I domain integrins.

Keywords: fibronectin/integrin/ligand binding/mutagenesis/VCAM-1

Introduction

Integrins are a superfamily of cell surface heterodimers, which mediate signal transduction through interactions with cellular or extracellular ligands (Yamada, 1991; Hogg *et al.*, 1992; Hynes, 1992; Hemler *et al.*, 1994; Springer, 1994). $\alpha 4\beta 1$ integrin recognizes vascular cell adhesion molecule (VCAM)-1 (Elices *et al.*, 1990), which is expressed on activated endothelial cells and constitutively on bone marrow stromal cells (Osborn *et al.*, 1989; Rice and Bevilacqua, 1989), and the alternatively spliced IIICS portion [connecting segment-1 (CS-1)] of fibronectin (Wayner *et al.*, 1989; Guan and Hynes, 1990; Mould *et al.*, 1990, 1991). Evidence is accumulating that $\alpha 4\beta 1$ plays a central role in leukocyte recruitment (for review, see Lobb and Hemler, 1994). $\alpha 4\beta 1$ integrin has been shown to initiate lymphocyte contract ('tethering') *in vitro* with vascular endothelial cells under shear and in the absence of a selectin contribution (Alon *et al.*, 1995; Berlin *et al.*, 1995). Anti- $\alpha 4$ monoclonal antibodies (mAbs) have shown therapeutic effects in numerous animal models of disease (e.g. experimental allergic encephalomyelitis, contact hypersensitivity, non-obese diabetes, allergic lung

inflammation, inflammatory bowel disease) (for review, see Lobb and Hemler, 1994). Therefore, the ligand- $\alpha 4\beta 1$ integrin interaction will be a therapeutic target for many diseases. Understanding of the ligand binding mechanism and identification of ligand binding sites are important for designing inhibitors that modulate the interactions. We recently localized the putative ligand binding site of the $\alpha 4$ subunit by mapping epitopes for function-blocking antibodies (Kamata *et al.*, 1995). Anti- $\alpha 4$ mAbs that block VCAM-1 and CS-1 to $\alpha 4\beta 1$ were mapped within residues 108–268 of $\alpha 4$, suggesting that VCAM-1 or CS-1 binding sites are close to or overlapping within $\alpha 4$.

In the present study, we have identified critical residues of $\alpha 4$ for VCAM-1 or CS-1 binding to $\alpha 4\beta 1$ within the putative ligand binding site of $\alpha 4$ using alanine-scanning mutagenesis (Cunningham and Wells, 1989). Mutations of Tyr187, Trp188 and Gly190 significantly reduced both VCAM-1 and CS-1 binding. These critical residues are clustered in a predicted β -turn structure (residues 181–190) of the third N-terminal repeat of the $\alpha 4$ subunit (Tuckwell *et al.*, 1994). The repeat does not involve the putative cation binding motifs. We show that mutations within the corresponding region of $\alpha 5$ significantly affect fibronectin- $\alpha 5\beta 1$ interaction, suggesting that the predicted β -turn structure of the α subunit may be ubiquitously involved in ligand binding of non-I domain integrins.

Results

Mutations of Tyr187, Trp188 and Gly190 of $\alpha 4$ in a predicted β -turn affect $\alpha 4\beta 1$ -specific cell adhesion to VCAM-1 and CS-1

To identify amino acid residues on the $\alpha 4$ subunit critical for ligand binding, we introduced multiple mutations within the previously located putative ligand binding site of $\alpha 4$ (residues 108–268) (Kamata *et al.*, 1995). CHO cells were co-transfected with either wild-type or mutant human $\alpha 4$ cDNA in an expression vector, together with the expression vector containing the neomycin resistance gene. Transfected cells were selected for G418 resistance. Typically 20–50% of the G418-resistant CHO cell populations stably express human $\alpha 4$, as detected by flow cytometry. Human $\alpha 4$ is expressed as a heterodimer with endogenous hamster $\beta 1$ (Kamata *et al.*, 1995).

Stably transfected cells were then tested for their ability to adhere to different substrates. CHO cells stably expressing human $\alpha 4$ were used without further enrichment for $\alpha 4$ expression. Untransfected CHO cells adhere to fibronectin well (>90 %) due to endogenous $\alpha 5\beta 1$ (Takada *et al.* 1992), but do not adhere significantly to bovine serum albumin, VCAM-1 or CS-1. In contrast, cells expressing wild-type $\alpha 4$ adhere to both VCAM-1 (Figure 1B) and CS-1 (Figure 1B). Most CHO cells expressing $\alpha 4$ mutants showed binding to either VCAM-1

Fig. 1. Effects of mutations of $\alpha 4$ on binding to VCAM-1 (A) or CS-1 (B). CHO cells expressing wild-type or mutant $\alpha 4$ were incubated for 1 h at 37°C with VCAM-1-Cx fusion protein (A) or CS-1-RSA (B) immobilized on to plastic wells (at a coating concentration of 0.05 and 0.025 $\mu\text{g}/\text{well}$, respectively). After rinsing the wells to remove unbound cells, the adherent cells were quantified using an endogenous phosphatase assay. Data are expressed as means (% of bound cells per % of $\alpha 4$ positive cells) $\pm$ SD of triplicate experiments. Transfected CHO cells after G418 selection were used without further enrichment of the $\alpha 4$ -positive population, except that CHO cells expressing wild-type $\alpha 4$ are clonal. Expression of $\alpha 4$ was determined by flow cytometry with mAb B5G10. More than 22% of the G418-resistant CHO cells were B5G10 positive. Some $\alpha 4$ mutants (F19A, R100A, K116A, D138A, T199A, F227A and D281A) were not expressed on the surface of cells.

or CS-1 at levels comparable with cells expressing wild-type $\alpha 4$. Interestingly, the Y187A and G190A $\alpha 4$ mutants showed significantly lower adhesion to both VCAM-1 and CS-1 than wild-type or the other mutant $\alpha 4$ s. The adjacent W188A mutant $\alpha 4$ also showed slightly lower binding to both ligands.

Interestingly, Tyr187, Trp188 and Gly190 are within the predicted β -turn structure (residues 181–190 of $\alpha 4$) of the third N-terminal repeat (Tuckwell *et al.*, 1994). CHO cells expressing $\alpha 4$ with mutations at positions 185–190 and 202 were cloned by cell sorting and further characterized. The adhesion of the cloned CHO cells expressing the $\alpha 4$ mutants to VCAM-1 or CS-1 was determined as a function of coating concentrations of

substrates (Figure 2A and B). The G190A mutant [mean fluorescent intensity (MFI) for $\alpha 4$ expression, 262] did not show significant adhesion to either ligand, even at high ligand coating concentrations (Figure 2). The Y187A mutant showed significantly lower adhesion to VCAM-1 or CS-1 than did wild-type $\alpha 4$, especially at a low coating concentration of substrates, although the expression level of the Y187A mutant (MFI for $\alpha 4$ expression, 516) is higher than that of the wild-type (MFI for $\alpha 4$ expression, 209). The Y187A mutant was significantly slower than wild-type $\alpha 4\beta 1$ in establishing adhesion to VCAM-1 or CS-1 (data not shown). The W188A mutant showed lower adhesion to both ligands at low substrate concentrations than did wild-type $\alpha 4$, but this mutation was much less

Fig. 2. Adhesion of CHO cells homogeneously expressing $\alpha 4$ mutant to VCAM-1 (A) and CS-1 (B) as a function of coating concentration of substrates. Clonal CHO cells expressing wild-type (●) or mutant $\alpha 4$ were incubated for 1 h at 37°C with VCAM-1-C κ fusion protein (A) or CS-1-RSA (B) immobilized at different concentrations (up to 0.1 μg per well). Y187A (▼), W188A (■), G190A (○), Y202A (◆), CHO cells (▲). After rinsing the wells to remove unbound cells, the adherent cells were quantified using an endogenous phosphatase assay (Prater *et al.*, 1991). The S185A, S186A, T189A and Y202A mutants showed similar adhesion profiles to that of the wild-type $\alpha 4$ (data not shown). MFI of the clones of CHO cells expressing mutant and wild-type $\alpha 4$ were 267 for S185A, 349 for S186A, 516 for Y187A, 406 for W188A, 412 for T189A, 262 for G190A, 238 for Y202A, 209 for wild-type $\alpha 4$ and 4.9 for untransfected CHO cells.

inhibitory than the Y187A or G190A mutations. The nearby S185A, S186A, T189A and Y202A mutants showed similar adhesion profiles to that of the wild-type $\alpha 4$ (Figure 2 and data not shown).

The Y187A, W188A and G190A mutations block soluble VCAM-1 binding to $\alpha 4\beta 1$

Cell adhesion is a complicated process including multiple receptor molecules and post-receptor events (e.g. cell spreading). To examine the direct effect of the mutations around Tyr187 on ligand interaction, soluble VCAM-1-C κ fusion protein was allowed to bind to CHO cells expressing wild-type or mutant $\alpha 4\beta 1$ on ice (to avoid

Fig. 3. The Y187A, W188A and G190A mutations block binding of soluble VCAM-1 to $\alpha 4\beta 1$. CHO cells were incubated with soluble VCAM-1-C κ fusion protein (900 ng) in DMEM for 30 min on ice. Bound VCAM-1 was detected with FITC-labeled goat anti-mouse C κ IgG using flow cytometry. The S185A, S186A, T189A and Y202A mutants showed binding profiles similar to that of wild-type $\alpha 4$. The binding of VCAM-1 to wild-type $\alpha 4\beta 1$ was blocked by 10 mM EDTA in the medium. The S185A, S186A and T189A mutant $\alpha 4$ s showed similar binding profiles to that of wild-type $\alpha 4$ (data not shown). The data suggest that the mutation reduces affinity for VCAM-1. Broken line shows VCAM-1 minus control.

internalization of the bound VCAM-1). Binding of VCAM-1-C κ was detected by FITC-labeled antibody against mouse C κ chain. VCAM-1 was shown to bind significantly to wild-type $\alpha 4$, the S185A, S186A, T189A and Y202A mutants, but not to the Y187A, W188A and G190A mutants (Figure 3 and data not shown). The data are consistent with the cell adhesion profiles of the mutants (Figure 2), and suggest that the mutations reduce the affinity of $\alpha 4\beta 1$ for ligands.

The Y187A, W188A and G190A mutations do not induce gross structural changes in $\alpha 4\beta 1$

In order to determine if the $\alpha 4$ mutations altered the receptor conformation, we first tested whether the mutant receptors could associate with endogenous $\beta 1$. Upon immunoprecipitation of the S186A, Y187A, W188A, G190A and Y202A mutants with anti-human $\alpha 4$ polyclonal antibodies (Takada *et al.*, 1989), protein bands corresponding to $\alpha 4$ with expected sizes (150 kDa, and

80 and 70 kDa fragments) (Hemler *et al.*, 1987) were detected in association with endogenous hamster $\beta 1$ (110 kDa) (Figure 4). The $\alpha 4$ and $\beta 1$ subunits have been known to be weakly associated (Hemler *et al.*, 1987). Although the recovery of the $\beta 1$ subunit was lower in the Y187A, W188A, G190A and Y202A mutants, this effect does not appear to correlate with ligand binding, since the Y202A mutant shows ligand binding function comparable with that of the wild-type $\alpha 4$. In addition, we found that multiple anti-human $\alpha 4$ mAbs [P4G9 and HP1/3 (epitope A); HP2/1, P4C2 and SG/73 (epitope B); B5G10 (epitope C)] (Pulido *et al.*, 1991; Kamata *et al.*, 1995) recognized most of the mutants used in this study, including the

Fig. 4. Immunoprecipitation of $\alpha 4$ mutants. CHO cells expressing wild-type or mutant $\alpha 4$ were surface-labeled with ^{125}I using Iodogen (Pierce, Rockford, IL) according to the manufacturer's instructions. Lysates were immunoprecipitated with rabbit polyclonal anti- $\alpha 4$ antibody (Takada *et al.*, 1989). Samples were analyzed in 7% gel under non-reducing conditions. The data show that wild-type and mutant $\alpha 4$ subunits are expressed on CHO cells as heterodimers with endogenous hamster $\beta 1$ subunit. Rabbit preimmune serum does not precipitate $\alpha 4$ or $\beta 1$ from the lysates of CHO cells used. Parent CHO cells do not express $\alpha 4$.

Y187A, W188A and G190A mutants, with some exceptions as described below (Table I and data not shown), indicating that the observed effects of these mutations on ligand binding are probably not due to gross structural changes.

Interestingly, the G190A mutation completely blocked binding of function-blocking mAbs P4C2 and SG/73 (Table I). Also, the K201A mutant does not react with mAbs HP2/1, P4C2 or SG/73, and the Y202A mutant does not react with mAb SG/73 among those mAbs tested. These data indicate that some function-blocking mAbs bind very close to the predicted β -turn structure.

The corresponding region of $\alpha 5$ is critically involved in the interaction between $\alpha 5\beta 1$ and fibronectin

To examine if the structure corresponding to the predicted loop region of $\alpha 4$ is similarly important in ligand binding in the $\alpha 5$ subunit, we introduced mutations of Tyr186, Phe187, Trp188 and Gly190 of $\alpha 5$ to Ala. We used wild-type or mutant $\alpha 5$ cDNA to transfect endogenous $\alpha 5$ -deficient CHO cells (B2 variant) (Schreiner *et al.*, 1989), together with the neomycin resistance gene. After selection for G418 resistance, cells expressing mutant or wild-type human $\alpha 5$ were cloned by cell sorting to obtain cells expressing high levels of wild-type or mutant human $\alpha 5$. Lysates from surface ^{125}I -labeled B2 cells expressing wild-type and mutant $\alpha 5$ were immunoprecipitated with anti-human $\alpha 5$ mAb BIIG2. We detected the $\alpha 5$ subunit with expected size (150 kDa) in association with endogenous hamster $\beta 1$ in each case (data not shown). These results suggest that mutations within this region do not affect the gross structure of integrin heterodimers, consistent with the results from the $\alpha 4$ mutants.

Figure 5 shows the fibronectin adhesion profiles of B2 cells expressing wild-type or mutant $\alpha 5\beta 1$. The Y186A, F187A and W188A mutants showed much lower adhesion than wild-type $\alpha 5$. The F187A mutation was most inhibitory. The F187A mutant was slower than wild-type in establishing adhesion and spreading on fibronectin. The G190A mutation did not show any effect on adhesion to fibronectin. The data suggest that the Y186A, F187A and W188A mutations of $\alpha 5$ block $\alpha 5\beta 1$ -specific cell adhesion to fibronectin. Interestingly, the most effective mutation of $\alpha 5$ (F187A) corresponds to the Y187A mutation of $\alpha 4$, suggesting that this region may be critical for ligand-integrin interaction.

Consistent with the cell adhesion data, B2 cells express-

Table I. Binding of anti- $\alpha 4$ mAbs to $\alpha 4$ with mutations within and around the predicted β -turn structure

mAbs	CHO cells	Wild-type $\alpha 4$	Y187A	G190A	K201A	Y202A
Mouse IgG	3.5	1.6	2.6	6.0	3.2	6.2
HP1/3	4.5	99.2/+	94.5/+	82.0/+	28.8/+	98.9/+
P4G9	3.3	83.9/+	70.7/+	56.7/+	34.9/+	90.4/+
HP2/1	5.1	98.1/+	90.1/+	75.3/+	2.6	95.5/+
P4C2	4.9	80.5/+	77.0/+	3.9	2.5	72.0/+
SG/73	4.1	98.9/+	96.7/+	3.8	2.4	3.7
B5G10	5.3	99.5/+	99.7/+	96.2/+	43.4/+	99.8/+

Binding of mAbs to $\alpha 4$ mutants was examined using flow cytometry. The data are shown as percent positive cells on flow cytometry. /+ indicates positive reactivity. Other $\alpha 4$ mutants including S185A, S186A, W188A, T189A, S191A, N200A and K203A mutants showed binding profiles similar to those of Y187A and wild-type $\alpha 4$. CHO cells are homogeneously expressing wild-type or mutant $\alpha 4$, except for CHO cells expressing S191A, N200A, K201A and K203A.

ing the F187A mutant of $\alpha 5$ showed [125 I]fibronectin binding at background level (data not shown). In contrast, fibronectin bound to wild-type $\alpha 5\beta 1$ with an apparent K_d of ~ 100 nM. This value is close to the published K_d for fibronectin binding to wild-type CHO-K1 cells (Faull *et al.*, 1993). Interestingly, function-inhibiting anti- $\alpha 5$ mAb 16 (Akiyama *et al.*, 1989) recognizes the Y186A, W188A

and F187A mutants, but not the G190A mutant. This suggests that mAb 16 binds very close to the predicted turn structure and probably blocks access of ligand to the region of $\alpha 5$ critical for ligand binding. These findings suggest that the predicted β -turn structure of $\alpha 5$ may be critically involved in fibronectin binding.

Discussion

The present study establishes the residues of $\alpha 4$ and $\alpha 5$ critical for ligand binding using alanine-scanning mutagenesis, which gives high-resolution epitope mapping (Cunningham and Wells, 1989). The data suggest that, among 39 mutations within the putative ligand binding site of $\alpha 4$ (residues 108–268) (Kamata *et al.*, 1995), Tyr187, Trp188 and Gly190 are critical for ligand interactions of $\alpha 4\beta 1$. These residues are clustered in the middle of the putative ligand binding sites. The effects of these mutations on the binding of VCAM-1 and CS-1 are comparable, suggesting that both ligands share a common binding site or mechanism. Interestingly, mutation of Tyr186, Phe187 and Trp188 in the corresponding region of $\alpha 5$ (Phe187 of $\alpha 5$ corresponds to Tyr187 of $\alpha 4$) significantly reduced binding of $\alpha 5\beta 1$ to fibronectin. These data strongly suggest that the corresponding region of $\alpha 5$ containing Phe187 may be critical for fibronectin binding. Loop regions are potential candidates for ligand binding sites. Tuckwell *et al.* (1994) recently predicted the secondary structure of the N-terminal repetitive domains of the integrin α subunit based on a large alignment of the seven repeats from 16 α subunits. Based on these predictions, the residues of $\alpha 4$ and $\alpha 5$ critical for ligand binding may be in a predicted β -turn structure between two β -sheets (probably anti-parallel) in the third N-terminal repeat (Figure 6) (Tuckwell *et al.*, 1994).

Fig. 5. Effects of $\alpha 5$ mutations on cell adhesion to fibronectin. CHO ($\alpha 5$ -deficient B2 variant) cells (Schreiner *et al.*, 1989) expressing wild-type or mutant human $\alpha 5$ were incubated with fibronectin-coated wells (at a coating concentration of $1 \mu\text{g}/\text{well}$) for 1 h at 37°C . Adhesion to BSA was $< 1.7\%$. MFI of the cells expressing $\alpha 5$ with mAb BIIIG2 (anti-human $\alpha 5$) was 99 for wild-type, 71 for Y186A, 84 for F187A, 66 for W188A, 46 for G190A and 3.2 for parent B2 cells, respectively. MFI with mAb PB1 (hamster $\alpha 5$) was 5.2–16.2 (much lower than wild-type CHO-K1 cells), and those with mouse IgG were 3–4.

Fig. 6. Critical residues for ligand binding are clustered in the third N-terminal repeat. Based on the recent prediction of the secondary structure of the repeated sequences of the integrin α subunits, these critical residues (Tyr187, Trp188 and Gly190 of $\alpha 4$, and Tyr186, Phe187 and Trp188 of $\alpha 5$) are clustered in the predicted C-terminal β -turn of the repeat between the two anti-parallel β -sheets. The amino acid sequences of the predicted β -turn (GAPGSSYWTG in $\alpha 4$) are relatively well conserved among α subunits. In the fifth to seventh repeats, putative divalent cation binding motifs [DX(D/N)XDGXXD] are located in the first predicted β -turn, but these motifs are absent in the third repeat. Vertical arrows indicate critical residues for ligands or antibodies.

Mutation of conserved Gly190 of $\alpha 4$ completely blocked ligand binding, but mutations of the other conserved nearby Gly residues at positions 168, 181 and 184 of $\alpha 4$ did not affect ligand binding (Figure 1). One possibility could be that mutation of Gly190 to Ala significantly changes the conformation of the predicted β -turn structure (residues 181–190), leading to disruption of ligand binding functions. Interestingly, critical residues for binding of function-blocking mAbs (HP2/1, SG/73 and P4C2) are also clustered within or around the critical predicted β -turn region of $\alpha 4$. Also, Gly190 of $\alpha 5$ is critical for binding of anti- $\alpha 5$ mAb 16 (Akiyama *et al.*, 1989) that blocks fibronectin binding to $\alpha 5\beta 1$. These findings support the idea that the predicted β -turn region of the integrin α subunits may be critical for ligand binding.

Ligand binding sites on the integrin α subunits have previously been identified by chemical cross-linking of ligand-derived peptides. The γ -peptide from fibrinogen cross-linked to the second metal binding site of αIIb (residues 294–314 of αIIb) (D'Souza *et al.*, 1990). Also, peptides from the second metal binding site and antibodies to the peptides block binding of fibrinogen to $\alpha IIb\beta 3$ (D'Souza *et al.*, 1991). The second metal binding site of αIIb corresponds to the first divalent cation binding site of $\alpha 4$ (residues 278–297) in the fifth repeat (Figure 6). However, a mutant (Asn283→Glu) within the first metal binding site of $\alpha 4$ still retains binding function to VCAM-1, CS-1 and invasin, despite their effect on the divalent cation requirement (Masumoto and Hemler, 1993). Interestingly, the RGD peptide chemically cross-links to the region of αV (residues 139–349 of αV) (Smith and Cheresh, 1990), which includes the predicted β -turn in the third repeat shown in this study. These reports suggest that: (i) there is a possibility that multiple binding sites may be involved in ligand binding; and (ii) the ligand binding sites might be specific to integrin species or ligand species. Detailed mapping of the ligand binding sites for the αV or αIIb subunits using site-directed mutagenesis has not been reported. The present study suggests that the predicted β -turn of the third repeat may be critical for ligand binding in two $\beta 1$ integrins with distinct ligand specificity and, therefore, the predicted turn structure of the integrin α subunit could be ubiquitously important for ligand binding to integrin α subunits. Interestingly, the third repeat of $\alpha 4$ and $\alpha 5$ does not contain the putative cation binding motifs (they are present in the fifth, sixth and seventh repeats in $\alpha 4$ and $\alpha 5$). The amino acid sequence of the predicted β -turn (GAPGSSYWTG in $\alpha 4$) is relatively well conserved among α subunits. It will be interesting to examine if this region of other integrin α subunits is related to ligand binding functions or specificity. Studies in this direction are in progress.

Materials and methods

Materials

mAb B5G10 (anti-human $\alpha 4$) was obtained from M.E.Hemler (Dana-Farber Cancer Institute, Boston, MA), HP1/3 and HP2/1 (anti-human $\alpha 4$) from F.Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain), P4C2 (anti-human $\alpha 4$) from E.Wayner (University of Washington, Seattle, WA) and SG/73 (anti-human $\alpha 4$) from K.Miyake (Saga Medical School, Saga, Japan). mAb 16 (anti-human $\alpha 5$) was obtained from K.Yamada (National Institute of Health, Bethesda, MD) and mAb BIIG2 (anti-human $\alpha 5$) from C.Damsky (University of California San Francisco,

San Francisco, CA). mAb PB1 (anti-hamster $\alpha 5$) was obtained from R.L.Juliano (University of North Carolina, Chapel Hill, NC). Recombinant VCAM-1–mouse C κ chain fusion protein was donated by D.Dottavio (Sandoz Pharmaceuticals, East Hanover, NJ) and CS-1 peptide–rat serum albumin conjugate (RSA–CS-1) was a gift from E.Wayner.

Construction of human $\alpha 4$ expression vectors and transfection

Wild-type human $\alpha 4$ cDNA (Takada *et al.*, 1989) was subcloned into pBJ-1 vector as described previously (Kamata *et al.*, 1995). Site-directed mutagenesis was carried out by unique site elimination with double-stranded vector (Deng and Nickoloff, 1992). The presence of mutations was confirmed by DNA sequencing. Twenty μ g of wild-type or mutant $\alpha 4$ cDNA construct were transfected into CHO cells (8×10^6 cells), together with 1 μ g of pFneo DNA containing the neomycin resistance gene, by electroporation. Transfected cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 6% CO₂ for 2 days. Then the cells were transferred to the same medium containing 700 μ g/ml G418 (Gibco). After 10–14 days, the G418-resistant colonies were harvested. The expression level of wild-type or mutant $\alpha 4$ was confirmed by flow cytometric analysis in FACScan (Becton-Dickinson) with mAb B5G10 that recognizes a non-functional epitope (Pulido *et al.*, 1991). Typically, 20–50% of G418-resistant cells are positive with mAb B5G10.

Adhesion assay

Wells of 96-well Immulon-2 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with RSA–CS-1 [up to 0.1 μ g/well in 100 μ l phosphate-buffered saline (PBS)] and human serum fibronectin (up to 1 μ g/well in 100 μ l PBS), and incubated at 4°C overnight. For VCAM-1, wells were first coated with anti-mouse C κ chain (100 μ l of 2 μ g/ml in PBS, Caltag Laboratories, South San Francisco, CA) overnight at 4°C. After washing with PBS, wells were then incubated with recombinant VCAM-1–mouse C κ chain up to 2 μ g/ml in 100 μ l PBS for 1 h at room temperature. The remaining protein binding sites were blocked by incubating with 1% bovine serum albumin (Calbiochem) for 30 min at room temperature. Cells (10^5 cells/well) in 100 μ l of DMEM (without serum) were added to the wells and incubated at 37°C for up to 1 h. After gently rinsing the wells three times with PBS to remove unbound cells, bound cells were quantified by assaying endogenous phosphatase activity (Prater *et al.*, 1991).

Binding of soluble VCAM-1–C κ fusion protein

CHO cells ($1\text{--}1.5 \times 10^6$ cells) were incubated with soluble VCAM-1–C κ fusion protein (900 ng) in 100 μ l of DMEM for 30 min on ice. Cells were washed with ice-cold DMEM and incubated with FITC-labeled goat anti-mouse C κ IgG (Caltag) for 20 min on ice. Cells were washed with DMEM and analyzed by FACScan (Becton-Dickinson).

Other methods

Immunoprecipitation and flow cytometric analyses (Takada *et al.*, 1992) and ¹²⁵I-labeled fibronectin binding (Faull *et al.*, 1993) were performed as described in the cited references.

Acknowledgements

We are grateful to Drs C.Damsky, D.Dottavio, M.E.Hemler, K.Miyake, F.Sanchez-Madrid, E.Wayner and K.Yamada for valuable reagents and J.Meredith (Scripps) for critical reading of the manuscript. This work was supported by National Institute of Health Grant GM47157, GM49899 and funding from Sandoz Pharmaceuticals. This work was done during the tenure of a Research Fellowship from the American Heart Association, California Affiliate to T.Kamata. This is Publication #9248-VB from The Scripps Research Institute.

References

- Akiyama, S.K., Yamada, S., Chen, W.-T. and Yamada, K.M. (1989) *J. Cell Biol.*, **109**, 863–875.
- Alon, R., Kassner, P.D., Woldemar Car, M., Finger, E.B., Hemler, M.E. and Springer, T.A. (1995) *J. Cell Biol.*, **128**, 1243–1253.
- Berlin, C. *et al.* (1995) *Cell*, **80**, 413–422.
- Cunningham, B.C. and Wells, J.A. (1989) *Science*, **244**, 1081–1085.
- Deng, W.P. and Nickoloff, J.A. (1992) *Anal. Biochem.*, **200**, 81–88.

- D'Souza,S., Ginsberg,M.H., Burke,T.A. and Plow,E.F. (1990) *J. Biol. Chem.*, **265**, 3440–3446.
- D'Souza,S., Ginsberg,M.H., Matsueda,G.R. and Plow,E.F. (1991) *Nature*, **350**, 66.
- Elices,M.J., Osborn,L., Takada,Y., Crouse,C., Luhowskyj,S., Hemler,M.E. and Lobb,R.R. (1990) *Cell*, **60**, 577–584.
- Faull,R., Kovach,N.L., Harlan,J. and Ginsberg,M.H. (1993) *J. Cell Biol.*, **121**, 155–162.
- Guan,J.L. and Hynes,R.O. (1990) *Cell*, **60**, 53–61.
- Hemler,M.E., Huang,C., Takada,Y., Schwarz,L., Strominger,J.L. and Clabby,M.L. (1987) *J. Biol. Chem.*, **262**, 11478–11485.
- Hemler,M.E., Weizman,J.B., Pasqualini,R., Kawaguchi,S., Kassner,P.D. and Berdichevsky,F.B. (1994) Structure, biochemical properties, and biological functions of integrin cytoplasmic domain. In Takada,Y. (ed.), *Integrins: The Biological Problems*. CRC Press, Boca Raton, FL, pp. 1–35.
- Hogg,N., Bennett,R., Cabanas,C. and Dransfield,I. (1992) *Kidney Int.*, **41**, 613–616.
- Hynes,R.O. (1992) *Cell*, **69**, 11–25.
- Kamata,T., Puzon,W. and Takada,Y. (1995) *Biochem. J.*, **305**, 945–951.
- Lobb,R.R. and Hemler,M.E. (1994) *J. Clin. Invest.*, **94**, 1722–1728.
- Masumoto,A. and Hemler,M.E. (1993) *J. Cell Biol.*, **123**, 245–253.
- Mould,A.P., Wheldon,L.A., Komoriya,A., Wayner,E.A., Yamada,K.M. and Humphries,M.J. (1990) *J. Biol. Chem.*, **265**, 4020–4024.
- Mould,A.P., Komoriya,A., Yamada,K.M. and Humphries,M.J. (1991) *J. Biol. Chem.*, **266**, 3579–3585.
- Osborn,L., Hession,C., Tizard,R., Vassallo,C., Luhowskyj,S., Chi-Rosso,G. and Lobb,R. (1989) *Cell*, **59**, 1203–1211.
- Prater,C.A., Plotkin,J., Jaye,D. and Frazier,W.A. (1991) *J. Cell Biol.*, **112**, 1031–1040.
- Pulido,R., Elices,M.J., Campanero,M.R., Osborn,L., Schiffer,S., Garcia-Pardo,A., Lobb,R., Hemler,M.E. and Sanchez-Madrid,F. (1991) *J. Biol. Chem.*, **266**, 10241–10245.
- Rice,G.E. and Bevilacqua,M.P. (1989) *Science*, **246**, 1303–1306.
- Schreiner,C.L., Bauer,J.S., Danilov,Y.N., Hussein,S., Sczekan,M. and Juliano,R.L. (1989) *J. Cell Biol.*, **109**, 3157–3167.
- Smith,J.W. and Cheresch,D.A. (1990) *J. Biol. Chem.*, **265**, 2168–2172.
- Springer,T.A. (1994) *Cell*, **76**, 301–314.
- Takada,Y., Elices,M.J., Crous,C. and Hemler,M.E. (1989) *EMBO J.*, **8**, 1361–1368.
- Takada,Y., Ylanne,J., Mandelman,D., Puzon,W. and Ginsberg,M. (1992) *J. Cell Biol.*, **119**, 913–921.
- Tuckwell,D., Humphries,M. and Brass,A. (1994) *Cell Adhesion Commun.*, **2**, 385–402.
- Wayner,E.A., Garcia-Pardo,A., Humphries,M.J., MacDonald,J.A. and Carter,W.G. (1989) *J. Cell Biol.*, **109**, 1321–1330.
- Yamada,K.M. (1991) *J. Biol. Chem.*, **266**, 12809–12812.

Received on June 8, 1995; revised on July 28, 1995