Monoclonal antibodies identify residues 199–216 of the integrin $\alpha 2$ vWFA domain as a functionally important region within $\alpha 2\beta 1$

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Integrin $\alpha 2\beta 1$ is the major receptor for collagens in the human body, and the collagen-binding site on the $\alpha 2$ subunit von Willebrand factor A-type domain (vWFA domain) is now well defined. However, the biologically important conformational changes that are associated with collagen binding, and the means by which the vWFA domain is integrated into the whole integrin are not completely understood. We have raised monoclonal antibodies against recombinant $\alpha 2$ vWFA domain for use as probes of function. Three antibodies, JA202, JA215 and JA218, inhibited binding to collagen, collagen I C-propeptide and E-cadherin, demonstrating that their function is important for structurally diverse $\alpha 2\beta 1$ ligands. Cross-blocking studies grouped the epitopes into two clusters: (I) JA202, the inhibitory antibody, Gi9, and a non-inhibitory antibody, JA208; (II) JA215 and JA218. Both clusters were sensitive to events at the collagen binding site, as binding of Gi9, JA202, JA215 and JA218 were inhibited by collagen peptide, JA208 binding was enhanced by

collagen peptide, and binding of JA202 was decreased after mutagenesis of the cation-binding residue Thr²²¹ to alanine. Binding of cluster I antibodies was inhibited by the anti-functional anti- $\beta 1$ antibody Mab13, and binding of Gi9 and JA218 to $\alpha 2\beta 1$ was inhibited by substituting Mn²⁺ for Mg²⁺, demonstrating that these antibodies were sensitive to changes initiated outside the vWFA domain. Mapping of epitopes showed that JA202 and Gi9 bound between residues 212–216, while JA208 bound between residues 199–216. We have therefore identified two epitope clusters with novel properties; i.e. they are intimately associated with the collagen-binding site, responsive to conformational changes at the collagen-binding site and sensitive to events initiated outside the vWFA domain.

Key words: adhesion, collagen, collagen I C-propeptide, epitope mapping, extracellular matrix.

INTRODUCTION

Integrin $\alpha 2\beta 1$ is the major receptor for collagens in the human body, being responsible for adhesion of platelets and other cells to collagens, modulation of collagen and collagenase gene expression, force generation and organization of newly synthesized extracellular matrix [1–8]. $\alpha 2\beta 1$ is also a receptor for laminins, collagen C-propeptides, echovirus 1 [1,9–12] and E-cadherin (J. D. Whittard, A. P. Mould, A. Koch, O. Pertz, J. Engel and M. J. Humphries, unpublished work).

The integrin $\alpha 2$ subunit, in common with the $\alpha 1$, $\alpha 10$, $\alpha 11$, αD , αE , αL , αM and αX subunits, is distinguished by the presence of a von Willebrand factor A-type domain (vWFA domain; also known as an A- or I-domain) in the 7-fold repeated N-terminal region otherwise common to all α subunits. The integrin vWFA domain, which is a member of a large family of similar domains [13–15], plays a central role in the binding of $\alpha 2\beta 1$ to all of its ligands, and is able to bind these ligands as a recombinant domain [11,16–21]. The interaction of the $\alpha 2$ vWFA domain with collagens has been studied in detail, and the ligand affinities and specificities seen for the vWFA domain closely resemble those reported for the whole integrin, as do the requirements for

bivalent cations [18–20,22–25]. Furthermore, $\alpha 2$ constructs lacking the vWFA domain do not bind collagen, despite forming stable heterodimers with the $\beta 1$ subunit [26]. Consequently the vWFA domain of the $\alpha 2$ subunit is the major, and probably the only, collagen-binding site in $\alpha 2\beta 1$.

Recently the structure of the $\alpha 2$ vWFA domain–collagen complex [27] has been solved by X-ray crystallography. This information, together with mutagenesis experiments [28,29], has defined the collagen-binding site in great detail, showing it to be located at the cation-binding metal-ion-dependent adhesion site (MIDAS), and to include residues adjacent to it. Central to this is an essential glutamate residue in the collagen molecule which co-ordinates to the MIDAS cation. Despite these advances, the biologically important conformational changes associated with ligand binding and the way in which the vWFA domain interacts with the other regions of the integrin are still not clear. To investigate these issues we raised monoclonal antibodies against the $\alpha 2$ vWFA domain for use as probes to identify functionally important regions within the domain. Using these antibodies we have identified two clusters of epitopes intimately associated with the binding of collagens and other ligands. In particular, residues 199–216 were shown to contain epitopes sensitive both to

Abbreviations used: ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); CB3(IV), integrin-binding fragment of collagen IV; COMP, cartilage oligomeric matrix protein; CPP-I, collagen I C-propeptide; FACS, fluorescence-activated cell sorting; GST, glutathione S-transferase; LIBS, ligand-induced binding site; MIDAS, metal-ion-dependent adhesion site; PBS[−], Mg²⁺- and Ca²⁺-free PBS; PBS⁺, PBS containing Mg²⁺ and Ca²⁺; TBS, Tris-buffered saline; vWFA domain, von Willebrand factor A-type domain.

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conformational changes at the collagen binding site, and to events initiated outside the vWFA domain. This region is therefore of major importance for the function of $\alpha 2\beta 1$.

EXPERIMENTAL

Materials

Acid-soluble rat tail type I collagen was obtained from Sigma (Poole, Dorset, U.K.) and was biotinylated as described by Calderwood et al. [20]. Collagen I C-propeptide (CPP-I) was prepared and biotinylated as described by Davies et al. [11]. Collagen peptide [Gly-Pro-Cys-(Gly-Pro-Hyp)₃-Gly-Phe-Hyp-Gly-Glu-Arg-Gly-Val-Gly-Pro-Hyp-Gly-Pro-Ala-(Gly-Pro-Hyp)₃-Gly-Pro-Cys] was prepared as described in Knight et al. [24]. Fibronectin 50 kDa fragment was a gift from Dr N. Schofield (Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, University of Manchester, Manchester, U.K.). The integrin-binding fragment of collagen IV [CB3(IV)] was a gift from Professor K. Kühn (Max-Planck-Institute for Biochemistry, Martinsreid, Germany) [30]. The E-cadherin-COMP construct comprising the five extracellular domains of mouse E-cadherin fused to the assembly domain of rat cartilage oligomeric matrix protein (COMP) was a gift from Dr O. Pertz (Biozentrum, University of Basel, Basel, Switzerland). The anti- $\beta 1$ integrin subunit antibody, Mab13, was a gift from K. Yamada (National Institute of Dental Research, National Institute of Health, Bethesda, MD, U.S.A.).

Production of $\alpha 2$ vWFA domain and integrins

The $\alpha 2$ vWFA domain-glutathione S-transferase (GST) fusion protein was generated as previously described by Tuckwell et al. [18,19].

HT1080 $\beta 1$ -integrins were prepared from the HT1080 fibrosarcoma cell line as described by Mould et al. [31]. This preparation contains predominantly $\alpha 2\beta 1$. Affinity-purified $\alpha 2\beta 1$ was isolated from HT1080 cells by the same method, using the anti- $\alpha 2$ antibody, 10A4 [32].

Production and purification of monoclonal antibodies

Monoclonal mouse anti-(integrin $\alpha 2$ vWFA domain) antibodies were produced essentially as described by Mould et al. [31,33]. BALB/c mice were immunized with recombinant $\alpha 2$ vWFA domain. Hybridomas were screened by ELISA against the immunogen and against GST. Clones reacting with GST were discarded.

Purification of antibodies was carried out as follows: 1 litre of culture supernatant was incubated with 3 ml of Protein G-Sepharose (Sigma), pre-equilibrated with 136.8 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl and 1.5 mM KH₂PO₄, pH 7.4 (PBS⁻), overnight at room temperature. Protein G-Sepharose was then removed using a sintered glass funnel and transferred on to a column. The column was washed with 20 volumes of PBS⁻, and the antibody was eluted with 100 mM glycine/HCl (pH 2.7). Samples were brought to near-neutral pH by the addition of 1 M Tris (pH 9.0). Antibody was then dialysed against PBS⁻, and the protein concentration was determined using mouse IgG as a standard.

Fluorescence-activated cell sorting (FACS) analysis was carried out as described in Tuckwell et al. [34]

Solid-phase binding assays

Solid-phase binding assays, to measure binding of biotinylated collagen to $\alpha 2$ vWFA domain or HT1080 $\beta 1$ integrins, were

carried out as follows: Immulon 4 microtitre plates (Dynex, Billingham, West Sussex, U.K.) were coated with 100 μ l of $\alpha 2$ vWFA domain fusion protein at 10 μ g/ml in PBS⁻ or with HT1080 $\beta 1$ integrins diluted 1:200 (v/v) into 136.8 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 0.9 mM CaCl₂ and 0.5 mM MgCl₂, pH 7.4 (PBS⁺), overnight at 4 °C. The following day, protein solutions were removed and wells were blocked with 200 μ l of 50 mg/ml BSA, 40 mM Tris/HCl and 150 mM NaCl, pH 7.4 (TBS), for 2 h at room temperature. Wells were then washed three times with 200 μ l of TBS containing 1 mg/ml BSA and 1 mM bivalent cation (MnCl₂ for $\alpha 2\beta 1$; MgCl₂ for vWFA domain) (buffer A) and to each well was then added 50 μ l of inhibitor (in buffer A at double the final concentration) followed by 50 μ l of biotinylated collagen I (also in buffer A at double the final concentration), for 3 h at room temperature. Wells were then washed three times with 200 μ l of buffer A, and 100 μ l of 1:200 (v/v) ExtrAvidin-peroxidase (Sigma) in buffer A was subsequently added for 10 min at room temperature. Wells were then washed three times with 200 μ l of buffer A, and colour was developed by the addition of 2 mM 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 0.03% (v/v) H₂O₂, 0.05 M NaH₂PO₄ and 0.1 M sodium acetate, pH 5.0 (ABTS reagent). Absorbance was measured at 405 nm on a plate reader.

For experiments studying the binding of biotinylated CPP-I, this was added in place of biotinylated collagen.

Solid-phase binding assays measuring binding of antibodies to purified $\alpha 2\beta 1$ in the presence of CPP-I, Mab13 or bivalent cations were carried out as described above, substituting antibodies for biotinylated collagen, and detecting with 1:1000 (v/v) peroxidase-conjugated rabbit anti-mouse antibody (DAKO Ltd., Ely, U.K.) in buffer A for 1 h at room temperature. The following modifications were also used.

For experiments studying the effects of collagen peptide and other $\alpha 2\beta 1$ ligands on antibody binding to $\alpha 2\beta 1$, assays were carried out in the presence of 1 mM MnCl₂. Antibody concentrations were as follows: JA202, 0.5 μ g/ml; JA208, 0.5 μ g/ml; JA215, 0.25 μ g/ml; JA218, 0.05 μ g/ml; Gi9, 0.05 μ g/ml; 16B4, 0.125 μ g/ml. Matrix protein ligands were added to give the following final concentrations: collagen peptide, 100 μ g/ml; collagen I and CB3(IV), 10 μ g/ml; CPP-I, 40 μ g/ml.

For experiments studying the effects of Mab13 on antibody binding to $\alpha 2\beta 1$, antibodies were added in the presence of 1 mM MnCl₂ and 5 mM EDTA. Antibody concentrations were as follows: JA202, 1 μ g/ml; JA208, 1 μ g/ml; JA215, 0.2 μ g/ml; JA218, 0.2–0.3 μ g/ml; Gi9, 0.2–0.3 μ g/ml. Mab13/non-immune rat IgG (Sigma) was used at 10 μ g/ml. Bound antibodies were detected with 1:1000 (v/v) peroxidase-conjugated goat anti-mouse serum (Sigma) in buffer A. This antiserum had been pre-adsorbed with rat serum, thereby preventing cross-reaction with Mab13.

For experiments studying binding of antibodies in the presence of different cations, TBS was pretreated with approx. 2 g/l Chelex (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.), for 18 h at 4 °C, to remove residual bivalent cations. Chelex was filtered out, and this TBS was then used to prepare all TBS-containing buffers. Washes preceding incubation with antibodies were carried out using buffer A without bivalent cations. Washes after incubation with antibodies and different cations were carried out using buffer A containing 1 mM MnCl₂.

Cell spreading assays

HT1080 cells were maintained, and spreading assays carried out, as described in Tuckwell et al. [19].

Epitope clustering ELISAs

Monoclonal antibodies were biotinylated by the addition of an equal mass of EZ-Link sulho-NHS-Biotin (Pierce, Chester, U.K.) to antibody diluted in 1 ml, final volume, of PBS⁻. Samples were left for 1 h at room temperature with occasional agitation and were then dialysed against PBS⁻ to remove unincorporated biotin.

Polyclonal anti-GST serum was raised by immunizing New Zealand White rabbits with GST, prepared as for $\alpha 2$ vWFA domain fusion protein [18–20] from bacteria transformed with pGEX plasmid. An anti-GST immunoglobulin fraction was prepared from this serum as described above for antibody purification.

Binding ELISAs were carried out after the method of Brookman et al. [35]. Immulon 4 microtitre plates were coated with 100 μ l of 5 μ g/ml anti-GST immunoglobulins in 50 mM Na₂CO₃ (pH 9.5) overnight at 4 °C. Wells were then washed twice with 200 μ l of PBS⁻ and blocked with 100 μ l of PBS⁻ containing 2% (w/v) fat-free milk powder for 1 h at 4 °C. Wells were then washed twice with 200 μ l of PBS⁻ containing 0.1% (v/v) Tween 20, and 100 μ l of 10 μ g/ml $\alpha 2$ fusion protein diluted in PBS⁻ containing 0.5% (w/v) milk powder and 0.1% (v/v) Tween 20 was subsequently added for 2 h at 4 °C. Wells were then washed twice with 200 μ l of PBS⁻ containing 0.1% (v/v) Tween 20, and biotinylated antibody was added in the presence of excess unbiotinylated antibody, both diluted in PBS⁻ containing 0.5% (w/v) milk powder and 0.1% (v/v) Tween 20, for 2 h at 4 °C. Wells were then washed three times with 200 μ l of PBS⁻ containing 0.1% (v/v) Tween 20, and 100 μ l of 1:200 (v/v) ExtrAvidin peroxidase in PBS⁻ containing 0.5% (w/v) milk powder and 0.1% (v/v) Tween 20 was subsequently added for 2 h at 4 °C. Wells were washed a further three times with PBS⁻ containing 0.1% (v/v) Tween 20, and colour was developed by addition of the ABTS reagent, as described above.

Biotinylated antibodies were used at the following concentrations: JA202, 0.75–3.25 μ g/ml; JA208, 1.25–2.5 μ g/ml; JA215 and JA218, 0.3 μ g/ml. Unbiotinylated antibodies were used at the following concentrations: JA202 and JA208, 20 μ g/ml; JA215, JA218 and Gi9, 10 μ g/ml. Zero binding was taken as binding of biotinylated antibody in the absence of fusion protein, except for biotinylated JA202 where, because of high background binding by the biotinylated antibody, binding in the presence of excess unlabelled JA202 was taken as zero binding. Binding of biotinylated antibody in the absence of competing antibody was taken as 100% binding.

Epitope mapping using human/mouse chimaeras

Mutant $\alpha 2$ vWFA domain constructs were generated using the mutagenesis protocols described in Kunkel [36] and the Bio-Rad Muta-Gene mutagenesis kit. Chimaeric vWFA domains were expressed and purified as described previously [18–20]. Binding of antibodies to chimaeric and mutant vWFA domains was measured as described in Brookman et al. [35].

RESULTS

Monoclonal antibodies against the $\alpha 2$ vWFA domain inhibit binding to $\alpha 2\beta 1$ ligands

Twenty-one mouse monoclonal antibodies were raised against recombinant human $\alpha 2$ vWFA domain and were designated JA201–JA221. Solid-phase binding assays were carried out to identify function-blocking antibodies. In assays measuring the binding of biotinylated collagen to $\alpha 2$ vWFA domain fusion

Figure 1 Characterization of inhibitory anti-($\alpha 2$ vWFA domain) antibodies

(A) Inhibition of binding of biotinylated collagen I (3 μ g/ml) to $\alpha 2$ vWFA domain fusion protein. The non-inhibitory antibody, 16B4, which binds outside the vWFA domain, is included as a negative control. ■, Gi9; □, JA202; ▲, JA208; △, JA215; ●, JA218; ○, 16B4. In the presence of 5 mM EDTA, binding was reduced to 3.0%. Data are presented as means $\pm$ S.D. from triplicate points of a representative experiment, except Gi9, where data are presented as means $\pm$ ranges from duplicate points of a representative experiment. (B) Inhibition of binding of biotinylated collagen I (0.3 μ g/ml) to $\alpha 2\beta 1$ (HT1080 $\beta 1$ integrins) by anti-($\alpha 2$ vWFA domain) antibodies. Collagen was added at 0.3 μ g/ml, antibodies at 50 μ g/ml. Data are presented as means $\pm$ S.D. ($n = 5$ from 2 experiments, except for Gi9, where a representative duplicate point is shown). (C) Inhibition of HT1080 spreading on collagen I (1 μ g/ml). Antibodies were added at 10–20 μ g/ml. Data are presented as means $\pm$ S.D. ($n = 12$ from 3 experiments).

protein or purified $\alpha 2\beta 1$, antibodies JA202, JA215 and JA218 were found to be inhibitory, using Gi9 (a previously characterized inhibitory antibody [37]) and JA208 as positive and negative controls respectively (Figures 1A and 1B). JA202, JA215 and JA218 were also able to inhibit HT1080 cell spreading on collagen I (Figure 1C), but had no effect on HT1080 spreading on the 50 kDa fragment of fibronectin, which is mediated by $\alpha 5\beta 1$ (results not shown). FACS analysis showed similar profiles for JA202, JA208, JA215 and JA218 binding to $\alpha 2\beta 1$ on HT1080 cells, indicating that they were able to recognize expressed integrins in an equivalent manner (results not shown).

$\alpha 2\beta 1$ has a number of ligands other than collagens, and the antibodies were therefore tested for their ability to inhibit other interactions of $\alpha 2\beta 1$. Spreading of HT1080 cells on CPP-I was

Figure 2 Anti-functional antibodies inhibit $\alpha 2\beta 1$ -ligand interactions

(A) Inhibition of HT1080 cell spreading on 10 μ g/ml CPP-I. Data are presented as means $\pm$ S.D. ($n = 8$ from 2 experiments). (B) Inhibition of binding of biotinylated CPP-I (7 μ g/ml) to purified $\alpha 2\beta 1$ by anti-($\alpha 2$ vWFA domain) antibodies. Data are presented as means $\pm$ S.D. ($n = 6$ from 2 experiments). (C) Inhibition of HT1080 cell spreading on 5 μ g/ml E-cadherin-COMP. Data are presented as means $\pm$ S.D. ($n = 8$ from 2 experiments).

inhibited by JA202, JA215 and JA218 (Figure 2A), and the same antibodies also inhibited binding of CPP-I to purified $\alpha 2\beta 1$ (Figure 2B). E-cadherin has recently been shown to be a ligand for the $\alpha 2$ vWFA domain (J. D. Whittard, A. P. Mould, A. Koch, O. Pertz, J. Engel and M. J. Humphries, unpublished work), and again JA202, JA215 and JA218 inhibited HT1080 cell spreading on a recombinant E-cadherin protein comprising the five extracellular domains of mouse E-cadherin fused to the assembly domain of rat COMP (Figure 2C). These data demonstrated that the function-blocking properties of these antibodies were important for matrix and cell surface ligands of $\alpha 2\beta 1$ and that they were not restricted to collagen.

Clustering of epitopes of the anti-(vWFA domain) antibodies

In order to determine the spatial relationships between the epitopes for the inhibitory antibodies, cross-blocking studies were carried out to cluster the antibodies. $\alpha 2$ vWFA domain fusion protein was captured on to ELISA plates using an anti-GST polyclonal antibody, and the binding of biotinylated Gi9,

Table 1 Epitope clustering by anti-GST serum capture ELISA

Data are presented as means $\pm$ S.D. ($n = 4$ from 2 experiments except for data from biotinylated JA202 where $n = 6$ from 3 experiments).

Inhibiting antibody	Percentage binding of detecting antibody (biotinylated)			
	JA202	JA208	JA215	JA218
Gi9	-26.7 ± 6.1	0.9 ± 2.7	95.1 ± 26.1	93.8 ± 2.7
JA202	0.0 ± 12.0	30.0 ± 1.8	126.3 ± 12.8	103.9 ± 5.9
JA208	4.5 ± 5.6	11.2 ± 16.6	118.3 ± 23.1	99.0 ± 4.9
JA215	42.0 ± 9.9	50.1 ± 5.0	2.4 ± 5.6	1.9 ± 0.9
JA218	47.8 ± 12.9	51.8 ± 7.6	2.2 ± 3.7	0.8 ± 0.3

Figure 3 Effects of ligand molecules on binding of antibodies to $\alpha 2\beta 1$

(A) The effect of collagen I peptide on antibody binding. The peptide sequence is Gly-Pro-Cys-(Gly-Pro-Hyp)₃-Gly-Phe-Hyp-Gly-Glu-Arg-Gly-Val-Glu-Gly-Pro-Hyp-Gly-Pro-Ala-(Gly-Pro-Hyp)₃-Gly-Pro-Cys [24,25]. Black bars, no inhibitor; white bars, 100 μ g/ml peptide. Data are presented as means $\pm$ S.D. ($n = 9$ from 3 experiments). (B) The effects of collagen I (CI), CB3(IV) and CPP-I on antibody binding; Mn, in the presence of 1 mM Mn^{2+} ions; EDTA, in the presence of 10 mM EDTA as a control. Black bars, JA202; grey bars, JA208; white bars, 16B4. Data are presented as means $\pm$ S.D. ($n = 6$ from 2 experiments).

JA202, JA215 or JA218 was subsequently examined in the presence of excess unlabelled antibodies. The control antibody JA208 was also included (Table 1). Gi9, JA202 and JA208 were found to inhibit the binding of biotinylated JA202 and JA208 (inhibition of biotinylated Gi9 could not be included in these experiments because of high background binding). JA215 and JA218 were found to inhibit each other. In contrast, the binding of biotinylated JA215 and JA218 was not affected by JA202, JA208 or Gi9. The antibodies therefore form two epitope clusters, cluster I, JA202/JA208/Gi9, and cluster II, JA215/JA218. A second approach was also used to study epitope clustering. The monoclonals were used to capture the $\alpha 2$ fusion protein, and then binding of a second biotinylated monoclonal was detected.

Figure 4 Mutagenesis of Thr²²¹ in the MIDAS motif affects binding of JA202

Binding of (A) JA202 or (B) JA218 to wild-type (○) or T221A mutated (□) recombinant $\alpha 2$ vWFA domains. Data are presented as means $\pm$ S.D. ($n = 6$ from 2 experiments).

This approach led to the same conclusion as above (results not shown).

Antibodies are sensitive to events at the vWFA domain ligand-binding site

Monoclonal antibodies have been extensively used in integrin studies, not only as reagents for blocking binding, but also as reporters of changes in integrin conformation and thus as means by which information may be gained about the way in which integrins function [38–40]. This approach has not, however, been previously used to study $\alpha 2\beta 1$. In order to understand further the events accompanying the binding of collagen to the $\alpha 2$ vWFA domain, the effect of collagen on the binding of inhibitory antibodies was examined using a collagenous peptide that contains the $\alpha 2\beta 1$ recognition site [24]. The peptide inhibited binding of Gi9 and JA202 to $\alpha 2\beta 1$ and partially inhibited binding of JA215 and JA218 (Figure 3A). These epitopes were therefore intimately associated with and responsive to events at the collagen binding site. Binding of the control antibody, 16B4, was not inhibited. In contrast with the inhibition seen above, binding of JA208 to the $\alpha 2$ vWFA domain was enhanced by collagen peptide, indicating that this epitope is not only associated with the ligand-binding site but is also sensitive to changes in conformation induced by ligand binding; this is the first example of a ligand-induced binding site (LIBS) in the $\alpha 2$ vWFA domain.

The effect of $\alpha 2\beta 1$ ligands, other than the collagen peptide, on JA202 and JA208 was also examined. JA202 binding was inhibited by collagen I, CB3(IV) and CPP-I, while these agents had little or no effect on 16B4 (Figure 3B). The enhanced binding seen for JA208 with collagen peptide was also seen with CB3(IV), but not with collagen I. This may be because the relative insolubility of collagen I compared with CB3(IV) meant that sufficient soluble collagen could not be provided to affect JA208 binding. In contrast with CB3(IV), CPP-I inhibited JA208

Figure 5 Mab13 inhibits binding of antibodies to $\alpha 2\beta 1$

Grey bars, Mab13; black bars, rat IgG. Data are presented as means $\pm$ S.D. ($n \geq 5$ from at least 2 experiments, except for JA215 where $n = 3$ from 1 experiment).

binding, suggesting that the JA208 epitope may respond in different ways to different $\alpha 2\beta 1$ ligands.

Evidence for conformational changes affecting epitopes in cluster I was also obtained using an $\alpha 2$ vWFA domain in which the co-ordinating Thr²²¹ of the MIDAS motif had been mutated to alanine. This mutant vWFA domain showed decreased binding of JA202, while binding of JA218 and the other antibodies was unchanged (Figure 4; results for Gi9, JA208 and JA215 are not shown). This data further demonstrates the ability of cluster I antibodies to respond to changes induced at the collagen-binding site, of which the MIDAS motif is an integral part.

Antibodies are sensitive to changes induced outside the vWFA domain

The inhibitory anti- $\beta 1$ antibody, Mab13, reduced the binding of biotinylated collagen to purified $\alpha 2\beta 1$ by $> 85\%$ (results not shown). It is believed to inhibit collagen binding at the $\alpha 2$ subunit vWFA domain by inducing an inhibitory conformational change that is transmitted from the β subunit to the α subunit vWFA domain. In solid-phase binding assays Mab13 inhibited binding of JA202 and JA208 to $\alpha 2\beta 1$, and slightly inhibited the binding of Gi9 (Figure 5). No effect was observed on JA215 or JA218. These data indicate that the JA208 and JA202 epitopes are responsive to the changes induced by Mab13.

The effects of bivalent cations on antibody binding were also investigated. Binding of collagen by $\alpha 2\beta 1$ is supported by Mg^{2+} and Mn^{2+} , with Mn^{2+} supporting greatly enhanced binding relative to Mg^{2+} [41]. The effects of cations on the isolated $\alpha 2$ vWFA domain are different, since Mg^{2+} and Mn^{2+} support similar levels of binding [18]. The enhancement induced in $\alpha 2\beta 1$ by Mn^{2+} is therefore mediated from outside the vWFA domain. The effects of Mg^{2+} and Mn^{2+} on antibody binding were studied to identify any changes occurring in the vWFA domain as a consequence of Mn^{2+} binding to sites in the rest of the integrin. Binding of Gi9 or JA202 to the recombinant vWFA domain was identical in the presence of Mg^{2+} or Mn^{2+} (Figure 6). Similarly, no effect was seen on JA208, JA215 and JA218 (results not shown). However, binding of Gi9 to $\alpha 2\beta 1$ was lower in the presence of Mn^{2+} than Mg^{2+} (Figure 6), and this was also seen for JA218 (results not shown). In contrast, binding of JA202 to $\alpha 2\beta 1$ was identical in the presence of either cation (Figure 6), and similar results were seen for JA208 and JA215 (results not shown). The Gi9 and JA218 epitopes were therefore sensitive to Mn^{2+} -induced changes initiated outside the vWFA domain,

Figure 6 Bivalent cations affect binding of antibodies to α2β1

Binding of antibodies Gi9 (A and B) and JA202 (C and D) to recombinant α2 vWFA domain (A and C) or α2β1 (B and D) was measured. ○, Mn²⁺; □, Mg²⁺. Data are presented as means ± S.D. [*n* = 4 from 2 experiments, except for (C), where data are means ± ranges of duplicate points from a representative experiment].

indicating that the conformation of these epitopes was modulated by the cation-induced changes.

The epitopes in cluster I map to residues 199–216

Binding of antibody Gi9 to α2 vWFA domain was found to be inhibited by the previously characterized anti-functional antibody 5E8 (results not shown). The epitope for 5E8 has been

mapped to residues 198–213 [42], which are located in the region of the α3 helix [43]. Regions topologically close to the α3 helix were therefore targeted as candidate sites for the epitopes for the cluster I antibodies, JA202, JA208 and Gi9. To identify the epitopes for these antibodies, chimaeric α2 vWFA domains were generated, in which short stretches of human sequence were replaced by the corresponding mouse amino acids. These chimaeras were then tested for their ability to support antibody

Figure 7 Mapping the epitopes of the anti-functional antibodies using human/mouse chimaeras

A diagrammatic representation of the α3 and α4 helix region of the α2 vWFA domain is shown. Directly below this is the corresponding human (hu) and mouse (mo) sequences, with amino acid numbers from the human α2 subunit sequence. Mouse sequence is only shown where it differs from the human sequence. Underlined regions indicate the sequences substituted to give chimaeras A–F. Beneath the sequence and structure representations are given the level of binding of each antibody to each of the chimaeras, expressed as a percentage of binding to wild-type human α2 vWFA domain. Values in **bold** indicate chimaeras where antibody binding was lost.

binding (Figure 7). Binding of JA202 and Gi9 was ablated only in construct D, mapping these epitopes to residues 212–216, at the C-terminal end of helix $\alpha 3$, close to the collagen-binding site [27–29]. The JA208 epitope covered constructs B–D, mapping between residues 199–216; this is larger than expected for an antibody epitope and suggests that JA208 binds to a epitope that is particularly sensitive to the changes introduced in the chimaeras. Binding of JA215 and JA218 was unaffected in all of the chimaeras, and the epitopes for these antibodies therefore lie outside the region chosen for mapping. The inhibitory functions of cluster I antibodies, the sites of conformational change and the sensitivities to events initiated outside of the vWFA domain therefore map to residues 199–216.

DISCUSSION

The conformational changes which are associated with integrin function are an area of considerable interest, as they hold the key to understanding the ways in which integrins convert binding events into cellular responses and regulate their activity. In this study, we have raised three novel antibodies, JA202, JA215 and JA218, directed against the vWFA domain of $\alpha 2$, and used them as probes of $\alpha 2\beta 1$ function. We have identified two epitope clusters, the first, residues 199–216, comprising the epitopes for JA202, the previously characterised antibody Gi9 and the control antibody JA208, and the second comprising the epitopes for JA215 and JA218. Both clusters define functionally important epitopes with novel properties: they are intimately associated with the collagen-binding site, responsive to conformational changes at the collagen-binding site and sensitive to regulatory events initiated outside the vWFA domain. The ability of JA202, JA215 and JA218 to inhibit $\alpha 2\beta 1$ binding to collagen I, CPP-I and E-cadherin indicates that these properties are relevant to the interactions of $\alpha 2\beta 1$ with a range of ligands.

Conformational change in integrin vWFA domains

This is the first report of anti-($\alpha 2$ vWFA domain) antibodies whose binding is enhanced by ligand or inhibited by changes at the MIDAS motif, and identifies residues 199–216, and perhaps the JA215/JA218 epitopes, as regions undergoing conformational changes associated with collagen binding. This report is also the first time that function-blocking antibodies have been unequivocally mapped close to the collagen binding site of the $\alpha 2$ vWFA domain, as examination of the vWFA domain–collagen crystal structure shows residues 212–216 to be close to the site at which collagen binds; D219, for example, binds Arg⁵⁰⁷ from the $\alpha 1$ chain of type I collagen, and vWFA domain Gln²¹⁵ forms a hydrophobic association with collagen Phe⁵⁰³ [27]. Each of these, whilst secondary to the crucial direct co-ordination of cation in the vWFA MIDAS by Glu⁵⁰⁶, has been shown to be essential for binding of the peptide to the $\alpha 2$ vWFA domain [28,29]. The proximity of JA202 and Gi9 to the collagen-binding site would account for their ability to inhibit the interactions of $\alpha 2\beta 1$ with collagen, CPP-I and E-cadherin, as well as their inhibition by collagen peptide.

Monoclonal antibodies are widely used in integrin studies as inhibitors of function, but little has been done to describe their method of action. Our data indicate that inhibitory antibodies can act by steric hindrance of the ligand-binding site. This is in contrast with the situation observed for anti-(β subunit) inhibitory antibodies, such as Mab13, which have been shown to act allosterically [44]. In another mapping study, binding of the anti- $\alpha 2$ antibody, P1H5, was found to be inhibited by mutagenesis of Asp¹⁶⁰ to alanine [45], and so this antibody could also function

by binding close to the collagen-binding site, although further characterization would be required to show this. We were unable to map JA215 and JA218 using the chimaeras described above, but their inhibition by collagen suggests that they also bind proximal to the ligand-binding site.

The recent solution of the crystal structure of the $\alpha 2$ vWFA domain complexed with collagen clearly demonstrates that the $\alpha 2$ vWFA domain undergoes conformational changes on binding ligand [27] and validated the proposals based on the two α M X-ray crystal structures of an ‘open’ (liganded) and ‘closed’ (unliganded) form [46]. Our antibody data both confirm and extend these observations, indicating that changes in the conformation of residues 199–216 have a biological role and can be detected by antibodies. Examination of the liganded and unliganded forms of $\alpha 2$ and the open and closed forms of α M suggest that conformational changes in the $\alpha 3$ helix could be responsible for the effects on the epitopes. Alternatively, movement of the adjacent $\alpha 1$ helix could affect epitope exposure in the $\alpha 3$ helix (and vice versa), and thus account for the differences in antibody binding that we report in the present study.

The conformational interplay between the ligand-binding site and residues 199–216 that we have demonstrated would account for the observation of Dickeson et al. [42], who showed that substitution of integrin $\alpha 2$ sequence with $\alpha 1$ sequence within helix $\alpha 3$ converted the vWFA domain from a weak collagen IV binding form (as normally seen with $\alpha 2\beta 1$) into a stronger collagen IV-binding form as seen in $\alpha 1\beta 1$; conformational change in helix $\alpha 3$ might propagate to the collagen-binding site and thus increase binding. Similarly, other observations of inhibitory or activating mutations in this region [47–49] or mapping of anti-functional antibodies to this locality [50] in $\alpha L\beta 2$ and $\alpha M\beta 2$ are accounted for by its major functional role.

JA208 is the first example of a LIBS antibody in the $\alpha 2$ vWFA domain. Despite its proximity to JA202 and Gi9, JA208 clearly has different properties from the other two antibodies, demonstrating that antibodies with overlapping or proximal epitopes need not behave in the same way. A similar phenomenon has been observed in the $\beta 1$ subunit, where the epitopes for both inhibitory and activating/LIBS antibodies map to within the same 11-amino-acid sequence [51]. Since certain regions within integrins undergo conformational changes on moving between the open and closed forms, it is likely that some antibodies could bind to the closed form, and thus inhibit, while others could bind to the open form and activate the integrin or report this state (i.e. a LIBS antibody). These situations reflect the importance of conformational changes in integrin function.

Conformational change and integrin function

Integrin function requires communication between the vWFA domain and the rest of the integrin; thus the signal initiated by ligand binding must be transmitted from the vWFA domain through the rest of the integrin and into the cell, while regulatory information is transmitted from within the cell into the vWFA domain to modulate binding. The mechanisms by which these processes occur are poorly understood, and little is known about the regions of the vWFA domain which are involved. Our report shows for the first time that residues 199–216 and the as yet undefined JA215/JA218 epitopes respond to signals initiated in $\alpha 2\beta 1$ outside the vWFA domain. Their proximity to, and intimate association with, the ligand-binding site suggests that these regions may be sites through which control of the vWFA domain is exercised by other regions of the integrin. Several potential sites for such regulation have been identified by the solution of the crystal structure of the collagen–vWFA domain complex

[27]. These include: (1) the C-helix, which unwinds upon ligation so that an extra turn is added to the $\alpha 6$ helix from the extension of the $\alpha C:\alpha 6$ loop so formed (around amino acids 284–290); (2) helices $\alpha 1$ and $\alpha 3$, which have been discussed above; (3) helix $\alpha 7$, which undergoes major perturbations affecting both the interface between the vWFA domain and the rest of the $\alpha 2$ subunit (around amino acid 330), and the adjacent $\alpha 1$ helix (around amino acid 160) in which novel epitopes are revealed by the same movement. These may be the sites of interaction of JA215 and JA218.

Our $\alpha 2\beta 1$ data show close and interesting parallels with the study of Oxvig et al. [40] on the conformational changes occurring in the αM vWFA domain: here the antibody CBRM1/5 bound to the αM vWFA domain of $\alpha M\beta 2$ in its activated state and inhibited ligand binding. The specificity for the activated form was shown to be due to sensitivity of the antibody to conformational change in the vWFA domain, induced from elsewhere in the integrin, and the epitope for this antibody was mapped to two discontinuous regions: the C-terminal end of the $\alpha 3$ helix (corresponding to residues 212–216 of $\alpha 2$) and the adjacent region comprising the C-terminal end of helix $\alpha 1$. CBRM1/5 thus resembles the cluster I epitope antibodies, which map to an equivalent site, in that it inhibited ligand binding, was sensitive to conformation and responded to changes initiated elsewhere in the molecule. Taken together, our data and that of Oxvig et al. and others [40,47–49] indicate that the $\alpha 3$ helix and adjacent regions are important in $\alpha 2$, αL and αM function, and therefore potentially in all vWFA-domain-containing integrins.

In conclusion, we have identified important regions in the vWFA domain of the $\alpha 2$ subunit, particularly residues 199–216, which are associated with ligand binding, respond to conformational change and are sensitive to events outside the vWFA domain. This greatly extends our knowledge of $\alpha 2$ vWFA domain function and has implications for our understanding of integrins in general.

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