Interaction between Collagen and the $\alpha_2$ I-domain of Integrin $\alpha_2\beta_1$

CRITICAL ROLE OF CONSERVED RESIDUES IN THE METAL ION-DEPENDENT ADHESION SITE (MIDAS) REGION*

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A docking model of the $\alpha_2$-I-domain and collagen has been proposed based on their crystal structures (Emsley, J., King, S., Bergelson, J., and Liddington, R. C. (1997) J. Biol. Chem. 272, 28512–28517). In this model, several amino acid residues in the I-domain make direct contact with collagen (Asn-154, Asp-219, Leu-220, Glu-256, His-258, Tyr-285, Asn-289, Asn-295, and Lys-298), and the protruding C-helix of $\alpha_2$ (residues 284–288) determines ligand specificity. Because most of the proposed critical residues are not conserved, different I-domains are predicted to bind to collagen differently. We found that deleting the entire C-helix or mutating the predicted critical residues had no effect on collagen binding to whole $\alpha_2\beta_1$, with the exception that mutating Asn-154, Asp-219, and His-258 had a moderate effect. We performed further studies and found that mutating the conserved surface-exposed residues in the metal ion-dependent adhesion site (MIDAS) (Tyr-157 and Gln-215) significantly blocks collagen binding. We have revised the docking model based on the mutagenesis data. In the revised model, conserved Tyr-157 makes contact with collagen in addition to the previously proposed Asn-154, Asp-219, His-258, and Tyr-285 residues. These results suggest that the collagen-binding I-domains (e.g. $\alpha_1$, $\alpha_2$, and $\alpha_1\beta_1$) bind to collagen in a similar fashion.

Several integrin $\alpha$ chains ($\alpha_1$, $\alpha_2$, $\alpha_10$, $\alpha_5$, $\alpha_6$, $\alpha_9$, and $\alpha_{10}$) have inserted I- or A-domains of about 200 amino acid residues (1–11). Integrins $\alpha_{1}\beta_1$ (1), $\alpha_2\beta_2$ (reviewed in Ref. 12), and $\alpha_10\beta_1$ (4) have been shown to bind to collagen and/or laminin. Several function-blocking antibodies map to the I-domains of $\alpha_{1}\beta_1$ (13) and $\alpha_2\beta_2$ (14). The recombinant $\alpha_2$-I-domain fragment binds to collagen (15, 16), and the recombinant $\alpha_1$-I-domain fragment binds to collagen and laminin (17). Conserved Asp and Thr residues in the $\alpha_2$-I-domain (Asp-151, Thr-221, and Asp-254) are critical for collagen binding (15). These lines of evidence suggest that the I-domain is critically involved in collagen binding.

The crystal structures of the I-domains of the integrin $\alpha_M$ (18), $\alpha_1$, (19), and $\alpha_2$ (20) subunits, and the A1 (21, 22) and A3 (23, 24) domains of von Willebrand factor (vWF) have been published. This domain adopts a classic “Rossmann” fold and consists of a hydrophobic β-sheet and an amphipathic α-helices on both sides. Interestingly, the integrin I-domain contains a Mg$^{2+}$/Mn$^{2+}$ coordination site at its surface, which is not present in proteins with similar structures (e.g. the NAD binding domain of lactate dehydrogenase) or the vWF A1 and A3 domains (21–24). The Asp and Thr residues in $\alpha_2$ that have been shown to be critical for ligand binding are involved in the coordination of a divalent cation in the crystal structure (20). The $\alpha_2$ I-domain has a unique helix (the C-helix) protruding from the metal ion-dependent adhesion site (MIDAS) that creates a groove centered on the magnesium ion. Emsley et al. (20) proposed a model in which a collagen triple helix fits into the groove and a Glu side chain from collagen coordinates the metal ion. In this model, the C-helix is a major determinant for collagen binding. It was predicted that the following I-domain residues make direct contact with collagen: Asn-154 (the $\beta_1$-$\alpha_1$ turn), Asp-219 and Leu-220 (the $\alpha_2$-$\alpha_3$ turn), Glu-256 and His-258 (the $\beta_2$-$\alpha_1$ turn), and Tyr-285, Asn-289, Asn-295, and Lys-298 (the C-helix, $\alpha_6$, and C-$\alpha_5$ turn). However, these residues are not well conserved among collagen-binding I-domains (e.g. $\alpha_1$, $\alpha_2$, and $\alpha_1\beta_1$), suggesting that different I-domains interact with collagen in different manners. Here we show that mutation of the residues proposed to be critical for ligand binding or deletion of the entire C-helix did not significantly affect collagen binding to whole $\alpha_2\beta_1$ expressed on mammalian cells except for Asn-154, Asp-219, and His-258. In contrast, mutating several conserved MIDAS residues including Tyr-157 significantly blocks collagen binding. We have revised the docking model based on the mutagenesis data. In the revised model, interaction between the $\alpha_2$ I-domain and collagen is mediated by relatively conserved residues in the MIDAS on the N-terminal side of the I-domain. Thus, it is suggested that the collagen-binding I-domains (e.g. $\alpha_1$, $\alpha_2$, and $\alpha_1\beta_1$) bind collagen in a similar fashion.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—HAS-3 and HAS-4 (25) are generous gifts from F. Watt (Imperial Cancer Research Fund, London, U.K.)

Adhesion of CHO Cells to Collagen—Wells of 96-well microtiter plates (Immulon-2, Dynatech Labs., Inc., Chantilly, VA) were coated with type I collagen (2 or 10 μg/ml) at 4 °C overnight. The other protein binding sites were blocked by incubating with 1% (w/v) bovine serum albumin (Calbiochem, CA) for 30 min at room temperature, and washing three times with phosphate-buffered saline (10 mM phosphate, 0.15 M NaCl, pH 7.4). Cells were harvested with 3.5 mM EDTA in phosphate-buffered saline and washed twice with Dulbecco’s modified Eagle’s medium. 105 cells (in 100 μl of Dulbecco’s modified Eagle’s medium) were added to each well and incubated for 1 h at 37 °C. The wells were rinsed three times with phosphate-buffered saline to remove unbound cells. Bound cells were quantified by assaying endogenous phosphatase activity (26).

Molecular Modeling—A model of a collagen triple helix was constructed from the crystal structure (Ref. 27, Protein Data Bank code...
1cag) as described previously (20). Side-chains from the sequence of the CB3(15/6 peptide containing the GER motif (28) were grafted onto the collagen in standard conformations using the program TOM (29). The glutamate of one of the GER motifs was attached to the Mg$^{2+}$ ion of the MIDAS motif via one of its carboxylate oxygens at a distance of 2.0 Å. Keeping the I-domain fixed, the collagen was then allowed to rotate around a fixed point (the glutamate oxygen) to minimize the distance between the collagen and the side chains of those residues which showed reduced collagen binding when mutated and which were exposed on the surface of the I-domain. Unfavorably close contacts (2.5 Å) between the collagen and the I-domain were monitored using the program TOM. Because the triple helical nature of collagen generates three chemically distinct strands even for a homo-tripeptide (which we call the leading, middle, and trailing strands) each of these was tested separately.

Other Methods—Swapping mutagenesis was carried out using the overlap extension polymerase chain reaction (30). The positions of the $a_2$ sequences replaced by homologous $a_1$ sequences are residues 152–157, 212–219, and 257–262 (designated $\beta_{a_2\alpha_1}$, $\alpha_{a_2\alpha_1}$, and $\beta_{a_2\alpha_1}$, respectively) (see Fig. 1). Deletion of residues 284–291 (designated $a_2$ del) and point mutations were created by site-directed mutagenesis using the unique site elimination method with a double-stranded vector (31). The presence of mutation was confirmed by DNA sequencing. Transfection of cDNAs into CHO cells by electroporation, selection of transfected cells with G418, and flow cytometry were carried out as described previously (32).

RESULTS AND DISCUSSION

The MIDAS of the $a_2$ I-domain is composed of four loops (the $\beta_{a_2\alpha_1}$, $\alpha_{a_2\alpha_1}$, $\beta_{a_2\alpha_1}$, and $\beta_{a_2\alpha_1}$) loops). The conserved residues Asp-151 and Thr-221, and Asp-254, which are conserved residues Asp-151, Thr-221, and Asp-254, which are critical for cation and collagen binding. Cells expressing mutants to collagen type I expressed as a percentage of cells used for adhesion assays. Fig. 2 shows the adhesion of the mutants to collagen type I at lower collagen coating concentrations. These swapping mutations did not change the CB3(15/6) sequence, which does not interact with collagen (Fig. 1). These swapping mutations did not change the conserved residues Asp-151, Thr-221, and Asp-254, which are critical for cation and collagen binding. Cells expressing mutant $a_2$ were tested for their ability to adhere to collagen. The expression of the $a_2$-swapping mutant was too low to produce reliable adhesion data (data not shown). Other mutants showed a surface expression level comparable with that of wild type and reacted with multiple monoclonal antibodies against $a_2$ (Fig. 3a). The $\beta_{a_2\alpha_1}$-swapping mutant showed collagen binding at a background level. Also, the $\beta_{a_2\alpha_1}$-swapping mutant showed significantly reduced collagen binding. These results are consistent with those obtained using alanine-scanning mutagenesis.

In contrast, mutation of amino acid residues in the $\beta_{a_2\alpha_1}$ loop, including the C-helix, did not have any inhibitory effect on collagen binding. Mutation of Tyr-285, Asn-289, Leu-291, and Asn-295, which are predicted to make direct contact with collagen, did not significantly affect collagen binding, even at low (2 μg/ml) collagen coating concentrations (Fig. 2). It is possible that single amino acid substitution may not be enough to induce a detectable effect on collagen binding. So, we deleted most of the $\beta_{a_2\alpha_1}$ loop, including the entire C-helix, to determine whether the C-helix is critical for ligand specificity. These mutant $a_2$ cDNAs were stably expressed on CHO cells and further cloned to obtain high expressors. The $a_2$ deletion mutant showed collagen binding at a level comparable with that of wild type (Fig. 3a). Adhesion of the $a_2$ deletion mutant as a function of collagen coating concentration was tested. Adhesion to collagen of both wild type and $a_2$ deletion mutant $a_2$ was saturated at about 1 μg/ml collagen coating concentration, indicating that the affinity to collagen is not affected by the $a_2$ deletion (Fig. 3b).

These results suggest that collagen binding is mediated by relatively conserved MIDAS residues, which are located on the N-terminal side of the I-domain. Ser-153 and Ser-155 are involved in metal coordination, and mutating these residues would disrupt metal binding to the I-domain. Glu-215 is part of the MIDAS face and makes the main hydrogen bond to D

![Fig. 1. Residues/loops chosen for mutagenesis in this study.](image)
binding by disrupting metal binding to the I-domain. Asn-154, Asp-219, and His-258 have been predicted to make direct contact with collagen in the previous model (20), although the effects of mutating these residues are moderate. Tyr-157 is totally exposed to the surface, and this residue may make direct contact with collagen. Tyr-157 has not been predicted to make direct contact with collagen. Mutating the other residues that are predicted to be critical for collagen binding in the proposed model (Leu-220, Glu-256, Tyr-285, Asn-289, Leu-291, Asn-295, and Lys-298) has no significant effect on collagen binding. Even deletion of the entire C-helix did not significantly affect collagen binding. These results support the role of the MIDAS motif in collagen binding because many of the mutants that affect collagen binding are predicted to disrupt the MIDAS motif. However, the present results are not fully consistent with the proposed model for collagen/\(\alpha_2\) I-domain binding (20).

We have modified the collagen/\(\alpha_2\) I-domain docking model based on the present mutagenesis data (Fig. 4). Recently, short synthetic triple-helical peptide corresponding to residues 502–516 of the collagen type I \(\alpha_1\) chain, has been shown to bind to purified \(\alpha_2\beta_1\) and recombinant \(\alpha_2\) I-domain (28). The Glu and Arg residues in the GER triplet were found to be essential for recognition by \(\alpha_2\) I-domain (28). In the current model, we first attached the glutamate side chain of the GER motif to the MIDAS Mg\(^{2+}\) ion. We then rotated the collagen to minimize the distance between the collagen and those surface-exposed residues implicated in collagen binding (His-258, Tyr-157, Asp-219, and Asn-154) while maintaining the 2-Å bond between the

**Fig. 2. Effects of point mutations on collagen binding.** Cells stably expressing wild type or mutant \(\alpha_2\) were used to determine adhesion to collagen (at a 10 or 2 \(\mu\)g/ml coating concentration, filled column and blank column, respectively). Data are presented as percent bound cells to collagen per percent human \(\alpha_2\) positive cells to normalize \(\alpha_2\) expression. Typically 40–60% of cells are positive after selection with G-418. Previously published function-negative mutations (D151A, T221A, and D254A) are included as negative controls. These results suggest that several relatively conserved residues in the \(\beta_\alpha\alpha_1\), \(\alpha_2\alpha_3\), and \(\beta_2\alpha_4\) loops are critical for collagen binding.

**Fig. 3. Effects of swapping/deletion mutations on collagen binding.** A, clonal CHO cells stably expressing wild type or mutant \(\alpha_2\) were incubated in the well coated with collagen type I or bovine serum albumin (negative control). After incubation at 37 °C for 1 h, non-adherent cells were removed and bound cells were determined by assaying endogenous phosphatase. Under the conditions used more than 80% of cells adhered to fibronectin as a positive control. Solid bar, BSA; hatched bar, collagen; MFI, mean fluorescence intensity. B, adhesion to collagen of wild type and the \(\alpha_2\) deletion mutant \(\alpha_2\) was determined as a function of collagen coating concentrations. The data suggest that the adhesive function of the \(\alpha_2\) deletion mutant is comparable with that of wild type.

**Fig. 4. A revised docking model of the \(\alpha_2\) I-domain and collagen.** All-atom representation of the \(\alpha_2\) I-domain, viewed looking down onto the MIDAS face. In the top panel, residues with mutations that reduce collagen binding are in red (surface-exposed) or pink (likely to disrupt MIDAS). Residues with mutations that have no effect on collagen binding are in cyan. The Mg\(^{2+}\) ion is shown as a gray ball. The new collagen model is shown as a colored triple helical coil (blue, green, and yellow) drawn through the Ca positions. The previous model (20) is shown as a transparent triple helical coil. Certain residues referred to under “Results and Discussion” are labeled. In the bottom panel, residues shown in red are invariant between \(\alpha_1\), \(\alpha_2\), and \(\alpha_{10}\) I-domains.
glutamate oxygen and the Mg$^{2+}$ ion and avoiding other close contacts (<2.5 Å) with the protein. It was not initially possible to make favorable hydrogen bonds with all four side chains simultaneously, so the side chains were allowed to rotate about their Ca-Cα bonds to make plausible hydrogen bonds with the collagen backbone carbonyl oxygens and amide nitrogens. The collagen orientation was then refined to optimize the hydrogen bonding geometry. This procedure allowed all four I-domain side chains to make reasonable hydrogen bonds to the collagen.

This model predicts that the side chain of Tyr-285, which projects from the C helix into the groove, makes unavoidable contact with the collagen and that further hydrogen bonds can be made between the Tyr hydroxyl and the collagen main chain. This revised model, which is rotated about 30 degrees from the previously published model, allows the arginine from the GER motif of the preceding strand to make a salt bridge to Glu-256. The previous model would not allow enough space for the arginine side chain without imposing unfavorable side chain torsion angles. Because mutation of Glu-256 does not significantly block collagen binding in the present study, this salt bridge might not be energetically important. The triple helical character of a symmetric collagen trimer generates three chemically distinct strands, which we call the leading, middle, and lagging strands. Attaching either the leading or middle strand glutamate to the Mg$^{2+}$ ion leads to the same conclusions. Attaching the trailing strand makes a difference because there is no arginine from the preceding strand to form a salt-bridge to Glu-256. The alternative orientation with the collagen rotated by 180 degrees is much less favorable because the arginine of the GER motif would clash sterically with the I-domain. As pointed out in our previous model, an aspartic acid side chain in place of the GER glutamate would be too short to reach the Mg$^{2+}$ ion without creating a large number of steric clashes. Because Asn-154, Tyr-157, His-258 are conserved in other collagen-binding integrin I-domains (α1 and α10), it is reasonable to assume that these residues are also involved in collagen binding in these integrins (Fig. 4). This model is consistent with the observation that tyrosine and arginine are enriched in hot spots of binding energy in the protein-protein interface (33).

In the present model, the highly conserved D151 and D254 residues in the α1 I-domain are buried underneath the Mg$^{2+}$ ion and cannot contact collagen directly. We have reported that mutating these residues only partially affects collagen binding to the recombinant α2 I-domain fragment (13, 15). Also, Bienkowska et al. (23) reported that mutating the corresponding residues in the recombinant fragment of the vWF A3 domain does not affect collagen binding. However, the same mutation in the whole α2 molecule completely blocks collagen binding to the α2β3 (13, 15). It is possible that cation coordination through these residues is critical for ligand binding in the I-domain of the integrin molecule but not in similar domains in non-integrin structures (e.g. vWF). Consistently, the cation-binding site is not present in the vWF A3 domain (23, 24). Further studies will be required to determine whether there are any common collagen binding mechanisms.

While this paper was under review, the crystal structure of a GER-containing collagen peptide/α1 I domain complex has been solved. Preliminary analysis reveals that the structure is very similar to the revised model described here. Thus, the orientation and location of the collagen is as predicted, with Glu residues from the collagen coordinating directly to the metal ion. In addition, there is an unexpected change in the C helix so that it no longer touches the collagen, in agreement with the mutagenesis results.

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REFERENCES

1. Ignatius, M. J., Large, T. H., Houde, M., Tawil, J. W., Burton, A., Esch, F., Cabomet, S., and Reichardt, L. F. (1990) J. Cell Biol. 111, 709–720
2. Briesewitz, R., Epstein, M. R., and Marcantonio, E. E. (1993) J. Biol. Chem. 268, 2898–2906
3. Takada, Y., and Hemler, M. E. (1989) J. Cell Biol. 109, 397–407
4. Camper, L., Hellman, U., and Lundgren-Akerlund, E. (1998) J. Biol. Chem. 273, 20383–20389
5. Larson, R., Corbi, A. L., Berman, L., and Springer, T. A. (1989) J. Cell Biol. 108, 703–712
6. Corbi, A. L., Kishimoto, T. K., Miller, L. J., and Springer, T. A. (1988) J. Biol. Chem. 263, 12403–12411
7. Arnaout, M. A., Gupia, S. K., Pierce, M. W., and Tenen, D. G. (1988) J. Cell Biol. 106, 2153–2158
8. Pytela, R. (1988) EMBO J. 7, 1371–1378
9. Corbi, A. L., Miller, L. J., O’Connor, K., Larson, R. S., and Springer, T. A. (1987) EMBO J. 6, 4023–4028
10. Van der Vieren, M., Trong, H. L., Wood, C. L., Moore, P. F., St. John, T., Staunton, D. E., and Gallatin, W. M. (1995) Immunity 3, 683–690
11. Shaw, S. K., Cepek, K. L., Murphy, E. A., Russell, G. L., Brenner, M. B., and Parker, C. M. (1994) J. Biol. Chem. 269, 6016–6025
12. Santoro, S. A., and Zutter, M. M. (1995) Thromb. Haemostasis 74, 813–821
13. Kamata, T., Puzon, W., and Takada, Y. (1994) J. Biol. Chem. 269, 9659–9663
14. Kern, A., Briesewitz, R., Bank, I., and Marcantonio, E. (1994) J. Biol. Chem. 269, 22811–22816
15. Kamata, T., and Takada, Y. (1994) J. Biol. Chem. 269, 26006–26010
16. Tuckwell, D., Calderwood, D. A., Green, L. J., and Humphries, M. J. (1995) J. Cell Sci. 108, 1629–1637
17. Calderwood, D. A., Tuckwell, D. S., Eble, J., Kuhn, K., and Humphries, M. J. (1997) J. Biol. Chem. 272, 12113–12121
18. Lee, J.-O., Rieu, P., Arnaout, M. A., and Liddington, R. (1995) Cell 80, 631–638
19. Qu, A., and Leahy, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10277–10281
20. Emsley, J., King, S. L., Bergelson, J. M., and Liddington, R. C. (1997) J. Biol. Chem. 272, 28512–28517
21. Emsley, J., Cruz, M., Mandin, R., and Liddington, R. (1998) J. Biol. Chem. 273, 10396–10401
22. Celikel, R., Varughese, K. I., Madhusudan, Yashiooka, A., Ware, J., and Ruggieri, Z. M. (1996) Nat. Struct. Biol. 3, 189–194
23. Bienkowska, J., Cruz, M., Atieno, A., Handin, R., and Liddington, R. (1997) J. Biol. Chem. 272, 25162–25167
24. Huzinga, E. G., Martijn van der Plas, R., Knoop, J., Sixma, J. J., and Gros, P. (1997) Structure 5, 1147–1156
25. Tenchini, M. L., Adams, J. C., Gilbert, C., Steel, J., Hudson, D. L., Malekowitz, M., and Watt, F. M. (1993) Cell Adhesion Comp. 1, 55–66
26. Prater, C. A., Plotkin, J. J., Daye, D., and Frazier, W. A. (1991) J. Cell Biol. 112, 1031–1040
27. Bella, J., Eaton, M., Brodsky, B., and Berman, H. M. (1994) Science 266, 75–81
28. Knight, C. G., Morton, L. F., Osnley, D. J., Peache, A. R., Messent, A. J., Smethurst, P. A., Tuckwell, D. S., Farnsdale, R. W., and Barnes, M. J. (1998) J. Biol. Chem. 273, 33287–33294
29. Camillia, H., Corbi, A. L., and Jones, T. A. (1994) Mol. Graphs 2, 53–54
30. Horton, R. M., and Pease, L. L. (1991) in Directed Mutagenesis: A Practical Approach (McPherson, M. J., ed), pp. 217–247, IRL Press, Oxford
31. Deng, W. P., and Nickleloff, J. A. (1999) Anal. Biochem. 200, 81–88
32. Takada, Y., Yianne, J., Mandelman, D., Puzon, W., and Ginsberg, M. (1992) J. Cell Biol. 119, 913–921
33. Bogan, A. A., and Thorn, K. S. (1998) J. Mol. Biol. 280, 1–9
34. Sasaki, T., Hohenester, E., Gohring, W., and Timpl, R. (1998) EMBO J. 17, 1625–1634
35. Symersky, J., Patti, J. M., Carmon, M., House-Pompeo, K., Teale, M., Moore, D., Jin, L., Schneider, A. D., lacas, L. J., Hook, M., and Narayana, S. V. (1997) J. Nat. Struct. Biol. 4, 833–838

2) J. Emsley, C. G. Knight, M. J. Barnes, R. W. Farnsdale, and R. Liddington, unpublished results.