Overlapping, but Not Identical, Sites Are Involved in the Recognition of C3bi, Neutrophil Inhibitory Factor, and Adhesive Ligands by the αMβ2 Integrin*

Li Zhang† and Edward F. Plow

From the Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Department of Molecular Cardiology, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

The αMβ2 (CD11b/CD18, Mac-1) integrin receptor binds numerous ligands, including neutrophil inhibitory factor (NIF), C3bi, and certain immobilized protein substrates, represented by denatured ovalbumin. These ligands share no obvious structural similarities, yet their interactions with receptor are inhibited by NIF and involve the I domain, a stretch of ~200 amino acids in the αM subunit. Recombinant wild-type and mutant forms of αMβ2 have been used to compare the recognition requirements of these ligands. The various constructs were expressed efficiently on the surface of human embryonic kidney 293 cells and formed αβ heterodimeric complexes. The wild-type transfectants bound the three ligands in a similar fashion to naturally occurring αMβ2. NIF inhibited these interactions, and deletion of the DaspGLPY from within the I domain abolished binding of all three ligands, suggesting an overlapping recognition specificity. A single point mutation of Ser136 to Ala in the β2 subunit abolished C3bi binding and cell adhesion but did not affect NIF binding. A switch of the R281QELNTI sequence in helix 6 of the αM I domain to the corresponding sequence in the I domain of the αL (QETLHKF) subunit completely abrogated adhesion while not affecting C3bi and NIF binding. The two mutant receptors also did not support activation-dependent adhesion to fibrinogen. Thus, the contact sites for NIF, C3bi, and adhesive proteins, represented by denatured ovalbumin and fibrinogen, in αMβ2 are overlapping but not identical.

---

*This work was supported by National Institutes of Health Grant HL38292 and the American Heart Association Grant 149-BG1A. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Joseph J. J Jacobs Center for Thrombosis and Vascular Biology, Dept. of Molecular Cardiology, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Mail Code: FF20; Tel.: 216-445-8213; Fax: 216-445-8204.

‡To whom correspondence should be addressed: Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Department of Molecular Cardiology, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Mail Code: FF20; Tel.: 216-445-8213; Fax: 216-445-8204.

---

The abbreviations used are: Fg, fibrinogen; dOva, denatured ovalbumin; EC3bi, C3bi-coated sheep erythrocytes; FACS, fluorescence activated cell sorting; HBSS, Hank’s balanced salt solution; mAb, monoclonal antibody; NIF, neutrophil inhibitory factor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
Ligand Binding Specificity of Integrin $\alpha_{\text{M}}\beta_2$

specific regions of the receptor involved in selective recognition of individual ligands.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human kidney 293 cells and the expression vector, pCI2SM were generous gifts from Dr. F. J. Castellino (Natre Dame, IN). The cDNA of CD11b and CD18 were obtained from Dr. B. Kamar-Tamir (Ammun, Thousand Oaks, CA). NIF was a gift from Dr. M. Moyle (Son, San Jose, CA), using the XbaI pCIS2M expression vector employing the I and $\beta$ sites; expression of $\beta_2$ subunit, F (pCIS2M-ogen, or NIF—

**Expression of $\alpha_{\text{M}}\beta_2$ in 293 Cells**—The expression vectors containing wild-type and mutated $\alpha_{\text{M}}\beta_2$ (pCI2SM-$\alpha_{\text{M}}\beta_2$ and pCI2SM-$\beta_2$) were purified using CsCl gradients and transfected, together with pRSVneo (neomycin-resistant gene), into 293 cells according to our established procedures (27). G418 ($600 \mu$g/ml)-resistant colonies were pooled, and transfected but not mock-transfected cells bound to NIF, $\alpha\beta$-integrin or NIF—

**FACS Analysis**—Approximately 10$^6$ cells in HBSS containing 1 mM $\text{CaCl}_2$, and 1 mM $\text{MgCl}_2$ were added to each well. The plate was incubated at 37°C for 60 min at 37°C, and excess paraformaldehyde was neutralized with 1% bovine serum albumin at 37°C for 2 h. Bound NIF $\alpha_{\text{M}}\beta_2$ were quantitated by addition of 300 $\mu$g of avidin-alanine phosphatase conjugate (1:2000 dilution) (Zymed Laboratory, San Francisco, CA) at 37°C for 90 min, followed by washing three times with PBS, and addition of 250 $\mu$g of 3 mg/ml poly-L-lysine phosphate. After 30 min incubation at 37°C, the absorbance at 405 nm was determined.

**RESULTS**

Wild-type Recombinant $\alpha_{\text{M}}\beta_2$. Is Similar to Naturally Occurring $\alpha_{\text{M}}\beta_2$.—To examine the ligand binding functions of $\alpha_{\text{M}}\beta_2$, the receptor was expressed as a recombinant protein in 293 cells. The wild-type recombinant and naturally occurring $\alpha_{\text{M}}\beta_2$ behaved similarly by the following criteria. First, the wild-type-transfected but not mock-transfected cells bound to NIF, EC3bi, and dOva-coated surfaces. These represent known ligands for $\alpha_{\text{M}}\beta_2$ on neutrophils and $\alpha_{\text{M}}\beta_2$-bearing cell lines (15,
is the immunoprecipitation of cellsurface-labeled wild-type αMβ2 on neutrophils (31). As shown in Fig. 1, mock-transfected cells failed to bind this αMβ2 specific ligand, and the cell-associated radioactivity was measured by a gamma counter. The data are representative of two independent experiments. B, αMβ2-expressing cells were incubated without mAb (open bar), with the activating mAb KIM185 (5 μg/ml), plus phorbol 12myristate 13-acetate (0.16 μM), in the absence (hatched bar) or the presence (solid bar) of the blocking mAb 2LP19c (5 μg/ml) and added to dOva or Fg-coated plates for 25 min at 37 °C. After three washes, the adherent cells were quantitated by their cellular acid phosphatase activity. The number of cells adherent to dOva in the absence of KIM185 was assigned a value of 100%. The means ± S.D. of two independent experiments are shown.

19). Data for NIF binding are shown in Fig. 1A. Whereas mock-transfected cells failed to bind this αMβ2-specific ligand, the cell transfected with wild-type αMβ2 bound NIF in a specific and saturable manner. The binding isotherm in Fig. 1A yielded a Kd of 7 nM with a Bmax of 4.2 × 108 sites per cell. This Kd value is similar to that for NIF binding to neutrophils, 5 nM (19). Second, the activating antibody KIM185 enhanced the ligand binding functions of the αMβ2 transfectants as it does for αMβ2 on neutrophils (31). As shown in Fig. 1B, adhesion of the transfectants to Fg was markedly enhanced by KIM185 in an αMβ2-dependent manner, i.e. adhesion was blocked by mAb 2LP19c (Fig. 1B), specific for αM, as well as by mAb MHM23 (see Fig. 5B), specific for β2. Adhesion to dOva also was αMβ2-mediated although maximal interaction did not require stimulation with mAb KIM185. Third, the recombinant wild-type αMβ2 formed a heterodimeric complex. Shown in Fig. 2, lane 1, is the immunoprecipitation of cell surface-labeled wild-type αMβ2 transfectants. The estimated molecular masses for the recombinant αM and β2 subunits were 165 and 95 kDa, similar to those of the naturally occurring subunits.

The Recognition Sites for NIF, C3bi, Fg, and dOva Are Overlapping—Evidence that the recognition requirements for these three αMβ2 ligands are distinct was derived from the characterization of two other mutant αMβ2 receptors. In the first mutant, designated β2(S138A), Ser138 residue in β2 subunit was changed to Ala, and, in the second mutant, designated αM2(H6), a segment in helix 6 of the I domain of αM, (R281QELNTI) was switched to its counterpart sequence in the I domain of αL (QETLHKF). Both mutants were stably expressed in 293 cells. By FACS analysis with mAbs to the αM subunit (OKM1, LM2/2, 2LP19c, 44, M1/70) or β2 (TS1/18, MHM23) subunits, wild-type and the two mutants were expressed at similar levels on the cell surface; the mean fluorescent intensities with OKM1 for mutant β2(S138A) was the same as for wild-type and was 2-fold higher for αM2(H6). Immunoprecipitations of 125I-surface-labeled cells are shown in Fig. 2. OKM1, a mAb to the αM subunit, precipitated bands of similar mobility on SDS-PAGE from the two mutant αMβ2 transfectants and from cells expressing the wild-type receptor. TS1/18, a mAb to the β2 subunit, also immunoprecipitated both subunits. Similar results were also obtained with mAbs LM2/2 to the αM subunit and MHM23 to the β2 subunit (data not shown). Taken together, the FACS and immunoprecipitation data indicate that both mutant receptors were cell-surface-expressed at similar levels and formed heterodimeric complexes.
As shown in Fig. 3A, both mutants bound NIF similarly to wild-type 

\( \alpha_{m} \beta_{2} \). With each transfectant, a 20-fold excess of 

unlabeled NIF inhibited 125I-NIF binding by more than 99%. The interactions of all three 

\( \alpha_{m} \beta_{2} \) transfectants with NIF depended upon divalent cations, as addition of 1 mm EDTA completely abolished their NIF binding activities. NIF binding was not affected by MHHM23, a mAb to the \( \beta_{2} \) subunit which is subsequently shown to block cell adhesion to dOva and Fg (see below). At a concentration of 10 \( \mu \)g/ml, 125I-NIF binding to all three 

\( \alpha_{m} \beta_{2} \) transfectants was inhibited by less than 2%. Moreover, NIF, at a concentration as high as 175 nm, did not inhibit binding of this mAb to the wild-type and mutant cells as assessed by FACS (not shown). NIF binding isotherms, similar to those shown in Fig. 1A, were constructed with each cell line. The \( K_{d} \) values derived from these analyses are summarized in Table I. These values were in a similar range although the affinity of the \( \alpha_{m}(H6) \) mutant for NIF was slightly lower than those of the other two mutants. The preservation of NIF binding function to the wild-type and mutant receptors also was demonstrable when NIF was presented as an immobilized ligand. When NIF was deposited onto a plastic surface, and cell adhesion was measured as described above for dOva and Fg, the two mutant receptors supported adhesion as well as wild-type \( \alpha_{m} \beta_{2} \) (Fig. 3B). The adhesion of the cells to NIF was completely inhibited by 10 \( \mu \)g/ml soluble NIF and was not affected by mAb MHM23. Two conclusions can be drawn from these results. 1) The mutations, \( \beta_{2}(S138A) \) and \( \alpha_{m}(H6) \), did not affect the structural integrity of \( \alpha_{m} \beta_{2} \) as both mutants maintained a conformation compatible with high affinity NIF binding, whether NIF was presented as a soluble ligand or as an adhesive substrate. 2) The NIF binding site does not involve the mutated areas of \( \alpha_{m} \) or \( \beta_{2} \).

EC3bi binding to the two mutant receptors is shown in Fig. 4. Mutation of the H6 segment did not alter recognition of this ligand. Several controls were performed to verify the specificity of the EC3bi interaction with this mutant as well as wild-type \( \alpha_{m} \beta_{2} \). No detectable binding to wild-type or the \( \alpha_{m}(H6) \) was observed with uncoated erythrocytes or IgM-coated erythrocytes; mock-transfected 293 cells did not rosette with EC3bi. As anticipated, EC3bi binding to both \( \alpha_{m}(H6) \) and wild-type cell lines required divalent cations, as no binding was observed in the presence of 1 mm EDTA (Fig. 4). In contrast, the \( \beta_{2}(S138A) \) transfectant lost more than 80% of its EC3bi binding activity. Thus, this mutant bound NIF, but not C3bi, indicating that the binding requirements for these two ligands are distinct.

The adhesion of wild-type and the two mutant receptors to dOva is shown in Fig. 5A. Wild-type \( \alpha_{m} \beta_{2} \) readily supported adhesion to dOva-coated surfaces in a divalent cation-dependent interaction, as inclusion of 1 mm EDTA abolished adhesion by 99%. Addition of 10 \( \mu \)g/ml NIF or 5 \( \mu \)g/ml MHHM23 blocked the cell adhesion by more than 95%. Mutation of the H6 segment of \( \alpha_{m} \) or Ser138 of \( \beta_{2} \) however, completely abrogated the adhesive activity of the receptors. A similar pattern was observed when the adhesive substrate was Fg. As shown in Fig. 5B, wild-type \( \alpha_{m} \beta_{2} \) adhered to Fg upon activation with KIM185, and this adhesion was blocked by mAb MHHM23 or NIF. However, the \( \alpha_{m} \) H6 and \( \beta_{2} \) Ser138 transfectants failed to adhere to Fg. In separate experiments, we verified that the activating mAb still bound to these mutant \( \alpha_{m} \beta_{2} \)-bearing cells by FACS. Thus, regions in both the \( \alpha_{m} \) and \( \beta_{2} \) subunits are critical for cell adhesion to dOva and Fg, and the \( \alpha_{m}(H6) \) mutant, which was capable of binding NIF and C3bi, could not adhere to either substrate.

**DISCUSSION**

One of the defining characteristics of integrins is the capacity of its family members to bind multiple ligands (1). \( \alpha_{m} \beta_{2} \) has one of the broadest ligand repertoires, recognizing no fewer than 12 ligands. To date, analyses of the ligand binding functions have emphasized that many \( \alpha_{m} \beta_{2} \) ligands share common binding requirements. However, as shown in this study, certain mutations, both in the I domain and in \( \beta_{2} \), can selectively inactivate the recognition of individual ligands. Thus, we propose that the binding sites for the ligand analyzed (NIF, EC3bi, and the adhesive substrates, Fg and dOva) are not identical.

Two regions of \( \alpha_{m} \beta_{2} \) have been implicated in providing the overlapping ligand contact sites, the I domain of \( \alpha_{m} \) and the \( \beta_{2} \)134–138 region. The role of the I domain has been demon-

**TABLE I**

| Protein          | \( K_{d} \) | S.D. |
|------------------|-----------|-----|
| wt               | 7.0       | 1.6 |
| \( \alpha_{m}(H6) \) | 22        | 4.0 |
| \( \beta_{2}(S138A) \) | 3.5   | 2.4 |

The \( K_{d} \) value was determined using 10^6 \( \alpha_{m} \beta_{2} \)-expressing cells in the presence of different concentrations of 125I-NIF and calculated from the binding isotherm using the method described.
strated by the capacity of NIF to inhibit binding of EC3bi and dOva to the receptor and the abolition of NIF, EC3bi, Fg, and dOva binding by deletion of D48PLGY. This conclusion is supported by previous data showing that several ligands bind to the expressed I domain (20–22, 25, 33–35), that several function blocking mAbs map to the I domain (28), and that NIF blocks intracellular adhesion molecule-1 (22) and EC3bi binding (20).

The crystal structure of the I domain shows that cation coordinating residues are provided from within noncontiguous loops, which connect α helices and β strands (23). As cations are intimately involved in the binding of most ligands to integrins (36), sequences within these loops also may provide several of the overlapping as well as ligand-selective contact sites.

While the β2134–138 is not required for NIF binding, the single point mutation of S138A abolished EC3bi, Fg, and dOva binding. Bajt et al. (26) had previously shown that Asp134 and Ser136 of β2 are required for C3bi binding to αMβ2, and these mutations in our study also block αMβ2-mediated cell adhesion to dOva (data not shown). In contrast to their experience with the S138A mutation in COS cells, we were able to express this mutant (as well as S134A and S136A) on the cell surface of 293 cells. Therefore, this β2 region, which also has been implicated in ligand and cation binding to β2 integrins (37), is involved in binding multiple ligands to αMβ2.

The nonidentical nature of the binding sites for NIF, EC3bi, and adhesive ligands (Fg and dOva) is demonstrated by two of the recombinant αMβ2 receptors analyzed. β2(S138A) bound NIF with a similar affinity as wild-type receptor but failed to bind EC3bi, Fg, or dOva. Hence, the NIF binding site cannot be identical to those of the other three ligands. NIF binds to the expressed I domain of αM with high affinity, and it may be that the entire binding pocket for NIF resides in the αM1 domain. The αM(H6) mutant bound EC3bi and NIF but not Fg or dOva. Thus, the binding sites for Fg and dOva cannot be identical to those of the other two ligands. In the crystal structure, helix 6 is placed at some distance from the cation binding site in the αM1 domain. Nevertheless, αMβ2-mediated adhesion to Fg or dOva is still divalent cation-dependent. Either the binding pocket for Fg and dOva extends over a broad region of the I domain to include both helix 6 and the cation binding site or one of the other cation binding sites (26) is required for interaction with these ligands.

To explain these data, we hypothesize that the binding sites for the ligands analyzed are overlapping but not identical. An alternative explanation of our data is that NIF, C3bi, and adhesive ligands share the same binding site but interact with different affinities. This possibility seems unlikely, since 1) the mutant β2(S138A) bound NIF with the same, if not higher affinity than the wild-type αMβ2 (Table I), suggesting that the mutations did not grossly alter the affinity of αMβ2 toward its ligands. Therefore, the failure of β2(S138A) to interact with C3bi, dOva, and Fg cannot simply be caused by a decrease in binding affinity. Moreover, the β2(S138A) mutant still bound NIF when it was presented in the same format, as an immobilized substrate, as D48PLGY. Activity each assay was performed in duplicate, and the data are representative of 2–3 independent experiments.

In summary, at least three functionally distinct sites in αMβ2 mediate adhesion and its interactions with C3bi and NIF. One site in β2, which includes Asp134, Ser136, and Ser138, is required for C3bi binding and adhesion to protein-coated surfaces. The second site, located in helix 6 of αM1 domain, is important for adhesion to Fg and dOva but not for C3bi and NIF binding. A third class of contact site, also in I domain, is shared by all three ligands, thereby accounting for the ability of NIF to block αMβ2 binding of the other test ligands. Thus, αMβ2 can be viewed as a mosaic in which certain regions of the receptor provide common recognition sites for NIF, EC3bi, Fg, and
dOva, whereas other regions mediate selective recognition of these ligands. As these molecules represent three general categories: soluble (NIF), cell-surface (EC3bi), and matrix-deposited (Fg and dOva)- of \( \alpha_M \beta_2 \) ligands, this model may apply to other \( \alpha_M \beta_2 \) ligands.

Acknowledgments—We thank Dr. F. J. Castellino, University of Notre Dame, South Bend, IN for providing the expression vector, pCIS2M; Dr. M. Moyle of Corvas International Inc., San Diego, CA for NIF; Dr. B. Karan-Tamir of Amgen, Thousand Oaks, CA for the cDNA for \( \alpha_M \) and \( \beta_2 \); and Dr. M. Robinson of Celltech, Berkshire, U.K. for mAb KIM185. We are very grateful to Xiaohua Wu for her excellent technical support.

REFERENCES

1. Springer, T. A. (1990) Nature 346, 425–434
2. Arnaout, M. A. (1990) Blood 75, 1037–1050
3. Smith, C. W., Marlin, S. D., Rothlein, R., Toman, C., and Anderson, D. C. (1989) J. Clin. Invest. 83, 2008–2017
4. Anderson, D. C., Miller, L. J., Schmalstieg, F. C., Rothlein, R., and Springer, T. A. (1986) J. Immunol. 137, 15–27
5. Shappell, S. B., Toman, C., Anderson, D. C., Taylor, A. A., Entman, M. L., and Smith, C. W. (1990) J. Immunol. 144, 2702–2711
6. von Asmuth, E. J., van der Linden, C. J., Leeuwenberg, J. F., and Buurman, W. A. (1991) J. Immunol. 147, 3869–3875
7. Schmalstieg, F. C. (1988) Pediatr. Infect. Dis. J. 867, 872
8. Ross, G. D. and Lambris, J. D. (1982) J. Exp. Med. 155, 96–110
9. Davis, M. S., Staunton, D. E., Marlin, S. D., and Springer, T. A. (1993) J. Immunol. 152, 4582–4589
10. Altieri, D. C., Agbanyo, F. R., Plescia, J., Ginsberg, M. H., Edgington, T. S., and Plow, E. F. (1990) J. Biol. Chem. 265, 12119–12122
11. Altieri, D. C., and Edgington, T. S. (1988) J. Biol. Chem. 263, 7007–7015
12. Wright, S. D., and Jorg, M. T. (1986) J. Exp. Med. 164, 1876–1888
13. Di Renzo, L., Yefenof, E., and Klein, E. (1991) Eur. J. Immunol. 21, 1755–1758
14. Russell, D. G., and Wright, S. D. (1989) J. Exp. Med. 169, 279–292
15. Davis, G. E. (1992) Exp. Cell Res. 200, 242–252
16. Orteipp, S., Stephens, P. E., Hogg, N., Figdor, C. G., and Robinson, M. K. (1995) Eur. J. Immunol. 25, 637–643
17. Bohnsack, J. F., and Zhou, X.-N. (1992) J. Immunol. 149, 1340–1347
18. Nathan, C., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gallit, J., and Wright, S. D. (1989) J. Cell Biol. 109, 1341–1349
19. Moyle, M., Foster, D. L., McGrath, D. E., Brown, S. M., Laroche, Y., De Meuter, J., Stanssens, P., Bogewitz, C. A., Fried, V. A., Ely, J. A., Soule, H. R., and Vlasuk, G. P. (1994) J. Biol. Chem. 269, 10008–10015
20. Ueda, T., Rieu, P., Brayer, J., and Arnaout, M. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10680–10684
21. Muchowski, P. J., Zhang, L., Chang, E. R., Soule, H. R., Plow, E. F., and Moyle, M. (1994) J. Biol. Chem. 269, 26419–26423
22. Rieu, P., Ueda, T., Haruta, I., Sharma, C. P., and Arnaout, M. A. (1994) J. Cell Biol. 127, 2081–2091
23. Lee, J.-G., Rieu, P., Arnaout, M. A., and Liddington, R. (1995) J. Immunol. 155, 631–638
24. Midchishita, M., Viden, V., and Arnaout, M. A. (1993) J. Biol. Chem. 269, 857–867
25. Zhou, L., Lee, D. H., Plescia, J., Lau, C. Y., and Altieri, D. C. (1994) J. Biol. Chem. 269, 17075–17079
26. Bajt, M. L., Goodman, T., and McGuire, S. L. (1995) J. Biol. Chem. 270, 94–98
27. Zhang, L., and Castellino, F. J. (1990) Biochemistry 29, 10828–10834
28. Diamond, M. S., Garda-Aguilar, J., Bickford, J. K., Corbi, A. L., and Springer, T. A. (1993) J. Cell Biol. 120, 1031–1043
29. Phillips, D. R., and Agn, P. P. (1977) J. Biol. Chem. 252, 2121–2126
30. Bilsland, C. A., Diamond, M. S., and Springer, T. A. (1994) J. Immunol. 152, 4582–4589
31. Andrew, D., Shoch, A., Ball, E., Orteipp, S., Bell, J., and Robinson, M. (1993) Eur. J. Immunol. 23, 2217–2222
32. McGuire, S. L., and Bajt, M. L. (1995) J. Biol. Chem. 270, 25866–25871
33. Randi, A. M., and Hogg, N. (1994) J. Biol. Chem. 269, 12395–12398
34. Kern, A., Briesewitz, R., Bank, I., and Marcantonio, E. E. (1994) J. Biol. Chem. 269, 22811–22816
35. Kamata, T., and Takada, Y. (1994) J. Biol. Chem. 269, 26006–26010
36. Hais, T. A., and Plow, E. F. (1994) Curr. Opin. Cell Biol. 6, 656–662
37. D’Souza, S. E., Hais, T. A., Piotrowicz, R. S., Byers-Ward, V., McGrath, D. E., Soule, H. R., Cieriewski, C. S., Plow, E. F., and Smith, J. W. (1994) J. Cell Biol 79, 659–667
38. McNally, A. K., and Anderson, J. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10119–10123