Identification and Reconstruction of the Binding Site within αMβ2 for a Specific and High Affinity Ligand, NIF*

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Engagement of the αMβ2 (CD11b/CD18, Mac-1) integrin on neutrophils supports adhesion and induces various cellular responses. These responses can be blocked by a specific ligand of αMβ2, neutrophil inhibitory factor (NIF). The molecular basis of αMβ2-NIF interactions was studied. The single chain αM subunit, expressed on the surface of human 293 cells, bound NIF with an affinity equivalent to that of αMβ2 heterodimer. This observation, coupled with previous data showing that the αM domain alone supported high affinity NIF binding, indicated that the binding site for NIF is restricted to the I domain. Guided by the crystal structure of the αM domain, 16 segments corresponding to the entire outer hydrated surface of αM domain were switched to their counterparts sequences in αM, which does not bind NIF. Surface expression and heterodimer formation were achieved for all mutants, and correct folding was confirmed. Of the 16 switches, only 5 affected NIF binding substantially, reducing affinity by 8- to 200-fold. These data confined the NIF-binding site to a narrow region composed of Pro147, Arg152, Pro201, Lys217, and Asp288 of αM. Verifying this localization, when these segments were introduced into the αM-I domain, the resulting chimeric receptor was converted into a high affinity NIF-binding protein.

αMβ2 (Mac-1, CR3, CD11b/CD18) is a member of the β2 integrin subfamily, which includes αMβ2 (LFA-1, CD11a/CD18), αMβ2 (p150, 95, CD11c/CD18), and αMβ2. These integrins share a common β subunit of 95 kDa which is noncovalently linked to distinct but homologous α subunits (1, 2). The physiological functions of αMβ2 include roles in adhesion and transmigration of leukocytes through endothelium (3), phagocytosis of foreign materials (4), and activation of neutrophils and monocytes (5). Excessive activation of αMβ2 contributes to sustained inflammation, reperfusion injury, and tissue damage (6). The importance of the β2 integrin subfamily is underscored by the severe phenotype of individuals with congenital deficiencies of these integrins (7).

NIF (neutrophil inhibitory factor), a novel glycoprotein isolated from canine hookworms, was originally identified as an inhibitor of a number of neutrophil functions, such as adhesion to endothelial cells and adhesion-dependent release of hydrogen peroxide (8). These functional effects resulted from its specific binding to αMβ2 but not to other β2 integrins. Subsequently, we and others have reported that NIF completely blocked αMβ2-mediated binding of C3bi (9), ICAM-1 (10), and adhesion to protein-coated surfaces (11), and partially blocked fibrinogen binding (12) to αMβ2-bearing cells. As an antagonist, NIF has been shown effective in attenuating the deleterious effects of excessive neutrophil activation, such as tissue damage and ischemia-reperfusion injury in animal models (13, 14).

The I domain, an inserted region of ~200 amino acids in the αM subunit (αM-I-domain), contributes to NIF binding to αMβ2 (10, 12). I domains with highly conserved amino acid sequences are also found in several other integrin α-subunits as well as in other proteins, such as vWF, and mediate a variety of protein-protein interactions, including ligand binding to integrins (15). Using blocking mAbs which map to the I domain or recombinant αM-I domain itself, this region has been implicated in the binding of ICAM-1, fibrinogen (16), and C3bi (9), as well as NIF, to αMβ2. Collectively, these data suggest that the αM-I domain is an independent structural unit, capable of interacting with many different proteins. Nevertheless, the ligand binding functions of αMβ2 are not solely a property of its I domain. We (11) and Bajt et al. (17) have shown that mutations of Asp134, Ser136, or Ser138 in β2 subunit abrogated the binding of αMβ2 to C3bi and adhesion to protein-coated surfaces, suggesting that, in addition to I domain, the β subunit also is involved in the recognition of certain ligands by αMβ2.

In this study, we have utilized NIF as a model αMβ2 ligand and have sought to delineate the molecular basis for its interaction with αMβ2. Homolog-scanning mutagenesis (18) in which sequences within the αM-I domain have been switched to the homologous sequences within the αM-I domain has been used to map the NIF-binding site in αMβ2. The NIF-binding site identified through the loss-of-function mutations was confirmed by a gain-in-function experiment, whereby the I domain of αM was converted into a NIF-binding protein. Given the ability of NIF to inhibit the binding of many ligands to αMβ2 and the structural similarities among I domains, the molecular details of NIF-αMβ2 interactions may also apply to other ligands of αMβ2 and to other I domain containing integrins.

EXPERIMENTAL PROCEDURES

Materials—Human kidney 293 cells and the expression vector, pCI32M, were generous gifts from Dr. F. J. Castellino (Notre Dame, IN). The cDNA of CD11b and CD18 were obtained from Dr. B. Karan-Timir (Amgen, Thousand Oaks, CA). NIF was a gift from Corvas Int., San Diego, CA. TSI1/18, OKM1, M1/70, IB4, 44a, 904, and LM2/1 were from ATCC (ATCC, Rockville, MD). G418, Dubecco’s modified Eagle’s
medium/F-12, HBSS (Hank's balanced solution), DH5α cell, and restriction enzymes were from Life Technologies, Inc. (Grand Island, NY). All other reagents were the highest grade available from Sigma unless otherwise noted.

**Segment Switches by Site-directed Mutagenesis—**Stretches of 7–12 amino acids within the αM domain were switched to the corresponding sequences in αL. All such segment switches were created by oligonucleotide-directed mutagenesis using uracil-containing single stranded M13mp18 DNA (19). To facilitate the mutagenesis, two unique restriction sites, Clal at position 535 (Ile127- and Nhel at position 1141 (Ala306-), flanking the I domain of αM, have been introduced into pCIS2M (11). To switch a segment of 7–11 amino acids from the mutant to αL, the mutagenic primer was designed to contain the corresponding mutations and 15 additional unchanged bases at each end. The length of the primer was typically 54 bases. The site-directed mutagenesis was performed according to our published procedure using T7 DNA polymerase (19). The mutant was identified by DNA sequencing of 5 randomly picked plaques. DNA sequencing of the entire I domain was conducted, confirming the presence of the desired mutations and the correctness of the rest of the I domain. The mutated I domain was transferred back to the expression vector pcIS2M-αM using the unique Clal and Nhel restriction sites. The cDNA of αM and β2 were inserted separately in the pcIS2M expression vector employing uracil-containing single stranded DNA and used as templates for site-directed mutagenesis using uracil-containing single stranded DNA.

To generate the chimera molecule, αMαL(Val340), where the αM I domain was replaced with αL domain, the fragment of the αM-I domain (Ile127- to Val146) containing Clal and Nhel restriction sites was prepared by reverse transcription and polymerase chain reaction using total RNA from polymorphonuclear cells and the following two primers: 5′-CC-CCCAAGACAAGAAGAAGCAGATG-3′ (forward) and 5′-TGTAGAAGCT- ACGCTGAAACCGCTC-3′ (reverse). The reverse primer contains a Nhel site (GCTAGC), and a naturally occurring Clal site exists in the αM cDNA at nucleotide position 530.

To graft the NIF-binding site in the αM domain, five segments of αM domain (I127-PHEHRR, K25-PITQLQG297, R297-HTHTATGI, D348PLGY, and E352-DVLP, and E352-DVLP) were substituted sequentially for their counterparts in αL, which was performed by oligonucleotide-directed mutagenesis using uracil-containing single stranded DNA. The site-directed mutagenesis was performed using T7 DNA polymerase (19). The mutant was identified by DNA sequencing of 5 randomly picked plaques. DNA sequencing of the entire I domain was conducted, confirming the presence of the desired mutations and the correctness of the rest of the I domain. The mutated I domain was transferred back to the expression vector pcIS2M-αM using the unique Clal and Nhel restriction sites. The cDNA of αM and β2 were inserted separately in the pcIS2M expression vector employing uracil-containing single stranded DNA and used as templates for site-directed mutagenesis using uracil-containing single stranded DNA.

**Expression of αMβ2 in 293 Cells—**The expression vectors containing wild-type and mutated αM (pcIS2M-αM) and β2 (pcIS2M-β2) were purified by CsCl gradients and transfected, together with the pcDNA3 (neomycin-resistant gene), into 293 cells according to our established procedures (19). G418 (600 μg/ml)-resistant colonies were pooled and αMβ2-expressing cells were sorted by FACS (FACStar, Becton-Dickinson, San Jose, CA), using αM-specific mAb, OKM1, which recognizes an epitope outside of the I domain (20).

**FACS Analysis—**Approximately 10⁶ cells in HBSS containing 1 mM Mg²⁺ were incubated with 5 μg of mAb for 30 min at 4 °C. A subtype-matched mouse IgG served as a control. After 3 washes with PBS (10 mM NaPO₄, 150 mM NaCl, pH 7.4), cells were incubated with fluorescein isothiocyanate goat anti-mouse IgG (H+L) (Fab' fragment, 1:20 dilution) (Zymed Laboratory, San Francisco, CA), kept at 4 °C for another 30 min, washed with PBS, and then resuspended in 600 μl of PBS. The FACS analysis was performed using FACSscan, counting 10,000 events. For dual color FACS analysis, the cells (10⁶) were stained with OKM1 (5 μg) and biotinylated NIF (0.5 μg) for 30 min at 4 °C, followed by three washes with PBS. These cells were then mixed with fluorescein isothiocyanate-avidin conjugate and phycocyanin goat anti-mouse IgG (H+L) (Fab' fragment, 1:20 dilution) (Zymed Laboratory), kept at 4 °C for another 30 min, washed with PBS, and then resuspended in 600 μl of PBS. FACS analysis was performed 30 min after the second staining. The same procedure was used for mAb 24 staining, except that 1 μM Mn²⁺ was substituted for the 1 mM Mg²⁺ and incubations were at 37 °C.

**Analytical Procedures—**The procedures used for NIF binding, and surface labeling and immunoprecipitation of cells have been described (11). Briefly, different concentration of NIF (0–20 μg) were incubated with 2 × 10⁶ αMβ2-expressing cells at 4 °C for 30 min. The bound NIF was separated from the free NIF by centrifugation through a 20% sucrose solution and counted with a γ-counter. The NIF titration data were fitted to a single site binding model using the equation: \[
K_a = \frac{[NIF]_b}{[NIF]_f} = \frac{B_{max}[NIF]/(K_d + [NIF])}{B_{max}}
\]
where \(B_{max}\) is the maximal NIF binding and \(K_d\) is the dissociation constant, using a program in Sigmaplot (Jandel Co., San Rafael, CA).

**RESULTS**

**The αM Subunit Is Capable of High Affinity NIF Binding—** When introduced into human kidney 293 cells in the absence of transfected β2, the αM subunit was expressed on the cell surface, albeit at low levels relative to the heterodimer. Immuno-precipitation of surface-labeled cells with OKM1 yielded a band of 165 kDa on polyacrylamide gels in SDS under both reducing and nonreducing conditions (Fig. 1A). No bands in the vicinity of 95 (β2) or 120 (β2) kDa were observed, suggesting that the surface-expressed αM is not complexed with its natural partner, β2, or with other typical integrin β subunits. No bands were observed for mock transfection with either OKM1 or TS1/18 (Fig. 1B), verifying the specificity of the immunoprecipitation. The presence of αM was confirmed by FACS analysis. The transfected cells were positive with OKM1, LM2/1, 2LP19c, 44a, and 904, mAbs to the α subunit, whereas mAbs to the β2 subunit, TS1/18, IB4, or MMH23 were unreactive. Bilsland et al. (21) also detected surface expression of the αM subunit in the absence of β2 in COS cells. The ability of the αM subunit to interact with soluble ¹²⁵I-NIF was assessed. As shown in Fig. 1C, the αM-expressing cells bound NIF in a dose-dependent and saturable fashion. The specificity of this binding was verified by addition of a 20-fold excess of unlabeled NIF or 1 mM EDTA: in both cases, ¹²⁵I-NIF binding was reduced by more than 99%. Scatchard plots of the NIF binding isotherms were consistent with a single class of binding sites with respect to affinity and yielded a dissociation constant (K_d) of 2.1 nM. This value is very similar to the K_d of 7 nM for NIF binding to heterodimeric αMβ2. To determine the nature of the molecule on the cell surface that binds NIF, lysate of surface-labeled αM-transfected cells were incubated with biotinylated NIF. The NIF-receptor complex was captured with avidin-agarose resin and analyzed on 7% SDS-PAGE. As shown in Fig. 1B, only one band of 165 kDa was present. In contrast, two bands (165 and 95 kDa) were observed for the αMβ2-expressing cells. The specificity of this assay was demonstrated by the absence of any band for the mock transfectant. These data indicate that, unlike other αMβ2 ligands (C3b and adhesion (11)), all major contributing elements of the binding site for NIF reside in the α subunit of αMβ2. Consistent with this conclusion, we also found that: 1) mutations in the β2 subunit, which abrogated binding of other ligands, did not affect NIF binding of αMβ2 complex (11); and 2) replacement of the αM with the αL I domain in the context of the αMβ2 heterodimer completely abrogated NIF binding (see below, Fig. 5).

The I Domain Peptide N502-³⁴⁹FALKILVVTIDGK Is Not Required for NIF Binding—Rieu et al. (10) had previously used synthetic peptides and implicated two candidate αM sequences in NIF binding: the A7 peptide, N399-³⁴⁹FALKILVVTIDGK, was the most potent inhibitor of NIF binding to the αM I domain (10). To test the role of this sequence in NIF binding, we created two switch mutants in which the amino- and carboxyl-terminal portions of this peptide were changed individually to the corresponding sequences in αL (αL I domain does not bind NIF (8)): (K517ALFALKILVVTIDGK)² to αL (PDATKVLHIITDGEATD). Thus, the first mutant, αM(K319NAF), changed the amino-terminal non-conserved KNAF to PDAT, and the second mutant, αM(K245FG), changed to non-conserved KFG to ATD. The hydrophobic cluster in the center of the peptide sequence is well conserved and was not altered. The mutant αM vectors were transfected together with β2 into 293 cells. Both mutants
Immunoprecipitation was performed as follows. A, \( \alpha_M \)-expressing cells were immunoprecipitated with 10 \( \mu \)g of OKM1, a mAb to \( \alpha_M \) (lanes 1 and 2), or TS1/18, a mAb to \( \beta_2 \) (lane 3), overnight at 4 °C. B, \( \alpha_M \)-expressing cells (lanes 1 and 2) or mock transfectant (lanes 3–5) were precipitated with 0.5 \( \mu \)g of biotinylated NIF (lanes 1 and 3); 10 \( \mu \)g of IV.3, an irrelevant mAb (lane 2); 10 \( \mu \)g of OKM1 (lane 4), and 10 \( \mu \)g of TS1/18 (lane 5). The wild-type heterodimer, \( \alpha_M \beta_2 \), precipitated with 0.5 \( \mu \)g of biotinylated NIF (lane 6) was included as a control. After washing, the immunoprecipitates were subjected to 7% SDS-PAGE, and the cell associated radioactivity was measured by \( \gamma \)-counter. The data are representative of two independent experiments.

were expressed well on the cell surface as judged by FACS analysis, and surface labeling and immunoprecipitation with OKM1 (data not shown). When stained with LM2/1 mAb, the mean fluorescence for the \( \alpha_M(K^{231}NAF)\beta_2 \) mutant was 392.0, compared with 398.4 for wild-type. A similar result was obtained for the \( \alpha_M(K^{245}FG)\beta_2 \) mutant. When stained with OKM1, the mean fluorescence was 377.2 for the mutant and 380.1 for the wild-type. Both mutants also bound NIF with high affinity (Fig. 2, and Table II), indicating that this peptide is not centrally involved in NIF binding when placed in the context of the intact receptor.

**Homolog-scanning Mutagenesis**—To systematically define the NIF-binding site in \( \alpha_M \), a homolog-scanning mutagenesis (18) strategy was implemented. Accordingly, guided by the crystal structure (15), the entire hydrated surface of the \( \alpha_M \) domain was replaced with sequences of the \( \alpha_I \) domain in segments of 7–11 amino acids. To apply this approach to the \( \alpha_M \) domain (100–200 amino acids), 16 segments were switched. The primers used are listed in Table I. The efficiency of mutagenesis was typically 60%, with the 7-amino acid segment switches having substantially higher efficiency (>90%) than the longer 10-amino acid segment switches (~30%). The appropriate DNA sequence of the entire \( \alpha_I \) domain (from Ile139 to Ala332) was confirmed for each mutant before transferring back into the \( \alpha_M \) subunit cDNA.

The functional consequences of these segment switches were initially investigated by transient expression in 293 cells. Forty-eight hours after transfection of \( \alpha_M \), together with \( \beta_2 \) subunit, the cells were detached from tissue culture dish and double stained with OKM1 and biotinylated NIF. A representative dual-color FACS analysis is shown in Fig. 3. With wild-type \( \alpha_M \beta_2 \) transfectant, 1.83% of the cells were positive with both OKM1 and NIF, whereas less than 0.02% of mock transfected cells were positive. Two distinct patterns were observed for the mutants. One, represented by \( \alpha_M(E^{178}_{\alpha M},T^{185}) \) in Fig. 3, in which a similar percentage (1.12%) of the cells were positive for both markers, i.e. exhibiting a pattern similar to the wild-type \( \alpha_M \beta_2 \) cells. The second pattern is represented by \( \alpha_M(\Delta D^{245}_{\alpha M}, Y^{252}) \), in which a substantial percentage of cells were positive with OKM1 (22.5%) but negative with NIF (<0.05%), indicating a
Fig. 3. Dual-color FACS analysis of cell transiently expressing wild-type or mutant αβ2 receptors. αβ2-expressing cells (10^6) were stained with OKM1 (5 μg) and biotinylated NIF (0.5 μg) in the presence of 1 mM Mg^2+ for 30 min, washed with PBS, and then mixed with fluorescein isothiocyanate-avidin and phycoerythrin goat anti-mouse IgG conjugate. After an additional 30 min, the cells were washed with PBS and analyzed using a FACSscan, counting 10,000 events. The percentage of cells falling within each quadrant is indicated. A, mock; B, αβ2-; C, ααΔΔ246-252β2; D, ααE178-T185β2.

The NIF-binding Site Is Composed of Segments P147–R152, P201–G207, and P326–335 To quantitate the binding affinities of each mutant receptor for NIF, stable cell lines were established. All 16 αβ2 mutants were cell surface expressed as heterodimers: immunoprecipitations of 125I-surface-labeled cells with OKM1 yielded a Kd of 5 nM reported for naturally-occurring αβ2 on neutrophils (8). The expression levels, reflected by the maximal NIF binding, were similar for all mutants, differing by less than 4-fold. The following mutants had loss in NIF-binding function. The following mutants belonged to the second category were: ααΔΔ246-252β2 (11). Identical patterns were obtained when the immuno-precipitations were performed with TS1/18, a mAb to the β2 subunit (data not shown). The specificity of this assay was reflected by the maximal NIF binding, were similar for all mutants, differing by less than 4-fold. The following mutants had similar Kd values calculated from these binding data summarized in Table II. The Kd of 7 nM for wild-type αβ2 was nearly identical to the Kd of 5 nM reported for naturally-occurring αβ2 on neutrophils (8). The expression levels, reflected by the maximal NIF binding, were similar for all mutants, differing by less than 4-fold. The following mutants had similar Kd values (<4-fold different than wild-type αβ2): ααM(153–T159), ααM(E162–L170), ααM(E278, T185), ααM(Q190–S197), ααM(K231NAF), ααM(K245FG), ααM(E253–R261), ααM(D273–K279), ααM(R281–I287), ααM(P201–G207), ααM(F297–T307), and ααM(E309–E314). The most dra-

![Image](image-url)

**Table I**

| Mutagenic primers used in the homolog-scanning mutagenesis of the αI domain |

| Mutagenic primers (from 5’ to 3’) |
|-----------------------------------|
| P147–R152 | 147–152 | PHDFRR | IEDFEL |
| M153–T159 | 153–159 | MKEFVST | ILDFMKD |
| E162–L170 | 162–170 | EQLLKSRTL | KKLNSNTSYQ |
| T207 | 175–185 | EEFHKHT | TSYKTEPD |
| G306–S317 | 190–197 | QKNIPFSR | VKWDIPPD |
| P201–G207 | 201–207 | PITQLLG | HKHVMLL |
| R208–K217 | 208–217 | RTHTATGIR | LTNTFGAINY |
| K231NAF | 231–234 | KNAF | PDAT |
| K245FG | 245–247 | KFG | ATD |
| M153–T159 | 147–152 | PHDFRR | IEDFEL |
| M(E278, T185), ααΔΔ246-252β2 |
| ααM(Q190–S197), ααM(K231NAF), ααM(K245FG), ααM(E253–R261), ααM(D273–K279), ααM(R281–I287), ααM(P201–G207), ααM(F297–T307), and ααM(E309–E314). |
FIG. 4. A, immunoprecipitation of $^{125}$I-surface-labeled stable cell lines expressing wild-type and mutant $\alpha_5\beta_2$, $\alpha_5\beta_2$-expressing cells (1 $\times$ 10$^6$) were surface labeled with $^{125}$I and lactoperoxidase, and immunoprecipitated with 10 $\mu$g of OKM1. After washing, the immunoprecipitates were subjected to SDS-PAGE (7% gels under nonreducing conditions) and exposed to Kodak XAR-5 film for 48 h. Lane 1, $\alpha_5\beta_2$; lane 2, $\alpha_5\beta_2$-expressing cells labeled with $^{125}$I and lactoperoxidase, and exposed to Kodak XAR-5 film for 48 h. Lane 1, $\alpha_5\beta_2$; lane 2, $\alpha_5\beta_2$-expressing cells labeled with $^{125}$I and lactoperoxidase, and exposed to Kodak XAR-5 film for 48 h. Lane 3, $\alpha_5\beta_2$-expressing cells labeled with $^{125}$I and lactoperoxidase, and exposed to Kodak XAR-5 film for 48 h. Lane 4, $\alpha_5\beta_2$-expressing cells labeled with $^{125}$I and lactoperoxidase, and exposed to Kodak XAR-5 film for 48 h. Lane 5, $\alpha_5\beta_2$-expressing cells labeled with $^{125}$I and lactoperoxidase, and exposed to Kodak XAR-5 film for 48 h. Lane 6, $\alpha_5\beta_2$-expressing cells labeled with $^{125}$I and lactoperoxidase, and exposed to Kodak XAR-5 film for 48 h. The selected patterns are representative of all 16 cell lines expressing the various $\alpha_5\beta_2$ mutants. B, binding of NIF to wild-type and mutant $\alpha_5\beta_2$ transfectants. Titrational analysis of NIF binding to wild-type and mutant $\alpha_5\beta_2$-expressing cells was conducted as described in Fig. 1C. The wild-type (●) and two representative mutants, $\alpha_5\beta_2$ (153-T159 MKEFVST) and $\alpha_5\beta_2$ (R208-K217 RTHTATGIRK), are shown. The data are representative of at least two independent experiments. C, reactivity of $\alpha_5\beta_2$ mutants with mAb 24. $\alpha_5\beta_2$-expressing cells were incubated with mAb 24 or OKM1 in the presence of 1 mM Mn$^{2+}$ for 30 min at 37 °C. Cells were washed, then mixed with fluorescein isothiocyanate goat anti-mouse IgG(F(ab')2 fragment for another 30 min, washed, and analyzed by FACScan, counting 10,000 events. The ratios of the mean fluorescence intensities of each mutant cell line with mAb 24 and OKM1 were quantitated. The ratio for wild-type $\alpha_5\beta_2$ receptor was assigned a value of 100%, and the OKM1 mAb fluorescence was used to normalize the expression levels of the $\alpha_5\beta_2$ receptors on the different cell lines. Addition of 1 mM EDTA abolished mAb 24 reactivity toward all five mutants. The data are the means + S.D. of two independent experiments.

### Table II

| Mutant | Segment switched | $K_d$ nm | S.D. |
|--------|------------------|----------|------|
| 1      | WT               | 7.0      | 1.6  |
| 2      | P147, R152      | 232.4    | 63.3 |
| 3      | M153, T159      | 11.5     | 0.5  |
| 4      | E162, L170      | 5.0      | 2.7  |
| 5      | E178, T186      | 28.9     | 11.0 |
| 6      | Q176, T185      | 14.3     | 8.0  |
| 7      | P191, Q195      | 2153.4   | 723  |
| 8      | R201, Q205      | 1451.1   | 578  |
| 9      | K279, NAF       | 11.8     | 0.9  |
| 10     | K245FG          | 5.4      | 1.3  |
| 11     | ΔD246, Y252     | 88.5     | 2.4  |
| 12     | E251, R255      | 55.9     | 8.5  |
| 13     | ΔE256, G258     | 21.0     | 2.7  |
| 14     | ΔD273, K279     | 15.3     | 5.4  |
| 15     | R281, G285      | 22.5     | 5.1  |
| 16     | E297, D297      | 12.2     | 4.6  |
| 17     | Q305, P311      | 14.3     | 4.9  |
| 18     | α5               | 2.1      | 0.5  |

The Defective Mutants Possess Correct Conformations—

Given the similarities in the three-dimensional structures of $\alpha_5\alpha_1$ and $\alpha_1\alpha_1$ domains (15, 22), it is unlikely that the defects in NIF binding of the five identified mutants arise from incorrect folding of their $\alpha_5\alpha_1$ domain. This assertion was supported by a series of additional experiments. First, FACS analyses were performed for all 16 mutants with a panel of mAbs against $\alpha_5\beta_2$, including OKM1, LM2/1, M1/70, TS1/18, and MHM23. The former three are $\alpha_5$-specific, and the latter two are $\beta_2$-specific. In all cases, the mAbs reacted well with wild-type and all five mutant receptors. Second, the reactivity of the five mutants with a conformation-dependent antibody, mAb 24, which has been used to probe the cation-dependent conformational integrity of $\alpha_5\alpha_1$ domain (23, 24), was assessed. FACS analyses were performed on the five mutants, together with the wild-type receptor. As a control, FACS analyses were performed in parallel with OKM1, a conformation-independent mAb. The ratios of the mean fluorescence intensities of the various cell lines with mAb 24 and OKM1 are shown in Fig. 4C. All of the mutants defective in NIF binding exhibited the capacity to bind mAb 24. Mutant $\alpha_5\beta_2$ (P201-G207) showed a slightly attenuated reactivity with mAb 24, suggesting a subtle perturbation of its structure. Nevertheless, binding of mAb 24 to this and the other mutants remained cation-dependent; addition of
mutants retained the cation-dependent conformation reported for their counterparts’ sequences in the site, we sought to impart NIF binding function to the chimeric fluorescence of the of the identified NIF-binding site into expressing the chimeric receptor, a domain in terms of primary structure but which still does not for NIF binding, essentially identical to that of the original molecule, was expressed in 293 cells, and NIF binding was as-

1 mM EDTA completely abrogated mAb 24 binding. Thus, all mutants retained the cation-dependent conformation reported by mAb 24.

Reconstruction of NIF-binding Site in αM Domain—When the I domain of αM (Ile139 to Ala342) was replaced with that of αX (Ile237 to Val340), the most closely related I domain to αM domain in terms of primary structure but which still does not bind NIF (8), the expressed heterodimeric receptor, αM(I/αX)β2, had no NIF binding capacity (Fig. 5), albeit expressed well on 293 cell surface. When stained with mAb 28/1/1, the mean fluorescence of the αM(I/αX)β2 chimera was 281.2, compared with 279.0 for wild-type αMβ2 receptor. This chimeric receptor continued to recognize the αMβ2 mAb, clone 3.9, and to rosette with C3bi (data not shown), indicating the retention of functional integrity. To provide direct evidence that the identified segments (Pro147-Arg152, Pro201-Gly207, Arg208-Lys217, Asp248-Thr252, and Glu253-Arg261) constitute a functional NIF-binding site, we sought to impart NIF binding function to the chimeric receptor, αM(I/αX)β2, by grafting the five segments from αM into their counterparts’ sequences in the αM I domain.

The chimeric αM(I/αX)β2 receptor, created by grafting of the identified NIF-binding site into αM I domain, acquires the ability to interact with NIF with high affinity. Cells (2 × 10⁶) expressing the chimeric receptor, αM(I/αX)β2, containing the wild-type αM I (○) or the five segments implicated in NIF binding (●) were incubated with different concentrations of ¹²⁵I-NIF in HBSS containing 2.5 mM Ca²⁺ for 30 min at 22 °C. Bound and free ligands were separated by centrifugation through 20% sucrose, and the cell associated radioactivity was measured. The data are representative of two independent experiments.

In this study, we have sought to map the binding site for NIF, a model ligand for the I domain of αMβ2. First, we have demonstrated that the binding surface for NIF is located exclusively in the I domain of the receptor. This conclusion is supported by the observations that αM alone (Fig. 1C) or the expressed I domain (10, 12) support NIF binding with an affinity similar to that of the intact αMβ2 heterodimer; that mutations in β2 subunit, known to abolish the binding of several other ligands to αMβ2 (11, 17), have no effect on NIF binding; and that a swap of the αM I domain for the αX I domain in the context of the intact αMβ2 receptor completely abolishes the NIF binding activity of the resulting chimeric receptor (Fig. 5). These observations also distinguish NIF binding from C3bi binding and adhesion to protein substrates mediated by αMβ2 interactions which require both subunits (11). Second, we have scanned the entire hydrated surface of I domain by substituting 16 αI segments of 7–10 amino acids for their corresponding sequences in the I domain of αMβ2. The approach of homolog-scanning mutagenesis had been previously employed to identify receptor and antibody epitopes in human growth hormone (18). Since the homologous segments of αM and αX adopted similar structures (15, 22), and the structurally important residues involved in cation coordination (Asp140, Ser142, Ser144, Thr209, and Asp242) are preserved, the structural integrity and the essential cation-binding site was maintained. Therefore, any functional changes resulting from the switches should reflect sequences that have a direct role in NIF binding. This strategy does not exclude a role for residues conserved among the two homologous proteins in NIF binding. Nevertheless, our study does identify segments that are required for a high af-

FIG. 5. The chimeric αM(I/αX)β2 receptor, created by grafting of the identified NIF-binding site into αM I domain, acquires the ability to interact with NIF with high affinity. Cells (2 × 10⁶) expressing the chimeric receptor, αM(I/αX)β2, containing the wild-type αM I (○) or the five segments implicated in NIF binding (●) were incubated with different concentrations of ¹²⁵I-NIF in HBSS containing 2.5 mM Ca²⁺ for 30 min at 22 °C. Bound and free ligands were separated by centrifugation through 20% sucrose, and the cell associated radioactivity was measured. The data are representative of two independent experiments.

FIG. 6. The NIF-binding site in the αM I domain. The structure of the I domain is modeled according to the crystal coordinates of the αM I domain (15) using the Biosym software. The backbone of the αM I domain is shown with oxygen atoms in red, hydrogen atoms in yellow, carbon atoms in green, and nitrogen atoms in blue. The NIF-binding site, as mapped in this study, is composed of three segments (P1⁴⁷-R1⁵², P2⁰¹-K2¹⁷, and D2⁴⁸-R2⁶¹) and is shown as a red ribbon.

DISCUSSION

In this study, we have sought to map the binding site for NIF, a model ligand for the I domain of αMβ2. First, we have demonstrated that the binding surface for NIF is located exclusively in the I domain of the receptor. This conclusion is supported by the observations that αM alone (Fig. 1C) or the expressed I domain (10, 12) support NIF binding with an affinity similar to that of the intact αMβ2 heterodimer; that mutations in β2 subunit, known to abolish the binding of several other ligands to αMβ2 (11, 17), have no effect on NIF binding; and that a swap of the αM I domain for the αX I domain in the context of the intact αMβ2 receptor completely abolishes the NIF binding activity of the resulting chimeric receptor (Fig. 5). These observations also distinguish NIF binding from C3bi binding and adhesion to protein substrates mediated by αMβ2 interactions which require both subunits (11). Second, we have scanned the entire hydrated surface of I domain by substituting 16 αI segments of 7–10 amino acids for their corresponding sequences in the I domain of αMβ2. The approach of homolog-scanning mutagenesis had been previously employed to identify receptor and antibody epitopes in human growth hormone (18). Since the homologous segments of αM and αX adopted similar structures (15, 22), and the structurally important residues involved in cation coordination (Asp140, Ser142, Ser144, Thr209, and Asp242) are preserved, the structural integrity and the essential cation-binding site was maintained. Therefore, any functional changes resulting from the switches should reflect sequences that have a direct role in NIF binding. This strategy does not exclude a role for residues conserved among the two homologous proteins in NIF binding. Nevertheless, our study does identify segments that are required for a high af-
finity interaction with this ligand. Of these 16 segment-switch mutants, only five (P147-R152, P201-G207, R208-K217, D248-Y252, and P255-R261) lost their ability to bind NIF with high affinity, suggesting that the NIF-binding site is composed of these five short segments, two of which are contiguous (Fig. 4B, Table II). The loss of NIF binding function in these five mutants is unlikely to arise from incorrect conformational folding since: 1) these, as well as all 16 mutants, were expressed on the cell surface and heterodimeric complexes were formed (Fig. 4A); and 2) they all reacted with a panel of αMβ2 mAbs, indicating the conformation-dependent antibody, mAb 24 (Fig. 4C). The reactivity of this mAb has been shown to depend on an intact conformation of the I domain (23). For mutant P201-G207, an attenuated reactivity with mAb 24 was observed, although its binding to mAb 24 remained cation-dependent. It is possible that this mutant has a slightly altered conformation; however, when this segment was grafted together with the other four segments into αI domain, cation-dependent NIF binding activity was observed.

Recently, the crystal structures of both the αI and αI domains have been solved (15, 22). These two I domains adopt segments into that this mutant has a slightly altered conformation; however, binding to mAb 24 remained cation-dependent. It is possible that the NIF contact site is dependent upon the swapped regions. Thus, the loss-in-function identified by the homolog-scanning mutagenesis is supported by this gain-in function experiment and is not due to disruption of conformation or loss of cation binding functions of the resultant αI domain mutants. The successful implantation of the homolog-scanning mutagenesis strategy marks the first time that a ligand-binding domain within any integrin molecule has been systematically probed over its entire surface. With these analyses serving as proof of principle, the homolog-scanning strategy should allow mapping of the ligand binding site for other αMβ2 ligands and other I domain-containing integrins. Experiments to test this hypothesis are in progress.

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