Solvent-accessible Residues on the Metal Ion-dependent Adhesion Site Face of Integrin CR3 Mediate Its Binding to the Neutrophil Inhibitory Factor*

(Received for publication, March 28, 1996, and in revised form, May 7, 1996)

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Neutrophil adhesion-dependent functions such as chemotaxis, spreading, and phagocytosis are inhibited by neutrophil inhibitory factor (NIF), a glycoprotein produced by the hookworm Ancylostoma caninum. The NIF binding site has been localized to the A-domain of integrin CR3 (CD11b/CD18) and shown to be metal-dependent. The recently solved crystal structure of the A-domain from CD11b revealed a putative metal ion-dependent adhesion site (MIDAS) on the top of the structure. To determine if NIF binds to the A-domain at its MIDAS face, amino acid substitutions involving 24 residues present in surface loops and adjacent helices in the structure were created. The expressed CD11b A-domain and CR3 heterodimers were then tested in a blinded manner for their ability to bind to biotinylated NIF. The solvent-exposed Gly143, Asp242, Glu178, Glu179, and Arg208, all located on the MIDAS face, in close proximity to the metal ion, were involved in CR3-NIF interaction. These data show that the natural integrin antagonist, NIF, binds to CR3 through the MIDAS region and identify putative contact residues in this region that could be targeted therapeutically.

The β2 integrin CR3 (CD11b/CD18, Mo-1, Mac-1) is a heterodimeric cell surface receptor formed by the noncovalent association of α (CD11b) and β (CD18) subunits (1). CR3 mediates leukocyte spreading, chemotaxis, adhesion to endothelium and transendothelial migration, phagocytosis, adhesion-dependent degranulation, and superoxide generation (2). The divalent cation-dependent binding of CR3 to several ligands (3–6). Neutrophil inhibitory factor (NIF)1 is a naturally occurring glycoprotein produced by the hookworm Ancylostoma caninum, which inhibits several neutrophil adhesion-dependent functions (7, 8). NIF binds in a divalent cation-dependent manner to the CD11b A-domain and blocks binding of the domain as well as the holoreceptor to several physiologic ligands (8, 9).

The crystal structure of the CD11b A-domain showed that the domain adopts a classic α/β fold, with seven α helices surrounding a mostly parallel β sheet, and contains an unusual MAl coordination site on its surface at the top of the β sheet (5). This cation is coordinated by the side chains of two serines (Ser142 and Ser144) and one threonine (Thr209) and the side chains of two aspartates (Asp140 and Asp242). A glutamate side chain from a second A-domain completes the octahedral coordination sphere of the metal. Since all known integrin ligands contain an acidic residue as a key feature of their binding motif (10), we have suggested that the glutamate may act as a ligand mimetic, with the metal assuming a direct role in ligand binding, and have referred to the metal coordinating region as MIDAS (metal ion-dependent adhesion site). Point mutations of metal-coordinating residues such as Asp140 and Ser142 abrogate cation binding and binding of the domain or the integrin to several protein ligands (3, 5, 11). In this report, we establish that the natural integrin antagonist NIF utilizes the MIDAS region in binding to CR3 and identify potential contact residues. The ability of NIF to block binding of physiologic ligands to CR3 suggests that identical and/or adjacent residues are also involved in CR3 binding to these ligands.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Modification and restriction enzymes were bought from Boehringer Mannheim, New England Biolabs, or Life Technologies, Inc. The murine monoclonal antibodies (mAbs) 44, 903, 904, LM2/1, and OKM10 directed against human CD11b were previously described (12–15). OKM10 was obtained from Ortho Inc. Recombinant NIF (rNIF) was expressed in Chinese hamster ovary cells and purified as described (9). It was kindly provided by Drs. Matthew Mayle and Howard R. Soule (Corvas International, Inc., San Diego, CA). rNIF was biotinylated with sulfo-NHS-biotin as detailed elsewhere (8).

Mutagenesis and Recombinant Protein Expression—The amino acids targeted for mutagenesis were located in loop structures and adjacent α helices in the A-domain (see Fig. 3b). All 11 loops in the structure were targeted with the exception of loops pC-1 and pC-2. The three amino acids (His235, Phe-Thr) comprising loop pC-2 had very low relative total side chain accessibility (17.4, 1.3, and 19%, respectively), as determined using the NACCESS Program (16). The pC-3 loop consisting of the four amino acids Asn192-Pro-Asn-Pro was avoided because of previous data showing that a P195A mutation impaired metal binding to the domain (11). The selected amino acids were replaced with residues occupying similar positions in the homologous CD11a (17) or CD11c (18) A-domains (which do not bind NIF) (8) (Table I). When the residues are conserved, alanine or lysine substitutions were made (Table I). Mutagenesis was carried out using polymerase chain reaction, the recombinant CD11b A-domain (r11bA)-containing expression vector PGEX-2TA as a template, mutant primers (Table I), and the Transformex site-directed mutagenesis kit (Clontech Laboratories, Inc., Palo Alto, CA) (19). Each mutation was confirmed by the presence of an introduced unique restriction site resulting from the base pair change (Table I) and by direct DNA sequencing (20). BamHI-BbsI- or BamHI-PvuII-restricted cDNAs encoding the mutant A-domain were cloned into BamHI-BbsI- or BamHI-PvuII-restricted pCDNA3 expression vec-

* The abbreviations used are: NIF, neutrophil inhibitory factor; MIDAS, metal ion-dependent adhesion site; mAb, monoclonal antibody; rNIF, recombinant NIF; WT, wild type; BSA, bovine serum albumin.

† The work was supported by grants from the National Institutes of Health, a fellowship grant from the American Heart Association, and the Philippe foundation. This work was presented at the 28th annual meeting of the American Society of Nephrology, November 1995, and the January 1996 Keystone Symposium on Cell Adhesion. 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.

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Role of MIDAS in NIF Binding

Table 1
Mutations and the nucleotide sequences used in their construction

| Mutant       | Mutagenic primer† | Restriction site |
|--------------|-------------------|-----------------|
| G143M        | GCTCTTCTGATGATGCTCGTACGGACATTACATCCATCCACATACG | BsaBl |
| D149K        | CTCCTGAGATGATGCTCGTACTCAAAATTGCGGATGAAGAGG | BglI |
| R152A/E155A  | CACCATGGATGATGCTCGTACTCAAAATTGCGGATGAAGAGG | SfI |
| K166A/S167A  | TACAGGATGATGCTCGTACTCAAAATTGCGGATGAAGAGG | AfII |
| E178A/E179A  | CTCCTTGTAGCAGTACGGCCTACATTCCATCCTTACC | EagI |
| T203K/Q204H  | TACCTGATGATGCTCGTACTCAAAATTGCGGATGAAGAGG | AvrII |
| R262A/D263A  | GAGGCCTAATACGCAGGCTACTCGGAAGGACCAGGGGATAC | AvrII |
| I225D        | GGCAAGAATGACGAGGCTACTCGGAAGGACCAGGGGATAC | Earl |
| E254K        | GGTGGTCATCACGGATGGg | |
| F254K        | GTTGGTCATCACGGATGGg | |
| E257K        | GTTGGTCATCACGGATGGg | |
| D273K        | GTTGGTCATCACGGATGGg | |
| E278A/K279A  | CGTCATTGGGGTGGGA | |
| D280A/R281A  | CGTCATTGGGGTGGGA | |
| D280A/R281A  | CGTCATTGGGGTGGGA | |
| T293A/K294A  | CGTCATTGGGGTGGGA | |
| N310K/Q311A  | CCATCGTCATTGGGGTGGGA | |

†The lowercase characters represent the mutagenized nucleotides, with those shown in bold encoding for the amino acid change. Underlined numbers refer to amino acid positions in the full-length CD11b subunit (24).

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FIG. 1. NIF binding to r11bA. a, reactivity of slot-blotted WT and r11bA mutants with biotinylated NIF and a polyclonal anti-CD11b antibody. All mutants reacted normally with anti-CD11b antibody (left panel). The reactivity of NIF with four mutants was significantly reduced when compared with WT and the other mutants. The lack of NIF binding to metal-defective mutant D140A/S142A (D140S142/AA) and to the WT in the presence of EDTA is shown for comparison. All mutants were tested in three independent experiments. A representative experiment is shown. Binding to T203K/Q204H (T203K/Q204H) mutant was performed at a different time. b, one representative experiment is shown. Binding, out of three, showing normal reactivity of slot-blotted NIF-defective r11bA with anti-11bAmAbs. c, binding of 35S-Mn to immobilized WT and mutant r11bA. Binding of the mutants to the labeled metal ion was indistinguishable from WT. The metal-defective mutant D140A/S142A (D140S142/AA) was used as a negative control. Similar results were obtained in a second experiment.

The mAb and NIF binding data from three or four independent experiments were then pooled and expressed as histograms representing mean ± S.E. before the mutants were decoded. Binding of NIF and the mAbs OKM10, 44, 904, and LM2/1 to CR3 mutants was normalized for the percentage of COS cells staining with mAb 903 in each experiment and for the percentage of binding obtained with WT as follows.

% binding = (% mutant binding) / (% WT binding) × 100 (Eq. 1)

Evaluation of the Effects of NIF-defective Mutants on Heterodimer Formation—This was assessed by immunoprecipitating recombinant receptors from the detergent-solubilized extracts of equal numbers of transfected COS cells with the anti-CD11b mAb 44 and then probing the SDS-polyacrylamide gel electrophoresis fractionated precipitate with a polyclonal anti-CD18 antibody (23), as described previously (11).
RESULTS AND DISCUSSION

Amino Acid Substitutions Impairing NIF Binding to r11bA—Slot blots of purified water-soluble mutant r11bA were probed with biotinylated NIF without prior knowledge of the precise location of these mutants in the structure (Fig. 1a). In these experiments, the reactivity of labeled NIF with WT in the presence of divalent cations served as a positive control. Binding of NIF to WT in the presence of EDTA and to the metal-defective mutant D140A/S142A served as negative controls. Of all the mutants generated, only four (G143M, D149K, E178A/E179A, and R208L) impaired the ability of labeled NIF to bind to r11bA (Fig. 1a). This reduction was not explained by differences in the amount of antigen blotted onto the nitrocellulose, as all mutants reacted equally with the polyclonal anti-CD11b antibody. The normal reactivity of the NIF-defective mutants with three mAbs directed against the CD11b A-domain (Fig. 1b) further excluded gross conformational changes in the structure as contributing factors. Since NIF binding to the domain is metal-dependent, the observed effects could be secondary to impaired metal coordination, especially in view of the fact that two of the mutated residues, Gly143 and Arg208, are located in the immediate vicinity of the metal coordinating residues Ser142, Ser144, and Thr209. We therefore assessed the direct binding of radiolabeled 54Mn to immobilized A-domains from the four mutants as well as WT and the metal-defective mutant D140A/S142A served as negative controls. Of all the mutants generated, only four (G143M, D149K, E178A/E179A, and R208L) impaired the ability of labeled NIF to bind to r11bA (Fig. 1a). This reduction was not explained by differences in the amount of antigen blotted onto the nitrocellulose, as all mutants reacted equally with the polyclonal anti-CD11b antibody. The normal reactivity of the NIF-defective mutants with three mAbs directed against the CD11b A-domain (Fig. 1b) further excluded gross conformational changes in the structure as contributing factors. Since NIF binding to the domain is metal-dependent, the observed effects could be secondary to impaired metal coordination, especially in view of the fact that two of the mutated residues, Gly143 and Arg208, are located in the immediate vicinity of the metal coordinating residues Ser142, Ser144, and Thr209. We therefore assessed the direct binding of radiolabeled 54Mn to immobilized A-domains from the four mutants as well as WT and the metal-defective mutant D140A/S142A. As previously reported (11), WT r11bA bound directly to 54Mn, whereas D140A/S142A did not (Fig. 1c). Binding of each of the four mutants to 54Mn was indistinguishable from WT, indicating that impaired NIF binding is not caused by a secondary effect on metal coordination in the domain. These data thus suggest that the identified mutants are directly involved in NIF binding to the isolated domain.

Effects of the Mutant Forms on Binding of CR3 to NIF—To determine if similar results are obtained in heterodimeric CR3, the holoreceptor from WT and each of the coded mutants were transiently expressed in COS cells. After co-transfection with
full-length CD18 cDNA, cell surface expression of CR3 was evaluated by radiomimunoassay, using mAbs 903 and OKM10, both of which lie outside the A-domain of CD11b and are therefore less likely to be affected by mutations within the domain. Expression of the associated CD18 subunit was independently evaluated using the anti-CD18-specific mAb TS1/18. All mutant receptors were found to be expressed at levels comparable with the WT ± 20% Binding of labeled NIF to WT and the mutant was then determined. After decoding, the same four mutant proteins that impaired NIF binding to the isolated domain exerted similar effects in the context of the heterodimer; no significant binding was detected to G143M and E178A/E179A mutants, and binding was significantly reduced to D149K and R208L mutants (Fig. 2a). Furthermore, each of these mutants reacted normally with several anti-CR3 mAbs directed to the CD11b A-domain, suggesting that the mutations exerted no gross structural effect on the domain in the context of the heterodimer (Fig. 2b). In addition, immunoprecipitation of the CD11b subunit from the mutant receptors followed by Western blotting using a polyclonal anti-CD18 antibody demonstrated that these mutants formed heterodimers with CD18 (Fig. 2c). These data thus indicate that the defective binding of each of the four mutants to NIF is not caused by reduced cell surface expression, gross structural changes in the domain, or by an inability of the respective receptors to form heterodimers. These results thus confirm the relevance of the identified mutations for NIF binding to the native receptor.

Mapping of the NIF-sensitive Residues to the MIDAS Region—The five amino acids involved in NIF binding were localized to the MIDAS region of the CD11b A-domain, in close proximity to the metal and lining one side of the metal (Fig. 3a). The specificity of the interaction was also reflected by the finding that amino acids with prominent side chains lining the other side of the metal (Glu144, Phe246, and Asp273) were not involved. Four of the five residues (Gly143, Glu178-Glu179, and Arg208) displayed prominent solvent-exposed side chains (percent accessibility of total side chains of 55.7, 46.5, 53.0, and 61.1, respectively), with Asp149 somewhat lower (at 26.2%), values that are also reflected in Fig. 3a. These data suggest that Gly143, Glu178-Glu179, and Arg208 may serve as contact residues. Of these, perhaps Gly143 and Glu178-Glu179 are most likely to be contact residues, since mutations involving these residues abolish NIF binding, whereas nonconservative substitutions involving Asp149 and Arg208 continue to retain some functional activity. Alignment of the CD11 A-domain region in human and mouse CD11a, b, c, and d (Fig. 3b) (17, 18, 24–27 revealed that Gly143, Asp149, Glu179, and Arg208 are identical in human and mouse CD11b (both of which bind to NIF) (8, 9), with only Glu178 in human CD11b replaced by the conserved aspartate residue in mouse. In contrast, only one of five residues is conserved at different sites in human and mouse CD11a and in human CD11c (Fig. 3b). In particular Glu178, Glu179, and Arg197 residues are not conserved in other CD11 A-domains, again pointing to a critical role of these residues in CD11b-NIF interactions. These data also offer a structural basis for the lack of binding of human CD11a and CD11c to NIF (8) and predict a similar outcome for the newly cloned CD11d receptor.

The fact that the particular amino acids involved in NIF binding lie in immediate proximity to the metal coordination site may also explain the crucial need for metal in NIF binding to CR3 and the inhibitory effects of linear peptides derived from this region of the structure (8). A previous study (28) found that several amino acids involved in CD11a binding to CD54 (ICAM-1) also cluster around the MIDAS face. Included in these residues are Met140 and Glu146. These are equivalent, respectively, to Gly143 and Asp149 in CD11b, which are involved in CD11b binding to NIF. Furthermore, the ability of NIF to inhibit the metal-dependent binding of physiological ligands such as fibrogin, complement iC3b, and CD54 to CD11b (8) suggests that some of the residues identified here are close to or identical with residues involved in binding of the CD11 A-domains to their respective ligands. The present data provide support to our proposed model for a direct role of the A-domain metal ion in coordinating an acidic residue that is uniformly expressed in integrin ligands (S). Since CR3 appears to mediate the inflammatory and tissue-destructive potential of leukocytes, identification of potential contact residues that are targeted by the natural CR3 antagonist NIF could have important therapeutic benefits.

Acknowledgment—We thank Colleen Laiselle for secretarial assistance.

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