

The A-Domain of $\beta 2$ Integrin CR3 (CD11b/CD18) Is a Receptor for the Hookworm-derived Neutrophil Adhesion Inhibitor NIF

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Abstract. The A-domain is a ~ 200 -amino acid peptide present within structurally diverse proadhesive proteins including seven integrins. A recombinant form of the A-domain of $\beta 2$ integrins CR3 and LFA-1 has been recently shown to bind divalent cations and to contain binding sites for protein ligands that play essential roles in leukocyte trafficking to inflammatory sites, phagocytosis and target cell killing. In this report we demonstrate that the neutrophil adhesion inhibitor, NIF produced by the hookworm *Ancylostoma caninum* is a selective CD11b A-domain binding protein. NIF bound directly, specifically and with high affinity (K_d of ~ 1 nM) to recombinant CD11b A-domain (r11bA). The binding reaction was characterized by rapid association and very slow dissociation, and was blocked by an anti-r11bA monoclonal antibody. No binding was observed to rCD11aA. The NIF-r11bA interaction required divalent cations, and was absent when the mutant r11bA D140GS/AGA (that lacks divalent cation binding capacity) was used. The NIF binding site in r11bA was mapped to four short peptides, one of which being an iC3b binding

site. The interaction of NIF with CR3 in intact cells followed similar binding kinetics to those with r11bA, and occurred with similar affinity in resting and activated human neutrophils, suggesting that the NIF epitope is activation independent. Binding of NIF to CR3 blocked its ability to bind to its ligands iC3b, fibrinogen, and CD54, and inhibited the ability of human neutrophils to ingest serum opsonized particles. NIF thus represents the first example of a disintegrin that targets the integrin A-domain, and is likely to be used by the hookworm to evade the host's inflammatory response. The unique structure of NIF, which lacks a disintegrin motif, emphasizes basic structural differences in antagonists targeting A^+ and A^- integrins, that should be valuable in drug design efforts aimed at generating novel therapeutics. Identification of the region in NIF mediating A-domain binding should also be useful in this regard, and may, as in the case of disintegrins, unravel a new structural motif with cellular counterparts mediating important physiologic functions.

MICROORGANISMS have evolved different strategies to subvert the host defense mechanisms in favor of their own well-being (see references 24, 34 for reviews). In many cases, infectivity is established by expression of gene products that modulate specific components of the host's innate or adaptive immune responses. Identification of these products is essential for a detailed understanding of the complex interactions between host and pathogens. It could also serve as a basis for development of novel drugs where such products are used as selective antagonists of specific components of the hosts' immune response to injury.

In contrast to bacteria and viruses, helminths are constrained by their slower replication rate. To begin and sustain the infection process, it is essential that these parasites avoid

the host's early reactions to injury that is often mediated by circulating leukocytes and chemoattractants derived from activated endothelial cells or the complement system. In this context, some of the remarkable adaptations include the secretion of an elastase inhibitor by *Echinococcus granulosus* which blocks C5a and PAF-induced chemoattraction in neutrophils (52), cleavage of PAF by an acetylhydrolase secreted by *Nippostrongylus brasiliensis* (12), and surface expression of several antioxidants by *Schistosomes* (19, 56) to counteract the neutrophil's oxidative burst.

The chemotaxins that are secreted or expressed at acute inflammatory sites in response to an invading microorganism, attract circulating neutrophils by activating a key cell surface receptor named complement receptor type 3 (CR3, CD11b/CD18, $\alpha M\beta 2$, Mac-1, Mol) (reviewed in reference 4). Activated CR3 plays an essential role in immune clearance by facilitating neutrophil adhesion to endothelium, transendothelial migration, and phagocytosis of serum-

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opsonized particles (reviewed in reference 5). CR3 is one of the four members comprising the $\beta 2$ integrin subfamily that share a common β subunit (CD18), but have distinct α (CD11) subunits, the other three members being LFA-1 (CD11a/CD18, $\alpha L\beta 2$, TA-1), p150,95 (CD11c/CD18, $\alpha X\beta 2$, Leu-M5), and the recently described macrophage-restricted CD11d/CD18 ($\alpha TM\beta 2$) (17) (reviewed in reference 4). CR3 binds in a divalent cation-dependent manner to several ligands including iC3b, the major complement opsonin (9, 10, 60), fibrinogen (1), and CD54 (ICAM-1) (21), through non-Arg-Gly-Asp-containing sequences (2, 53, 55). Recently, the A-(I) domain, a ~ 200 -amino acid segment in the extracellular region of the α subunits of seven integrins has been shown to be a metalloprotein, containing a divalent cation binding site(s), formed by discontinuous hydroxylated amino acids (36). Mutations involving the conserved amino acids D140GS and D242 within the CD11b A-domain impair CR3 binding to iC3b, and mutations of the equivalent residues in CD49b also inhibit binding of CD49b/CD29 ($\alpha 2\beta 1$) to collagen (29). Recombinant forms of the $\beta 2$ integrin A-domain have been found to bind directly to iC3b (58), fibrinogen, and CD54 (46, 61), indicating that this domain is a major binding site for physiologic ligands involved in leukocyte extravasation and phagocytosis.

The hookworm *Ancylostoma caninum* produces a 41-kD heavily glycosylated protein called NIF, that inhibits neutrophil spreading and adhesion to endothelial cell monolayers, and binds to CR3 (40). In this communication we show that CR3 is the sole protein recognized by NIF in neutrophils, and demonstrate that the interaction of NIF with CR3 is divalent-cation dependent and mediated through the A-domain. The binding site for NIF in the A-domain is comprised of non-contiguous peptides, one of which serves as the major iC3b binding site (58). NIF is the first example of a selective integrin A-domain antagonist. Its unique structure, selectivity and binding properties should be useful in the design of therapeutically effective anti-inflammatory agents, and suggest novel therapies to treat hookworm infections.

Materials and Methods

Reagents and Antibodies

Restriction and modification enzymes were bought from New England Biolabs (Beverly, MA), Boehringer Mannheim Biochemicals (Indianapolis, IN) or BRL. The bacterial expression vector pGEX-2T vector was obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). The murine mAbs directed against human CD11b [44 (9), 904 (20), OKM9 (60)], CD11a [TS1/22] and CD18 [TS1/18] (48), and CD11c [L29] (32) have been previously described. The mAb 107 (IgG1) was raised in our laboratory by immunizing BALB/c mice with pure recombinant human CD11b A-domain. This mAb reacts with CD11b but not CD11a A-domain by ELISA immunoprecipitates CR3 from neutrophil extracts, and binds to neutrophils by FACS analysis (unpublished observations).

Generation and Purification of CD11 A-domain Recombinant Proteins

Recombinant proteins encoding wild-type CD11a and CD11b A-domains and the non-metal binding CD11b A-domain mutant D140GS/AGA were expressed as GST-fusion proteins in *Escherichia coli* as described elsewhere (36, 58). The fusion proteins were purified by affinity chromatography, used as such or cleaved with thrombin to release the rA-domains (54). Recombinant purified NIF (rNIF)¹ was kindly provided by Drs. Matthew Moyle

1. **Abbreviations used in this paper:** EA, erythrocytes; NEM, N-ethylmaleimide; ORO, opsonized oil red O; rNIF, recombinant purified NIF; RT, room temperature; STI, soybean trypsin inhibitor.

and Howard R. Soule (Corvas International Inc., San Diego, CA). A recombinant soluble form of human CD54 (containing all five Ig domains but lacking the intramembranous and cytoplasmic regions) was the kind gift of Dr. Jeffrey Greve (Miles Research Center, West Haven, CT) (26). Recombinant protein concentrations were determined using the protein assay kit from BioRad Laboratories (Melville, NY), and analyzed by Coomassie staining after electrophoresis on denaturing polyacrylamide gels (31).

Peptides

Synthetic peptides were obtained commercially or made at the Howard Hughes Biopolymer facility at Massachusetts General Hospital. All peptides were purified on HPLC, and selective ones were subjected to amino acid analysis. All peptides were soluble in water at 1 mg/ml.

Immobilization of Recombinant Proteins and Peptides

Purified rA-domain preparations (1 μ g/well), soluble CD54, human fibrinogen (Sigma Chemical Co., St. Louis, MO), gelatin (BioRad Laboratories) or BSA (Calbiochem-Behring Corp.) (each at 10 μ g/well) or selected A-domain-derived peptides (10 μ g) were added to Immulon-2 96-well microtiter plates (Dynatech Labs, Chantilly, VA) overnight. Quantitation of adsorbed wild-type and mutant A-domain and synthetic peptides was done using the mAb 44 in an ELISA, and the BCA kit (from Pierce Chemical Co., Rockford, IL), respectively. Wells were then washed with PBS, pH 7.4, without metals, and blocked with 1% BSA for 1 h, washed again in binding buffer, and used immediately in the functional assays.

Preparation of Complement C3-coated Erythrocytes

Sheep erythrocytes were incubated with 1:240 dilution of rabbit anti-sheep erythrocyte antiserum (Diamedix Corp. Miami, FL) for 30 min at 37°C to generate IgM-coated sheep erythrocytes (EA). EAiC3b was prepared using C5-deficient human serum (Sigma Chemical Co.) at 1:10 dilution (60 min at 37°C). EAiC3b cells were washed and stored in isotonic VBSG⁺⁺ (veronal-buffered saline, pH = 7.4, containing 0.1% gelatin [VBSG⁻] and 1 mM each of CaCl₂ and MgCl₂) and to which soybean trypsin inhibitor (STI; Worthington Biochemical Co., Freeton, NJ) was added at 1 mg/ml. EAiC3b (at 1.5×10^8 cells/ml) were labeled with 5-(and-6)-carboxy fluorescein (Molecular Probes, Eugene, OR) at 1:100 dilution of a 10 mg/ml stock for 5 min on ice and washed before use in the binding studies.

Recombinant CR3 Binding to EAiC3b

Binding of EAiC3b to recombinant membrane-bound CR3 expressed on COS cells was performed as previously described (36). To assess the effect of NIF on this interaction, EAiC3b binding was performed in the absence and presence of increasing amounts of NIF. After incubation, cells were washed, examined briefly by light microscopy, and then solubilized with 1% SDS-0.2 N NaOH. Fluorescence was quantified (excitation wavelength, 490 nm, emission wavelength, 510 nm) on each sample using a SLM 8000 fluorometer (SLM Instruments, Urbana, IL) (36).

Neutrophil Binding to Fibrinogen and CD54

Human neutrophils were purified as described (14). Binding of neutrophils to CD54- or fibrinogen-coated 96-well microtiter plates was performed as follows: neutrophils (8×10^6 /ml) were labeled with 5-(and-6)-carboxy fluorescein (Molecular Probes) at 1:100 dilution of a 10 mg/ml stock for 5 min on ice and washed in M199 medium containing an additional 1 mM MgCl₂, 1 mM CaCl₂ and 0.1% BSA (MB) before use. Fluoresceinated neutrophils (25 μ l of 8×10^6 /ml) were added to each well containing 25 μ l of buffer alone or containing 2×10^{-6} M f-met-leu-phe. The plates were centrifuged at room temperature (RT) (800 rpm in a Sorvall RT 6000B) for 30 s, and incubated for only 5 min at RT, to avoid cell spreading, a fact confirmed by visual inspection of the cells at the end of this incubation period. Wells were washed three times with 100 μ l of MB each, examined by light microscopy, and then solubilized with 1% SDS/0.2N NaOH, and fluorescence quantified. To evaluate the effects of mAbs and NIF on binding, mAbs (each used at 1:100 dilution of ascites) or NIF (used at 5 μ g/ml final concentration) were preincubated with fluoresceinated neutrophils for 15 min at 4°C prior to the binding reaction.

Phagocytosis Assays

Phagocytosis of serum opsonized oil red O (ORO) particles was performed essentially as described elsewhere (7). To determine the effect of rNIF or

the anti-CD11b mAb 44 on phagocytosis, rNIF (at 4 $\mu\text{g/ml}$) or 44 (at 10 $\mu\text{g/ml}$) were preincubated with neutrophils for 10 min at RT prior to addition of opsonized ORO. The reactants were prewarmed for 2 min at 37°C before mixing. Incubation was then commenced for 5 min at 37°C with continuous shaking in a water bath. The reaction was stopped by addition of 1 ml of ice-cold PBS containing 1 mM *N*-ethyl-maleimide (NEM), followed by two washes. The cell pellet was examined visually for its red color (reflecting ingestion of the red oil droplets), and then solubilized with 0.5 ml of dioxane, and the amount of ORO in the extract quantified by measuring absorption at 525 nm and converted to milligrams of ORO ingested/ 10^5 cells/minute. Specific uptake of ORO was determined by subtracting the background (uptake in the presence of 1 mM NEM).

rNIF Binding, Competition, and Displacement Studies

100 μg of rNIF were labeled with sulfo-NHS-biotin as described by the manufacturer (Pierce Chemical Co.). rNIF binding to resting or stimulated human neutrophils (pretreated with 10^{-6} M *f*-met-leu-phe, for 15 min at 37°C, and then washed) was measured. Increasing amounts of biotinylated rNIF in the absence or presence of 100-fold molar excess of unlabeled rNIF were incubated on ice for one hour with 1×10^6 neutrophils in VBSG⁺⁺ in a total volume of 50 μl . Cells were then washed and incubated with phycoerythrin-coupled avidin (Sigma Chemical Co.) under similar conditions, washed again, fixed in 1% paraformaldehyde in PBS, and analyzed using FACScan® (Becton Dickinson Co., Mountain View, CA). Mean channel fluorescence for each sample was then expressed as a function of the amount of biotinylated rNIF used. Background binding of phycoerythrin-streptavidin alone to neutrophils was subtracted (2.8 fluorescent units). Specific binding was obtained by subtracting total binding from that seen in the presence of excess unlabeled rNIF, and the values plotted according to Scatchard (49). To determine the effect of unlabeled fluid-phase r11bA or GST on rNIF binding to neutrophils, each was preincubated at varying concentrations with biotinylated rNIF (20 ng/ml, final concentration) for 15 min on ice before addition of the mixture to neutrophils. The effect of mAbs on biotinylated NIF binding to neutrophils was assessed by preincubating the neutrophils with 100 $\mu\text{g/ml}$ of each mAb at 4°C for 15 min before addition of biotinylated NIF (20 ng/ml). The incubation then continued for 1 h, followed by processing of cells for FACScan® analysis as described above.

To measure binding of biotinylated rNIF to immobilized r11bA, increasing concentrations of biotinylated rNIF in VBSG⁺⁺, in the absence or presence of 100-fold unlabeled rNIF, were added to rA-domain-coated 96-well microtiter wells, and incubated at RT for 60 min. Wells were then washed, incubated with alkaline phosphatase-coupled avidin, washed again, developed with substrate, and quantified colorimetrically using a microplate reader. To evaluate the ability of anti-CD11b A-domain mAbs to block biotinylated NIF binding to immobilized r11bA, coated wells were preincubated with the mAbs (each at 100 $\mu\text{g/ml}$ or 1:100 dilution of ascites) for 15 min at RT. Biotinylated NIF (50 ng/ml final concentration) was then added, and incubation continued for an additional hour. To assess the ability of fluid-phase r11bA or GST to block biotinylated NIF binding to immobilized r11bA, each was preincubated at 7 $\mu\text{g/ml}$ with biotinylated NIF (50 ng/ml final concentration) in a total volume of 50 μl for 15 min at RT, followed by incubation of this mixture with the r11bA-coated wells for an additional hour. In experiments where the effects of divalent cations on biotinylated rNIF binding to immobilized rCD11b A-domain were measured, VBSG⁻ buffer was used as such, containing 1 mM of Ca^{2+} , Mg^{2+} , Mn^{2+} , EDTA, EGTA, EGTA plus 1 mM MgCl_2 , or 1 mM MnCl_2 . In these experiments, BSA-blocked A-domain-containing wells were first washed with buffer containing 10 mM EDTA (to remove protein-bound cations), and then washed with the respective binding buffer. The effect of temperature was evaluated in the presence of the standard divalent cation mixture at 37°, 22°, and at 4°C with saturating amounts of biotinylated rNIF (200 ng/ml).

The kinetics of rNIF-neutrophil or rNIF-A-domain interactions were determined as described (33). Neutrophils or immobilized rA-domains were each incubated with half-saturating concentrations of biotinylated rNIF (20 ng/ml and 40 ng/ml for neutrophils and immobilized rA-domain, respectively), in the absence or presence of 100-fold molar excess of unlabeled rNIF at 4°C (with neutrophils) or at RT (with immobilized rA-domain). The specific binding of biotinylated rNIF was determined at various times as described above, and plotted versus time. The time required to reach equilibrium was one hour. The value for $t_{1/2}$ of association was determined graphically from the association plot. To determine dissociation rates, neutrophils or immobilized A-domain was incubated for 1 h with the respective half-saturating concentrations of biotinylated rNIF mentioned above, in the absence or presence of 100-fold molar excess of unlabeled rNIF, at 4°C (for neutrophils) or at RT (for immobilized A domain). Afterwards, neutrophils were washed twice in VBSG⁺⁺ and incubated in 4 ml of this buffer on ice

with shaking. At various time points, aliquots were removed, centrifuged and the amount of specifically bound rNIF measured. For immobilized r11bA, wells were washed twice and incubated with 300 μl of VBSG⁺⁺ per well at RT with shaking. At various time points, the buffer was removed and specific binding measured. The dissociation rates in each case were determined by plotting $-\ln(B/B_{\text{eq}})$ versus time, where B and B_{eq} represent, respectively, the fraction of rNIF bound to cells (or to immobilized r11bA) at time t , and at equilibrium. The value for $t_{1/2}$ of dissociation was calculated according to the formula $t_{1/2} = \ln 2/K_{\text{off}}$ (33).

Surface Biotinylation, Immunoprecipitation, and Western Blotting

Surface biotinylation of purified human neutrophils was performed on ice by incubating the cells ($3 \times 10^7/\text{ml}$ in PBS) with 0.1 mg/ml final concentration of sulfo-NHS-biotin (Pierce Chemical Co.) for 30 min at 4°C. Afterwards, cells were washed twice in PBS, quenched for 15 min in RPMI on ice and washed once again in PBS. The NP-40-soluble fraction from unlabeled or biotin-labeled cells was used to immunoprecipitate $\beta 2$ integrins with the anti-CD11a, b, c-specific mAbs (TS1/22, 44, L29, respectively). Immunoprecipitates were electrophoresed on gradient 4–16% polyacrylamide gels in Laemmli buffer (31), electroblotted onto Immobilon-P membranes, and blocked with BSA. Membranes containing immunoprecipitates from surface-biotinylated cells were then probed with HRP-coupled avidin (Sigma Chemical Co.), while those with immunoprecipitates from unlabeled cells were first probed with biotinylated rNIF (at 1 $\mu\text{g/ml}$), washed then re-probed with HRP-coupled avidin (Sigma Chemical Co.). Membranes were developed using the enhanced chemiluminescence system from Amersham Corp. (Arlington Heights, IL).

Results

Binding Characteristics of rNIF to Human Neutrophils

The optimal parameters of neutrophil binding to biotinylated rNIF were determined. Measurement of the time course of association of biotinylated NIF with neutrophils at 4°C (to avoid endocytosis) revealed a rapid uptake, with maximum levels achieved within 60 min, and with a $t_{1/2}$ at 15 min (Fig. 1 A), as previously shown (40), and was completely inhibited in the presence of 100-fold molar excess of unlabeled NIF at each time point.

Upon washing and dilution of cells preincubated for 1 h at 4°C with biotinylated rNIF, the cell-associated rNIF slowly dissociated with a $t_{1/2}$ of ~ 7.6 h (Fig. 1 A, inset). Thus, the association of rNIF with neutrophils is reversible and characterized by rapid binding and very slow dissociation. The slow dissociation rate permitted the use of biotinylated rNIF under the conditions described to evaluate its interaction with whole cells and with protein fragments. The slow dissociation observed here, together with the fact that Moyle et al. performed their binding studies to *f*-met-leu-phe-activated neutrophils at RT where endocytosis of CR3 can take place (16, 45), may account for the previous suggestion that binding of NIF to neutrophils is irreversible (40).

Incubation of increasing concentrations of biotinylated rNIF with resting or activated neutrophils at 4°C, revealed a predominantly saturable component (Fig. 1 B), with the non-saturable (nonspecific) fraction (obtained in the presence of 100-fold molar excess of unlabeled rNIF) accounting for less than 10% of the total binding. A Scatchard plot of the binding data (Fig. 1 B, insets) indicated a linear relationship in both resting and activated cells. Both cell types bound NIF with approximately similar affinities (K_d ranging from 0.35 to 1.3 nM), suggesting that the 12-fold increase in NIF binding to activated vs resting cells is primarily due to an in-

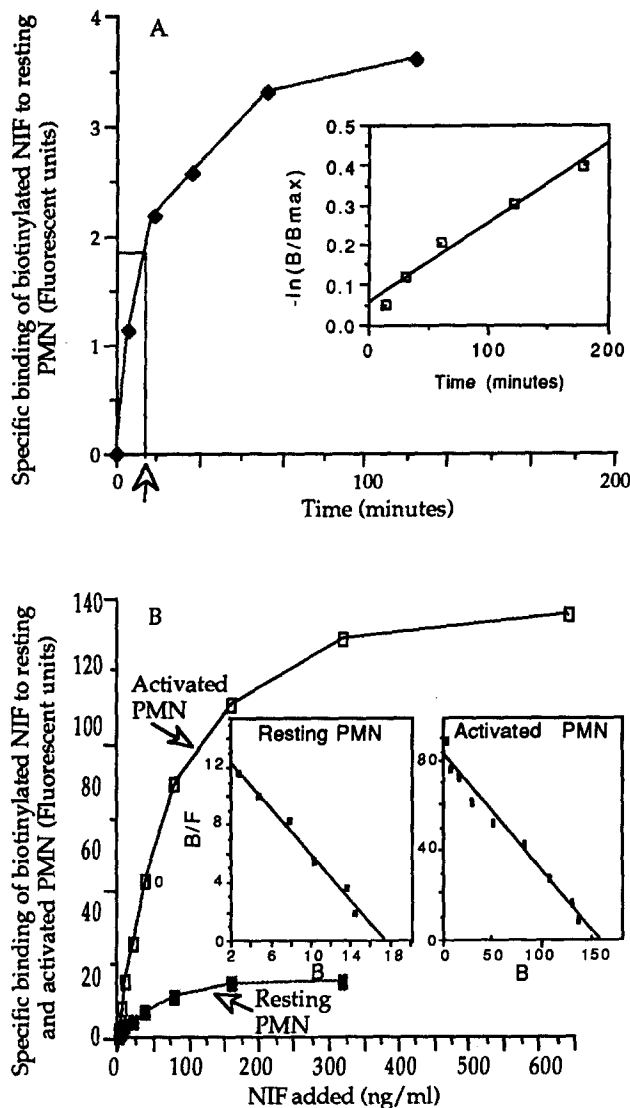


Figure 1. (A) Time course of the specific association of biotinylated NIF (20 ng/ml) with neutrophils. Association was maximal by 1 h at 4°C, with a $t_{1/2}$ of association of 15 min. The mean \pm SE of $t_{1/2}$ from this and two additional experiments was 15 ± 5 min. (*Inset*) Time course of the release at 4°C of biotinylated rNIF from neutrophils following one hour of binding, removal of the unassociated label, and a 100-fold dilution in buffer alone. The K_{off} for rNIF binding to neutrophils (given by the slope of the curve) was $\approx 2.5 \times 10^{-5} \text{ s}^{-1}$, with a dissociation $t_{1/2}$ of ≈ 460 min. (B) Specific binding of increasing concentrations of biotinylated rNIF to purified resting and f-met-leu-phe activated human neutrophils. Specific binding was obtained by subtracting non-specific binding (in the presence of 100-fold molar excess of unlabeled rNIF) from total binding (in the absence of unlabeled NIF). (*Inset*) Specific binding to resting and activated neutrophils plotted according to Scatchard. The results shown are from one experiment, with similar results obtained in two others.

crease in the number of NIF binding sites induced by cell activation (4).

Effects of rNIF on Neutrophil Ligand Binding and Phagocytosis

The effects of rNIF on CR3-mediated neutrophil binding to the physiologic ligands complement iC3b, fibrinogen and

CD54 were measured. rNIF inhibited binding of EAiC3b to recombinant human CR3 (expressed in COS cells) in a dose-dependent manner with complete inhibition achieved at 3 $\mu\text{g/ml}$ (IC_{50} of $\sim 5 \text{ nM}$) (Fig. 2 A). rNIF also abolished iC3b-dependent phagocytosis of serum-opsonized oil red O particles by human neutrophils (Fig. 2 A, *inset*).

Binding of f-met-leu-phe-activated fluoresceinated neutrophils to microtiter wells coated with human fibrinogen or soluble CD54 was also inhibited significantly in the presence of NIF (5 $\mu\text{g/ml}$) (Fig. 2, B and C). Inhibition of neutrophil binding to fibrinogen was incomplete even at high NIF concentrations (50 $\mu\text{g/ml}$) (Fig. 2 B). CD54 binds to both LFA-1 and CR3 (21, 35). Complete inhibition of neutrophil-CD54 interactions therefore requires the simultaneous use of mAbs directed against both antigens (Fig. 2 C). Although NIF did not inhibit neutrophil binding to CD54 when used alone, it abolished this binding when combined with an anti-CD11a mAb (Fig. 2 C).

CR3 is the Receptor for NIF in Neutrophils

The structural basis for the ability of rNIF to inhibit CR3-mediated functions in neutrophils was explored. Although a previous study showed that NIF binds to neutrophil CR3, it did not evaluate the subunit binding specificity, and did not establish whether CR3 is the only receptor for NIF on the neutrophil surface. To address the first question, we probed western blots of heterodimeric CR3 immunoprecipitated from unlabeled neutrophils with biotinylated rNIF, and compared the pattern with biotinylated CR3 (generated by surface biotinylation of neutrophils). This analysis showed that rNIF binds to the CD11b but not the CD18 subunit of the CR3 heterodimer (Fig. 3, A and B). rNIF did not bind to the other two $\beta 2$ integrins CD11a or CD11c expressed on neutrophils (Fig. 3, A and B).

To determine if CR3 is the only receptor on the neutrophil surface that binds to NIF, we evaluated several anti-CD11b mAbs known to inhibit CR3 functions for their ability to block the binding of biotinylated NIF to neutrophils. As shown in Fig. 3 C, the mAb 107 inhibited NIF binding to neutrophils completely. Two other anti-CD11b mAbs 44 and 904 and the anti-CD11a mAb (TS1/22) had no inhibitory effects.

rNIF Binds to the CD11b A-domain

Recent evidence has shown that r11bA binds directly to iC3b (58), fibrinogen and CD54 (61). The fact that NIF inhibited neutrophil binding to these ligands (Fig. 3) and that NIF binding to neutrophils was blocked by a mAb raised against r11bA (Fig. 3 C) led us to examine the ability of NIF to bind directly to this domain. r11bA and r11aA were expressed as GST fusion proteins, used as such or after thrombin cleavage (36, 58). Each recombinant protein reacted with several blocking monoclonal antibodies (44, 904, OKM9 and 107 in the case of the r11bA, and TS1/22 and L1 in the case of the r11aA (58; and data not shown).

Biotinylated rNIF bound directly and specifically to immobilized r11bA (Fig. 4, A and B). Binding of rNIF to this domain was characterized by a rapid on rate (Fig. 4 A), and a slow off rate (Fig. 4 A, *inset*), that were almost identical to those characterizing NIF binding to whole neutrophils (Fig. 1 A). NIF binding to immobilized rA-domain was specific and saturable. Scatchard analysis of this binding

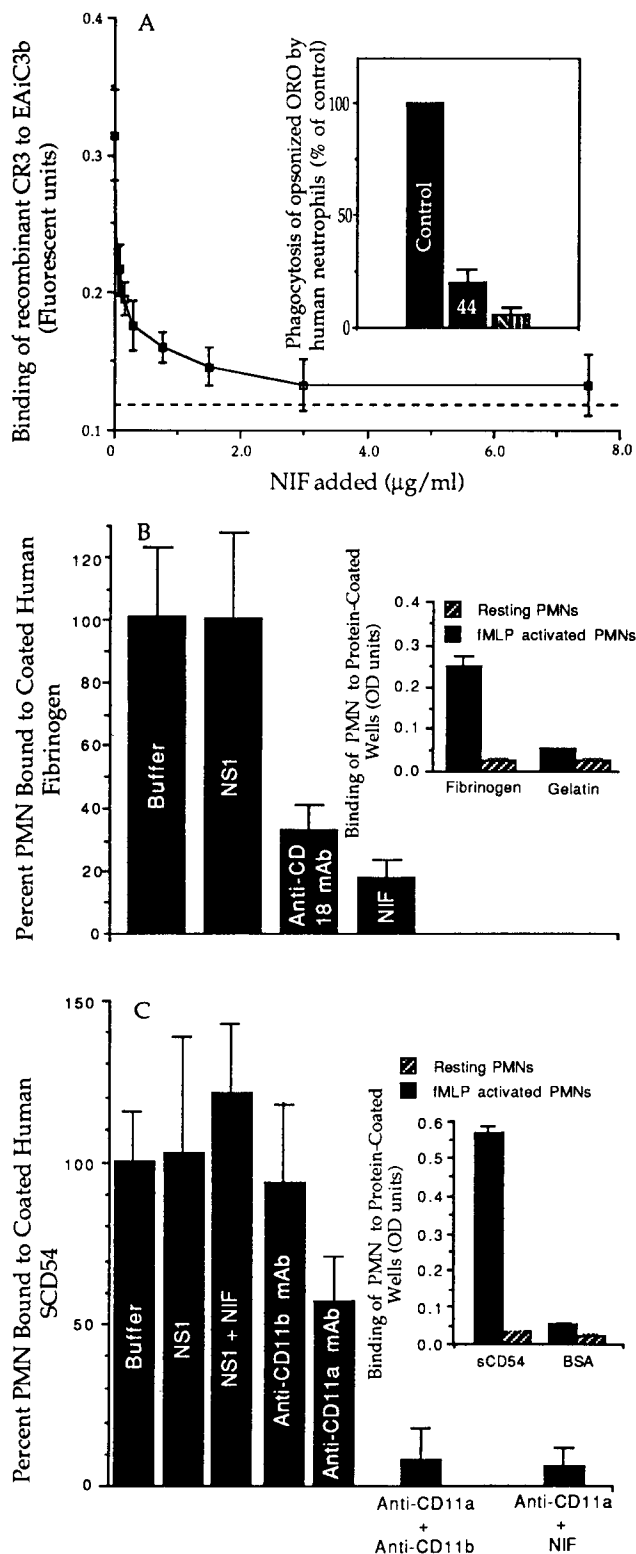


Figure 2. (A) rNIF-induced inhibition of binding of EAiC3b to COS cells expressing rCR3. Values represent the mean \pm SD of three independent experiments each carried out in triplicate. Dotted line represents background binding of EA to rCR3. (Inset) Histograms (mean \pm SD, $n = 4$ determinations) showing the inhibitory effects of rNIF or an anti-CD11b mAb (44) on ingestion of serum-opsonized paraffin oil red O (ORO) by human neutrophils. Ingestion rate in buffer alone was 0.44 ± 0.12 mg ORO/ 10^5 cells/min, mean \pm SD, $n = 4$ determinations). (B) Histograms (mean \pm SD of three independent experiments each carried out in triplicate)

yielded an apparent K_d of ~ 1 nM (Fig. 4 B, inset), similar to that obtained when the neutrophil-bound native CR3 was used (Fig. 1 B). In western blots, biotinylated NIF bound directly to r11bA but not to r11aA (Fig. 4 C), and binding to r11bA was inhibited completely by the mAb 107, and partially by OKM9, but not by 44, 904 or TSI/22 (Fig. 4 D), indicating the specificity of r11bA-NIF interactions.

NIF Binding to Recombinant 11b A-domain Is Metal Dependent

The CD11b A-domain has been shown to contain a discontinuous divalent cation binding site(s) that is essential for the binding of CR3 to iC3b (36, 58). To determine if NIF interaction with r11bA is cation dependent, we examined the role of divalent cations on this interaction. As can be seen in Fig. 5 A, binding of NIF to immobilized r11bA required divalent cations, as it was blocked in the presence of EDTA. EDTA was also able to completely reverse r11bA-NIF interaction even when added 1 h after the complex is formed (Fig. 5 A). NIF bound to r11bA in VBSG $^-$ buffer under these conditions, and binding was not significantly affected by Chelex treatment of VBSG $^-$ (not shown) or by addition of Ca^{2+} , Mg^{2+} , or Mn^{2+} each at 1 mM (Fig. 5 A). Addition of EGTA at 1 mM to the VBSG $^-$ buffer reduced NIF binding only marginally, indicating that binding can occur in the absence of Ca^{2+} . As the other cations (e.g., Mg^{2+} , Mn^{2+}) cannot be selectively chelated, we cannot exclude that binding of NIF to r11bA can occur in presence of Ca^{2+} alone. Since binding is abolished by EDTA, trace amounts of other divalent cations (derived from the buffer salts, gelatin, glucose, or BSA) are essential. The divalent cations appear to be required at least at the level of the A-domain, since the mutant r11bA (D140GS/AGA) that lacks the metal binding site(s) did not bind NIF even in the presence of 1 mM each of Ca^{2+} and Mg^{2+} (Fig. 5 A). Binding of NIF to the A-domain was not affected by temperature (Fig. 5 A) as in whole cells. Fluid-phase r11bA, but not its fusion partner GST, abolished biotinylated rNIF binding to human neutrophils (Fig. 5, B and C) or to immobilized r11bA (Fig. 4 D; and data not shown) in a dose-dependent manner, with half-maximal inhibition seen at ~ 1 nM in each case, reflecting the lack of significant structural differences between the adsorbed and soluble forms of r11bA.

showing the effect of NIF or the anti-CD18 mAb (TS1/18) on binding of activated neutrophils to immobilized fibrinogen. Binding in the presence of buffer alone is expressed as 100%. The ascites control NS1 had no effect on neutrophil binding to fibrinogen. (Inset) Histograms showing the binding of unstimulated (hatched histograms) or f-met-leu-phe-treated neutrophils (black histograms) to immobilized fibrinogen and gelatin. (C) Effect of the mAbs 44 (anti-CD11b), TSI/22 (anti-CD11a), the negative control ascites NS1 and NIF, on activated neutrophil binding to immobilized soluble CD54. Each histogram represents mean \pm SD of three independent experiments each in triplicate. (Inset) Comparison of the binding of resting (hatched histograms) and f-met-leu-phe-treated neutrophils (black histograms) to CD54 and to BSA. Each histogram represents the mean \pm SD of three independent experiments each carried out in triplicate.

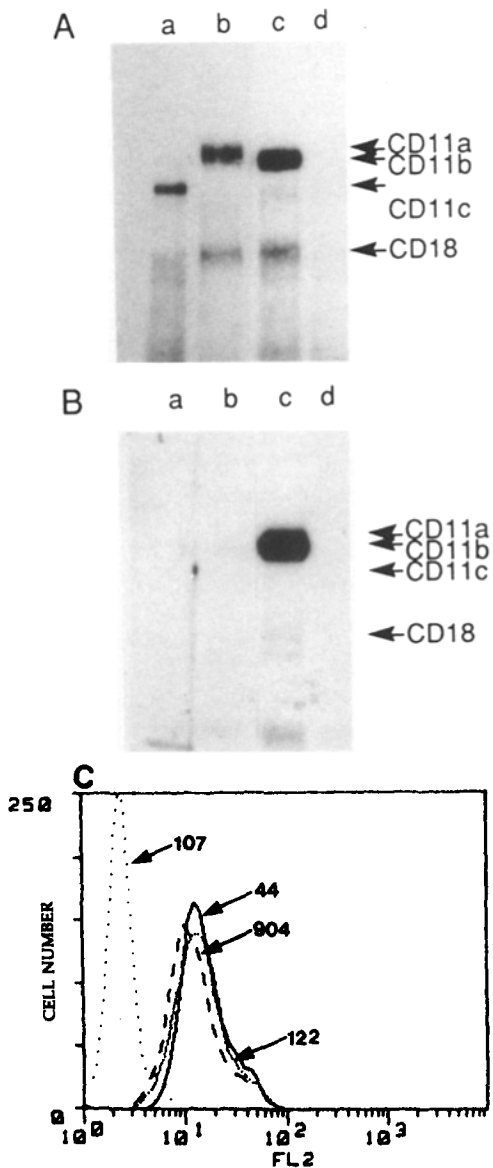


Figure 3. (A) $\beta 2$ integrins expressed on neutrophils. Radioautograph of a Western blot showing the biotinylated $\beta 2$ integrin α subunits (CD11a (lane b), CD11b (lane c), CD11c (lane a), and the common β subunit CD18 (lanes a-c) immunoprecipitated from surface-biotinylated neutrophils. Lane d is a negative control representing immunoprecipitates from biotinylated neutrophils using the nonreactive NS-1 ascites. (B) Radioautograph of a Western blot run in parallel to the one in A, in which unlabeled $\beta 2$ integrins were immunoprecipitated from neutrophil lysate and probed with biotinylated rNIF. The latter bound to CD11b (lane c) but not CD11a, CD11c, or the common CD18 subunit (arrows). Binding was done in the presence of the divalent cations CaCl_2 plus MgCl_2 each at 1 mM. (C) FACS[®] analysis showing that binding of biotinylated rNIF to resting human neutrophils in the presence of the control anti-CD11a mAb TS1/22 [122], and the anti-CD11b A-domain mAbs 44, 904, and 107. 107 reduced staining to the background levels obtained by omission of biotinylated rNIF (tracing not shown).

Mapping of the rNIF Binding Site in the CD11b A-domain

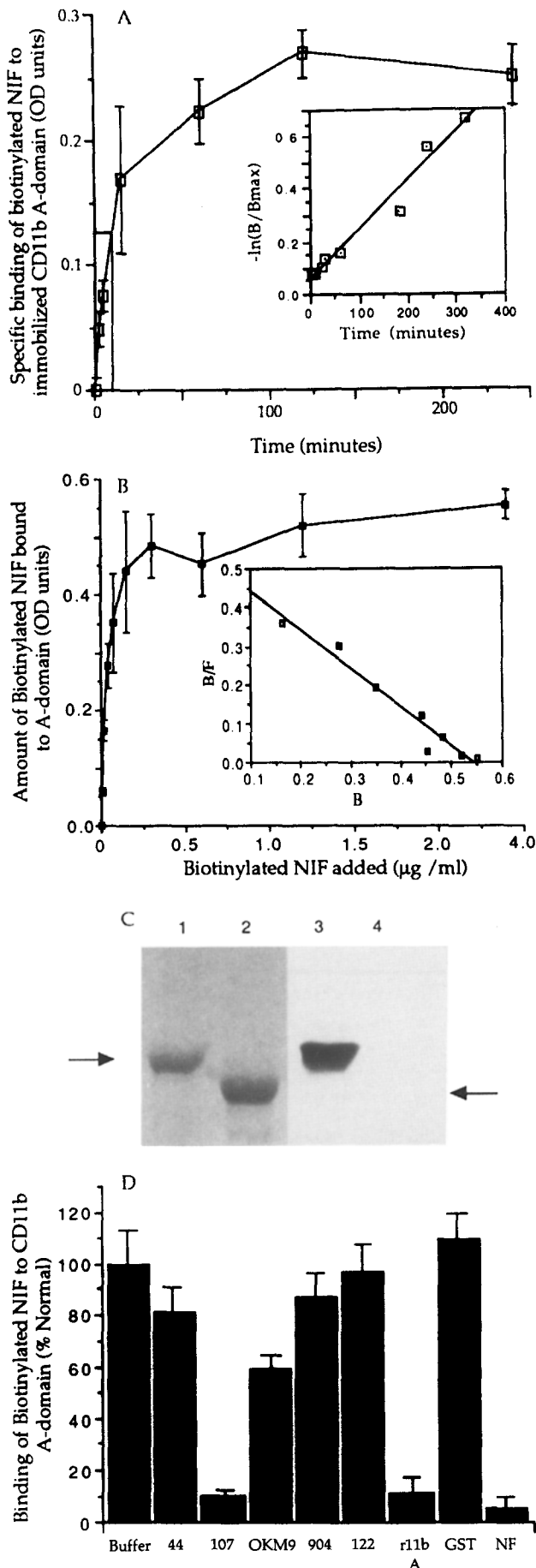
To identify the site in r11bA involved in NIF binding, 11 overlapping peptides spanning the A-domain were synthesized and tested for their ability to inhibit NIF binding to immobilized r11bA. We found that the two contiguous peptides (A6 and A7) inhibited binding of rNIF to rCD11b A-domain dramatically (Fig. 6 B). A scrambled form of A7 had no such effect (Fig. 6 B). Two additional peptides (A1 and A12), located at the beginning and end of the domain had moderate and weak inhibitory effects respectively (Fig. 6 B). Dose-response curves (Fig. 6 C) revealed that while combining A6 and A7 each at 161 $\mu\text{g/ml}$ (80–115 μM) achieved complete inhibition of biotinylated NIF binding to r11bA, addition of A1 (but not A12) produced a shift in the binding curve to the left suggesting that A1 within the recombinant A-domain also contribute to NIF-r11bA interaction (Fig. 6 C). Some peptides (A7, A7M, A3, A11) adsorbed well to microtiter plates, allowing an assessment of the direct binding of rNIF to these peptides. As can be seen in Fig. 6 C (inset) biotinylated rNIF bound to immobilized A7 peptide but not to A3 and A11. Binding of NIF to A7 was not affected when the aspartate residue at position 242 (involved in metal coordination in r11bA and CR3) is replaced with alanine (Fig. 6 C, inset). Direct binding of rNIF to A6, A1, A12 could not be tested because these peptides did not adsorb adequately to plastic wells.

Discussion

The major finding in this report is that the hookworm-derived NIF is a specific CR3 antagonist, that binds to neutrophils through the CD11b A-domain and inhibits their ability to recognize several CR3 ligands and to mediate phagocytosis. The binding of NIF to the CD11b A-domain is selective, of high affinity and divalent cation-dependent. The NIF binding site in r11bA partially overlaps that of human iC3b, the major complement C3 opsonin.

Evidence supporting that CR3 is the sole receptor on the neutrophil surface for NIF is based on four types of experiments. First, binding of biotinylated NIF to intact cells was completely blocked by an anti-CR3 mAb (Fig. 3 C). Second, probing western blots of detergent extracts from normal or $\beta 2$ integrin-deficient (3) neutrophils with biotinylated NIF revealed a single specific band, that of CD11b, in normal cell lysates, that was lacking in the genetically deficient cells (not shown). Third, of the three $\beta 2$ integrins immunoprecipitated from normal neutrophils, only the CD11b subunit reacted with biotinylated NIF in Western blots (Fig. 3, A and B). NIF bound to neutrophil CR3 with high affinity (nM range) and inhibited the binding of neutrophils to the CR3 ligands iC3b, fibrinogen and CD54 (Figs. 1 and 2). Fourth, soluble r11bA completely blocked the binding of biotinylated NIF to neutrophils. These findings indicate that NIF is a highly selective CR3 antagonist.

Previous studies have identified several naturally occurring proteins, so-called disintegrins, that bind to other integrins with high affinity and block integrin-mediated adhesion (reviewed in reference 43). Disintegrins isolated from leeches and snake venoms inhibit adhesion-dependent functions such as platelet aggregation when present in low



nanomolar concentrations. The majority of disintegrins contain the tripeptide Arg-Gly-Asp and have so far been shown to bind to integrins lacking the A-domain (for example some members of the $\beta 1$, $\beta 3$, and $\beta 5$ integrins). Disintegrins interact with their respective receptors through a disintegrin domain, a ~ 60 -amino acid motif with a characteristic cysteine-rich profile (25). NIF neither contains an Arg-Gly-Asp sequence, nor the disintegrin motif (40). The unique structure of NIF probably reflects different structural requirements for antagonists targeting A-domain-containing integrins. It is interesting to note that the physiologic ligands of CR3 such as iC3b, fibrinogen, and CD54 do not contain or do not require an Arg-Gly-Asp sequence (2, 53, 55). The disintegrin motif has been recently found in several cellular proteins expressed on sperm cells (13), epididimal epithelial cells (42), monocytes (39), and appears to play important roles in fertilization and tumor suppression (22). NIF may similarly contain a novel motif with cellular counterparts functioning perhaps in regulating important physiologic interactions. Identification of the active site in NIF involved in integrin binding should be very useful in this regard.

The binding site of NIF in CR3 is the A-domain. This conclusion is based on the following observations. First, NIF bound to r11bA directly, specifically and with kinetics and affinity very similar to that in whole neutrophils (compare Fig. 1 and 4, A and B). Second, binding of NIF to immobilized r11bA was blocked by the anti-CD11b A-domain mAb 107 or with excess unlabeled fluid-phase r11bA (Fig. 4 C). Third, fluid-phase r11bA completely blocked the binding of biotinylated NIF to intact neutrophils (Fig. 5, B and C). The A-domain was first identified in von Willebrand factor, a multimeric protein that plays essential roles in platelet adhesion and hemostasis (59). Cloning studies later showed that this domain is also present in single or multiple copies in several otherwise structurally unrelated proteins including matrix proteins (cartilage matrix protein, collagen VI, XII, and XIV), plasma proteins (complement factors C2 and B, inter- α -trypsin inhibitor), and cell surface proteins (CD11a, b, c, d/CD18, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha \text{IEL}\beta 7$, malarial TRAP and hypothet-

Figure 4. (A) Time course of the specific association at RT of biotinylated rNIF with immobilized CD11b A-domain. Specific binding was obtained as described in the legend to Fig. 1 B. Each time point represents mean \pm SD of triplicate determinations. Similar results were observed in a second experiment. (Inset) Time course of dissociation of bound rNIF from immobilized CD11b A-domain. See Materials and Methods for experimental protocol and calculations. (B) Specific binding (total binding minus binding in the presence of 100-fold molar excess of unlabeled NIF) of biotinylated rNIF to immobilized r11bA. (Inset) Scatchard plot of the specific binding data shown in B. (C) Coomassie stain (lanes 1 and 2) and radioautograph of a Western blot (lanes 3 and 4) showing the binding of biotinylated rNIF to rCD11b- (lanes 1 and 3), but not to rCD11a (lanes 2 and 4) A-domain fusion protein. Lanes 1 and 2 show that equivalent amounts of both fusion proteins were used. Binding was done in the presence of the divalent cations CaCl_2 plus MgCl_2 each at 1 mM. (D) Effect of mAbs or peptides on the binding of biotinylated NIF to immobilized r11bA. Each histogram represents the mean \pm SD of three independent experiments each carried in triplicate. The mAbs used were 44, 904, 107, OKM9 (against CD11b), and TSI/22 (anti-CD11a, as a negative control). Fluid-phase r11bA and GST were used at 7 $\mu\text{g/ml}$. Unlabeled NIF was used at 100-fold molar excess.

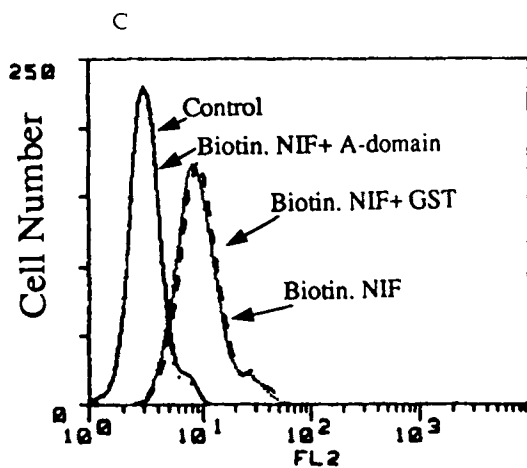
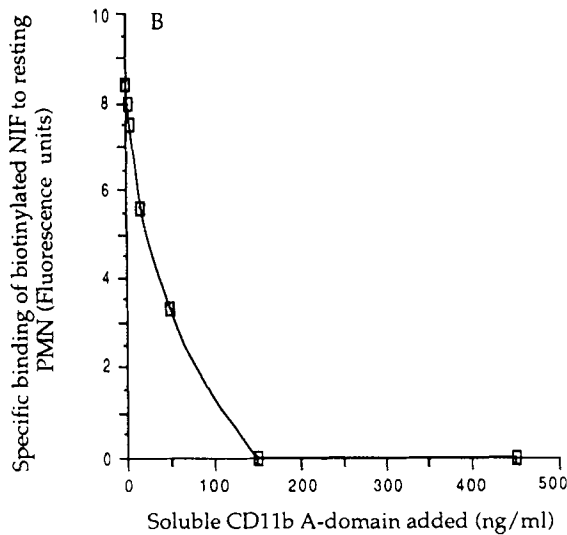
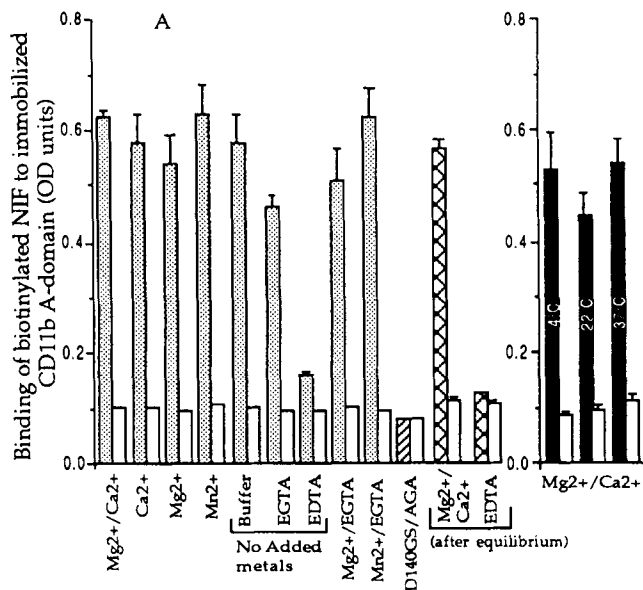


Figure 5. (A) Histograms (mean \pm SD) from three experiments each done in triplicate, showing the effects of divalent cations and temperature on binding of biotinylated rNIF to immobilized r1IbA wild-type or mutant D140GS/AGA. In each case binding in the

ical protein 337.6 of *Caenorhabditis elegans* (reviewed in references 18, 47). The A-domain thus appears to be the product of a primordial gene that served some primitive recognition function, and later incorporated into several proteins to mediate specialized adhesive functions. The studies reported here are the first to identify a naturally occurring integrin antagonist directed exclusively to the integrin CD11b A-domain. It has recently been shown that *Echo virus I*, a picorna-virus, uses the A-domain-containing integrin CD49b/CD29 ($\alpha 2\beta 1$) for cell attachment (11). Given the fact that several ligand binding activities in $\beta 2$ integrins map to their A-domains, it will be of interest to determine if *Echo virus I* also uses the CD49b A-domain to infect the host.

The interaction of NIF with the r1IbA (Fig. 5 A) as well as with whole cells (data not shown) requires divalent cations. Binding in either case did not occur in the presence of EDTA, and was reversed even when EDTA was added after the receptor-ligand complexes have formed (Fig. 5 A). A recent study has shown that the CD11b A-domain is a metalloprotein containing a divalent cation binding site(s) formed by two noncontiguous segments of the protein (36). Point mutations (D140GS/AGA and D242A) affecting two separate regions in r1IbA blocked ^{54}Mn binding to this protein and interfered with iC3b binding to mutant CR3 (36, 58). As in the case of iC3b, biotinylated NIF did not bind to mutant D140GS/AGA r1IbA (Fig. 5 A), indicating that binding of these two ligands requires an intact metal binding site in the A-domain. While the divalent cation requirements for fibrinogen and CD54 binding to the A-domain remain to be determined (46, 61), binding of several mAbs (such as 44, 904, and OKM9) to r1IbA is unaffected by removal of cations (data not shown). The fact that divalent cations are required for the binding of NIF as well as iC3b to r1IbA, and that this requirement disappears when synthetic peptides are used, suggest that cations play an indirect role in ligand binding, perhaps stabilizing a specific permissive conformation. Solution of the three dimensional structure of the A-domain will be important in evaluating this possibility.

CR3 exists in multiple cellular compartments in phagocytic cells. These include the specific (8, 57) and tertiary

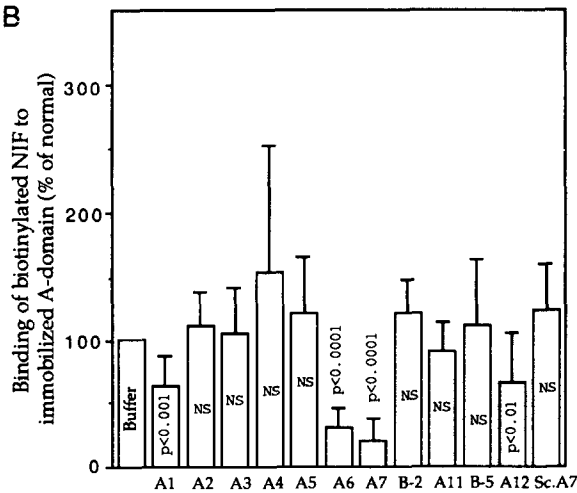
presence of 100-fold excess of unlabeled NIF is shown as white histograms. The dotted histograms show binding in VBSG⁻ (buffer) alone, or containing 1 mM Ca²⁺ plus 1 mM Mg²⁺ (Mg²⁺/Ca²⁺), 1 mM Ca²⁺, Mg²⁺, or Mn²⁺, 1 mM EDTA, 1 mM EGTA, 1 mM Mg²⁺ and 1 mM EGTA, or 1 mM Mn²⁺ and 1 mM EGTA. Crossed histograms show that preformed rNIF-immobilized r1IbA complexes are maintained as long as cations are present but rapidly dissociate if EDTA is added. Hatched histogram represent biotinylated NIF binding to immobilized mutant D140GS/AGA r1IbA (in Mg²⁺/Ca²⁺ buffer). The black histograms represent binding of biotinylated NIF to r1IbA at different temperatures in the presence of Mg²⁺/Ca²⁺-containing buffer. (B) Dose-dependent inhibition of biotinylated NIF binding to neutrophils by increasing amounts of fluid-phase r1IbA. Labeled NIF was used at a concentration that produced 50% binding to resting neutrophils. Similar results were obtained in a second experiment. (C) FACS[®] analysis showing the binding of biotinylated rNIF (used at the half-saturation concentration) to resting human neutrophils (Biotin. rNIF) and its complete inhibition by fluid-phase r1IbA (150 $\mu\text{g}/\text{ml}$) (dotted lines). No inhibition is observed with GST (slashed line). The negative control was obtained by omission of biotinylated rNIF.

A

Peptide	Amino acid sequence	Residue No.
A1:	CPQEDSDIAF LIDGSGSITP	(128-147)
A2:	IIPHFERRMK EIVSTVMEQL	(145-164)
A3:	EQLKKSITLF SLMQYSEEF	(162-181)
A4:	EFRIHETFE FQNNPNRSL	(179-198)
A5:	RSLVKEITQL LGRTHIATGI	(196-215)
A6:	TGIRKWRREL FNTINGARKN	(213-232)
A7:	NAFKILWIT DGEK	(232-245)
B-2:	DGEKFGDPLG YEDVIPEADR	(242-261)
A11:	DREGVIRYVI GVGDAFR	(260-276)
B-5:	FRSEKSRQEL NTIASKPPRD HV	(275-296)
A12:	HVFQVNNFEA LKTIQNQLRE	(295-314)

A7M: NAFKILWIT aGEK
Scram. A7: TVDLKFGIKN IEAV

B



C

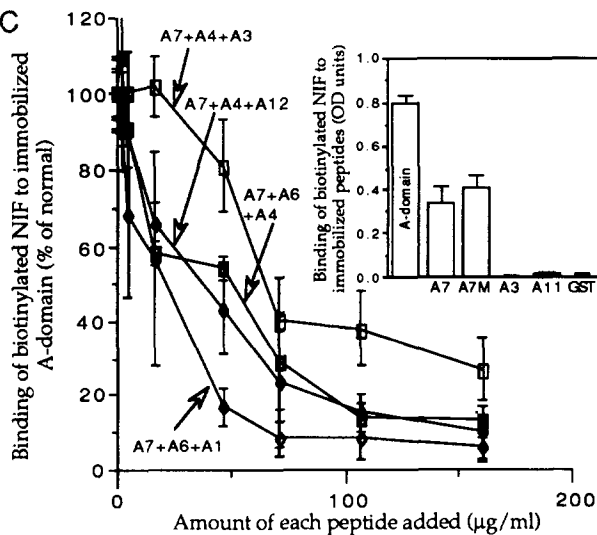


Figure 6. (A) Sequence of the synthetic peptides used in this study. The name and position of the overlapping peptides synthesized based on the amino acid sequence of CD11b (6) is shown. Scram. A7 is scrambled sequence of A7. A7M is a mutant form of A7 where the Asp. residue is replaced synthetically with Ala. (B) Histograms (mean \pm SD, $n = 3$, each in triplicate) showing the effects of CD11b A-domain-derived synthetic peptides (at 140 μ M) on binding of biotinylated rNIF (2.5 nM) to immobilized r11bA. (C) Dose-

granules (gelatinase granules) (28) in neutrophils, peroxidase-negative granules in monocytes (37), and secretory vesicles in both cell types (30, 50). In unstimulated neutrophils, $\sim 5\%$ of CR3 is present on the plasma membrane, $\sim 75\%$ is in the specific and tertiary granules, and $\sim 20\%$ is in secretory vesicles which also contain cytochrome b_{558} , CR1, f-met-leu-phe receptors, alkaline phosphatase and plasma proteins such as albumin (51). Activation of phagocytic cells with several agonists leads to the rapid translocation of CR3 from these pools to the cell surface, and an increase in its numbers by up to 10-fold (8). In addition, CR3 is also internalized in macrophages (15) and in transfected COS cell lines (45), and actively recycles in human neutrophils (16). The rapid redistribution of CR3 may be important in replenishment of receptors lost (perhaps through endocytosis) from the leading front to the cell's uropod during neutrophil migration (23). Our findings that monomeric fluid-phase rNIF binds to both the inactive and active forms of CR3 with equal and high affinity, and in an EDTA-reversible manner are unique. These properties could be invaluable in probing the dynamics of CR3 recycling, in determining if some of the contents of secretory vesicles accumulate through a CR3-dependent endocytic pathway, similar to the one described in platelets (27), and in evaluating the competence of the A-domain metal binding state in internalized CR3.

The binding site for NIF in the CD11b A-domain is broad, comprised primarily of two centrally located overlapping peptides A6 and A7, with additional contribution by two peptides located at the beginning (A1) and end (A12) of the domain. These data predict that A6, A7, A1 and A12 are spatially close in the three-dimensional structure of the domain, perhaps lying on one side of the protein. A7 has previously been found to bind to iC3b, suggesting that the NIF binding site overlaps with that of iC3b. This may explain the ability of NIF to completely block the binding of this ligand to rCR3 (Fig. 2 A) as well as to r11bA (data not shown). The fact that NIF inhibits CD54 binding to neutrophil CR3 completely (Fig. 2 C) also suggests common or overlapping binding sites for iC3b, CD54, and NIF. Inhibition of CR3-fibrinogen binding by NIF was partial (Fig. 2 B). This finding could reflect differences in the binding sites for iC3b, CD54, and fibrinogen in r11bA. It remains possible however that fibrinogen may bind to CR3 at more than one site, or through an additional receptor. Detailed studies evaluating the kinetics, metal requirements and conditions under which the binding of CD54 and fibrinogen to r11bA takes place, and mapping and mutagenesis studies to define the binding sites will be required to evaluate these possibilities. The broad interactive region between r11bA and NIF may account for the high selectivity of this protein to CD11b but not CD11c (which binds iC3b) (41): although the A7 region is highly conserved in both receptors, the regions encoding A1, A6, and A12 are less conserved.

response curves (mean \pm SE, three experiments each in triplicate) showing the effects of combinations of A6, A7, A1, and A12 on biotinylated NIF binding to immobilized r11bA. (Inset) Histograms (mean \pm SD, $n = 3$, each in triplicate) showing the direct binding of biotinylated rNIF (400 nM) to the immobilized peptides A7, A7M. Binding to r11bA is shown for comparison. Controls consisted of GST, and the A-domain-derived peptides A3 and A11.

Hookworms infect more than 700 million people and cause loss of ~7 million liters of blood daily worldwide (44). The larvae penetrate skin, migrate through the lungs, are coughed up, and then swallowed. In the intestine, they mature into adult worms, and persist within the host for many years. The major clinical manifestations of hookworm disease are due to chronic gastrointestinal blood loss. Gross and microscopic examination of the bowel itself reveals conspicuously little damage (44). The intestinal wall is usually thickened by edema, principally localized to the submucosa, with minimal infiltration of the infested region by neutrophils (38), suggesting that hookworms are able to evade the host's immune defenses. By locally producing a factor that blocks CR3-mediated functions in neutrophils, hookworms may be able to prevent neutrophil extravasation into infected regions, and the destruction of the parasites through their phagocytic and killing abilities. Because r1IbA inhibits NIF binding to leukocytes in the low nM range whereas its inhibition of iC3b binding to the same cells requires micromolar concentrations (58), it is conceivable that the r1IbA may be useful as such or in a modified form in treating hookworm infections, without producing generalized immunosuppression. In addition, the present study highlights the integrin A-domain as a specific target in development of novel anti-inflammation therapeutics, that could be very beneficial in mitigating phagocyte and complement-induced tissue injury during immune and inflammatory disorders (3), a lesson long learned by the hookworm.

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