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Antibodies That Selectively Inhibit Leukocyte Function-associated Antigen 1 Binding to Intercellular Adhesion Molecule-3 Recognize a Unique Epitope within the CD11a I Domain*

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Several studies indicate that the I domain located in the α chain (CD11a) of leukocyte function-associated antigen-1 (LFA-1; CD11a/CD18) plays an essential role in ligand recognition. We recently identified three distinct epitopes (IdeA, IdeB, and IdeC) within the CD11a I domain, recognized by antibodies that block binding of LFA-1 to intercellular adhesion molecules (ICAM) 1, 2, and 3. In the present study, we used a series of human/murine CD11a I domain chimeras, to localize a fourth I domain epitope (IdeD), recognized by three independently derived anti-CD11a antibodies that selectively block the binding of LFA-1 to ICAM-3, but not to ICAM-1. The IdeD epitope depended on human CD11a residues Asp[182] and Ser[184] and was not present in CD11b or CD11c. Although mutation of Asp[182] and Ser[184] failed to abolish ICAM-3 adhesion of LFA-1 transfectants, alignment of these residues with the crystal structure of the CD11a I domain suggested that the IdeD epitope is located in close proximity to residues (Ile[126] and Asn[129]) recently implicated in the ICAM-3 binding site (1). Interestingly, the IdeB and IdeC epitopes appeared to be in close proximity of a divalent cation binding pocket within the CD11a I domain that regulates both ICAM-1 and ICAM-3 adhesion. Taken together, these data indicate that distinct regions of the CD11a I domain contain epitopes for antibodies that either selectively inhibit binding of LFA-1 to ICAM-3, or interfere with both ICAM-1 and ICAM-3 binding of LFA-1.

The leukocyte integrin LFA-1[1] (CD11a/CD18) is a cell surface receptor that mediates adhesive interactions and signal transduction in the immune system (2–5). LFA-1 is expressed by leukocytes and belongs to the β_2 family of integrins, in which a common β subunit (CD18) is associated with any of three distinct, but structurally homologous, α subunits; α_L (CD11a, LFA-1), α_M (CD11b, Mac-1), and α_X (CD11c, p150, 95) (2). The extracellular domain of the LFA-1 α subunit contains two domains thought to be of functional significance. These include a putative divalent cation binding region, consisting of three tandem repeats of an EF-hand motif, also found in other integrins (6, 7) and a 200-amino acid inserted or "I" domain (8), which is also present in α_M, α_X, α_1, α_2, and α_E subunits (2, 9). The I domain contains sequences homologous to the type A domains of von Willebrand factor, cartilage matrix-binding protein, and complement factor B (8).

LFA-1 is known to recognize three ligands: ICAM-1 (10), ICAM-2 (11), and ICAM-3 (12–14), all of which are members of the Ig superfamily, and have five, two, and five Ig-like domains, respectively. ICAM-1 is expressed on many cells, including lymphocytes and endothelial cells, and its expression is cytokine inducible (15). ICAM-2 is expressed on lymphocytes, endothelial cells, and platelets (16, 17), whereas ICAM-3 is only expressed on leukocytes (18). In contrast to ICAM-1, expression of ICAM-2 and -3 is not induced by cytokines (18, 19).

LFA-1-mediated adhesion requires activation of the LFA-1 molecule (20–22). Activation can be induced by intracellular signals generated upon cross-linking of cell surface receptors (T cell receptor/CD3) (21, 22), upon binding of activating anti-LFA-1 antibodies (23–27), or divalent cations, such as Mn^{2+} (28). Activation of LFA-1 and subsequent ligand binding is thought to result from conformational changes in the α/β heterodimer and requires binding of divalent cations, such as Mg^{2+} and Ca^{2+}, an intact cytoskeleton, and a physiological temperature (29). While Ca^{2+} binding supports clustering of LFA-1 on the cell surface, presumably resulting in enhanced ligand binding avidity, Mg^{2+} binding to LFA-1 has been suggested to alter the affinity of LFA-1 for its ligands (28, 30).

Recent findings indicate that the I domains of CD11a, CD11b, and CD11c, as well as I domain sequences of the α_1 and α_2 chains of β_1 class of integrins, are involved in ligand binding. Evidence comes from the finding that purified α_L (CD11a), α_M (CD11b), and α_2 I domains directly bind their respective ligands, ICAM-1, fibrinogen and iC3b, or collagen (31–33). Second, mutation of aspartic acid or threonine residues within the I domains of α_M, α_L, α_1, and α_2 affects cation binding and impairs adhesion (33–37). Furthermore, we identified residues Ile[126] and Asn[129] within the CD11a I domain to be critical for adhesion to ICAM-3, but not for ICAM-1 binding, indicating that the CD11a I domain contains distinct binding sites for different ligands (1, 27). Finally, most anti-CD11a, CD11b, and CD11c antibodies that block ligand interactions recognize the I domain (38–40). Previous investigations have revealed that anti-human CD11a antibodies do not cross-react with murine LFA-1, implying that sequences in the I domain important for mAb binding can be located by replacing human CD11a sequences with the murine homologues (40). Using human/mouse I domain mutants in which sequences from the human CD11a

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[1] The abbreviations used are: LFA-1 (CD11a/CD18), leukocyte function-associated antigen 1; ICAM, intercellular adhesion molecule; mAb, monoclonal antibody; H/M, human/murine; MIDAS, metal ion-dependent adhesion site; I domain, inserted domain.

I domain were substituted into murine I domain residues, we recently demonstrated that anti-CD11a I domain antibodies that inhibit the interaction of LFA-1 with ICAM-1, -2, and -3[2] (*e.g.* TS1/22, 25.3, and MHM.24) (39, 40), recognized three distinct epitopes within the CD11a I domain (IdeA, residues 126–129; IdeB, residues 143–148; IdeC, residues 198–204) (40).

In the present studies, we identified a fourth epitope within the CD11a I domain (IdeD), that is recognized by three anti-CD11a antibodies that selectively inhibit the binding of LFA-1 to ICAM-3. Alignment of the IdeD epitope with the recently solved crystal structure of the CD11a I domain (41) suggested that it is located in close proximity to I domain residues Ile[126] and Asn[129], critical for ICAM-3, but not ICAM-1 binding of LFA-1 (1).

EXPERIMENTAL PROCEDURES

Antibodies—A panel of mouse monoclonal antibodies (mAbs) was used, MEM-83 (24), YTH81.5 (39, 42), 122.2A5 (39), NKI-L15 (43), and NKI-L16 (23) directed against CD11a, MHM.23 (44), KIM185 (26), and MEM-48 (27) directed against CD18, REK-1 directed against ICAM-1 (27), AZN-IC3.1 directed against ICAM-3,[3] and T3b directed against CD3 (45).

Generation of CD11a I Domain Mutants—The construction of chimeric human/mouse CD11a I domain variants (H/M48-H/M54) and the generation of the D137A mutant has been described previously (1, 37, 40). The H/M48-H/M54, D137A, and D239A mutants were generated by oligonucleotide-directed mutagenesis using a plasmid containing the entire human CD11a chain (pRKLFAαm), as a template. After each polymerase chain reaction step, clones were checked by sequencing for correct incorporation of oligonucleotides.

Expression of CD11a Mutants in 293 Cells and Immunofluorescence Analysis of Transfectants—Chimeric CD11a and wild type CD18 cDNAs were cloned into the RK 5 and RK 7 expression plasmids and transfected into the 293 human kidney adenocarcinoma cell line, using a standard calcium phosphate coprecipitation method (46). Transfection efficiencies ranged from 30 to 70%. Three days after transfection, transfectants were harvested by EDTA (5 mM) treatment and expression of CD11a/CD18 on the transfectants was determined by immunofluorescence. Cells (2×10^5) were incubated for 1 h at 4 °C with appropriate dilutions of the different mAbs (2 μg/ml) in Hepes buffer (0.02 M Hepes, 0.14 M NaCl, 0.2% glucose, 1 mM $MgCl_2$, and 1 mM $CaCl_2$), followed by incubation with fluorescein isothiocyanate-labeled goat $(Fab')_2$ anti-mouse (Cappel, Inc., West Chester, PA) or anti-rat IgG (Caltag, Los Angeles, CA) antibodies, for 1 h at 4 °C. The percentage of positive cells was determined by FACScan analysis (Becton Dickinson, Mountain View, CA).

Immunoprecipitation of CD11a Variants Expressed in 293 Cells—The method used for immunoprecipitation of CD11a variants has been described in detail elsewhere (37, 40). Briefly, 2 or 3 days after transfection of CD11a chimeras into 293 cells, transfectants were metabolically labeled in methionine-free Dulbecco's modified Eagle's medium with 100 μCi/ml [^{35}S]methionine for 4–5 h. The medium was removed, and the cells were lysed in 1 ml of lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 2 mM $CaCl_2$, and 2 mM $MgCl_2$)/10-cm dish. Cell debris and nuclei were pelleted, and the supernatant was used for subsequent immunoprecipitations. Antibodies (1–2 μg/immunoprecipitation) were incubated with 100 μl of labeled lysate and 2 μg of rabbit anti-mouse IgG (Cappel) for 2 h at room temperature. The antibody/antigen complexes were precipitated using 2 mg of Protein A-Sepharose CL-4B beads (Pharmacia Biotech, Inc., Uppsala, Sweden), the beads were washed two times in wash buffer (0.5% Nonidet P-40, 0.01% sodium dodecyl sulfate (SDS), 400 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2 mM $CaCl_2$, 2 mM $MgCl_2$) and resuspended in 30 μl of 2 × concentrated SDS-polyacrylamide gel electrophoresis sample buffer, boiled for 3 min, and centrifuged briefly. The clarified sample was then resolved on 6% SDS-polyacrylamide gels.

Adhesion Assay—ICAM-1 and ICAM-3 fusion proteins consisting of the five domains of ICAM-1 or ICAM-3 fused to a human IgG1 Fc fragment (ICAM-1Fc and ICAM-3Fc, respectively) were isolated from supernatants of L-cell cultures stably transfected with pICAM-1Fc and pICAM-3Fc, respectively (13, 47). Culture supernatant was purified by protein A column affinity chromatography and eluted by 0.01 M Hepes buffer at pH 7.0, containing 0.15 M NaCl, 3.5 M $MgCl_2$, and 10% (w/v) glycerol. 96-well flat-bottomed plates (Maxisorb, Nunc, Roskilde, Denmark) precoated with 4 μg/ml goat anti-human Fc (Jackson Immunoresearch Laboratories, Inc., Westgrove, PA) for 2 h at 37 °C, and blocked with 1% bovine serum albumin (Boehringer Mannheim) (1 h at RT), were coated overnight at 4 °C with ICAM-1Fc or ICAM-3Fc proteins (300 ng/ml, 50 μl/well). A stable human LFA-1 transfected 293 cell line (293-LFA), or transiently transfected 293 cells (100,000/well or 200,000/well, respectively), were added in adhesion buffer (0.14 M NaCl, 0.2% glucose, 0.02 M Hepes, 1 mM $CaCl_2$, 1 mM $MgCl_2$) in the presence of the indicated mAbs (10–50 μg/ml) and incubated for 1.5 h at 37 °C. Nonadherent cells were removed by three washes with adhesion buffer, and cell attachment was measured using the PNAG method of Landegren (48) Results are expressed at mean OD_{405} values of triplicate wells. For adhesion of the human T cell line HSB (obtained from ATCC), cells (40,000/well) were labeled with ^{51}Cr for 45 min at 37 °C and incubated on ICAM-1Fc- or ICAM-3Fc-coated plates for 30 min at 37 °C. Nonadherent cells were removed by three washes with adhesion buffer, adhering cells were lysed with 1% Triton X-100, and radioactivity was quantified. Results are expressed as the mean percentage of adhesion of triplicate wells.

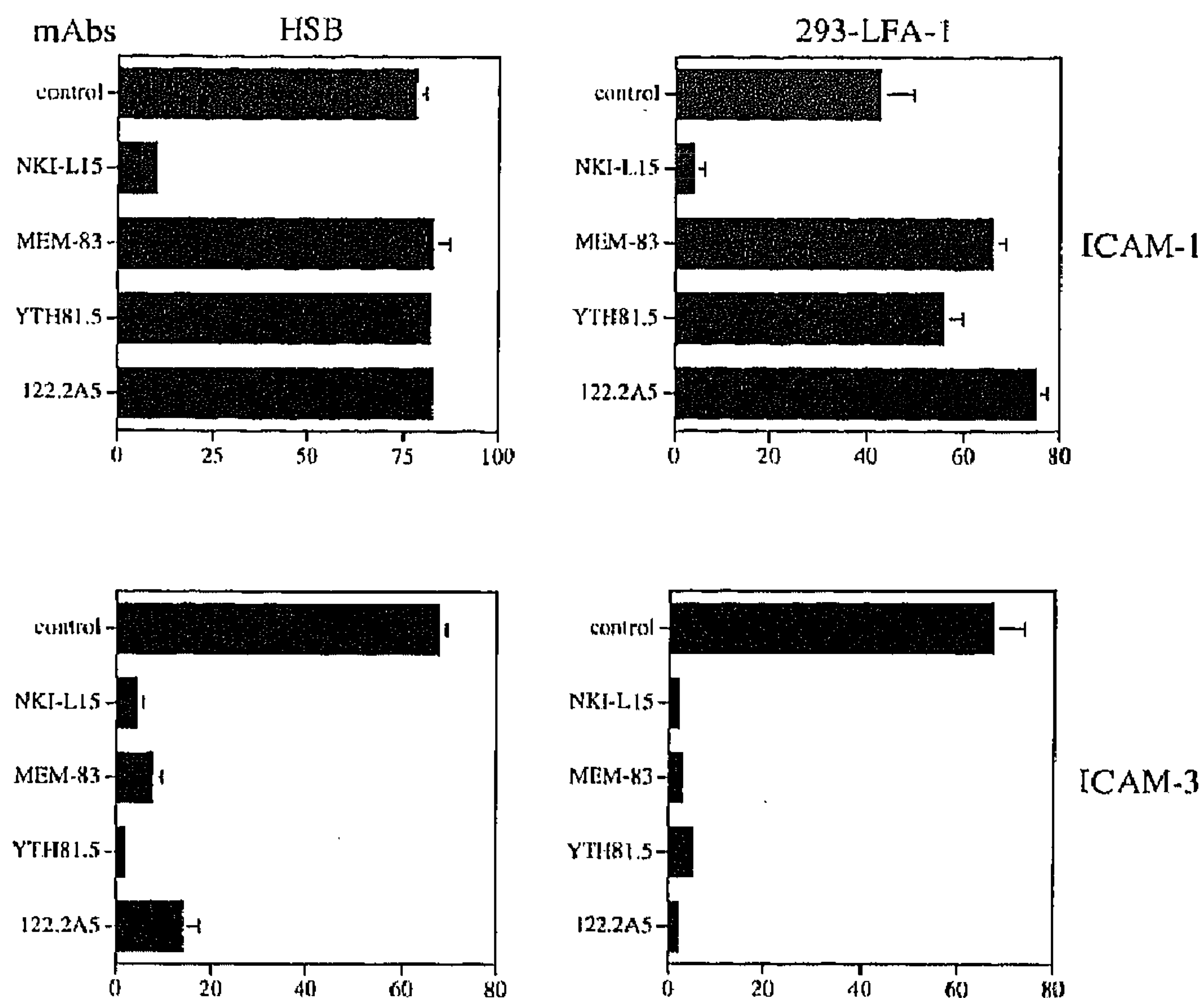

FIG. 1. **The CD11a I domain-specific mAbs MEM-83, YTH81.5 and 122.2A5 selectively inhibit the LFA-1/ICAM-3 interaction.** After activation of LFA-1 with the CD18-activating antibody MEM-48 (5–20 μg/ml), the capacity of HSB T cells or 293 cells transfected with CD11a/CD18 constructs, to bind purified ICAM-1Fc or ICAM-3Fc proteins (300 ng/ml; 50 μl/well) was determined, in the absence of presence of mAbs MEM-83, YTH81.5, 122.2A5, or NKI-L15 (CD11a) at 10–50 μg/ml. Results are expressed as the mean percentage of adherent cells from triplicate wells. One representative experiment out of three is shown.

Proliferation Assay—96 flat-bottomed wells (Maxisorb, Nunc, Roskilde, Denmark) were coated with suboptimal concentrations of anti-CD3 antibodies (T3b, 30 ng/ml, 100 μl/well, 1 h 37 °C), followed by goat anti-human Fc (Jackson Immunoresearch Laboratories, Inc.; 4 μg/ml, 100 μl/well, 1 h at 37 °C), 1% BSA (100 μl/well, 30 min at 37 °C), and ICAM-1Fc or ICAM-3Fc proteins (300 ng/ml, 50 μl/well, 1 h at 37 °C). Resting peripheral blood lymphocytes obtained by Ficoll-Hypaque density centrifugation and subsequent depletion of monocytes by adherence to plastic, were added (100,000 cells/well) and cultured for 3 days. On day 3 cells were pulsed for 16 h with [^{3}H]thymidine (1.52 TBq/mmol, 0.5 μCi/well; Amersham, Buckinghamshire, United Kingdom), to measure ICAM-1- or ICAM-3-dependent proliferation. To determine whether induced proliferation was LFA-1- and ICAM-1- or ICAM-3-specific, cells were cultured in the presence of function blocking antibodies at a concentration of 10 μg/ml.

RESULTS

MEM-83, YTH81.5, and 122.2A5 Selectively Block the Binding of LFA-1 to ICAM-3, but Not to ICAM-1—To characterize

[2] M. E. Binnerts, Y. van Kooyk, and C. G. Figdor, unpublished observation.

[3] M. E. Binnerts, Y. van Kooyk, and C. G. Figdor, manuscript in preparation.

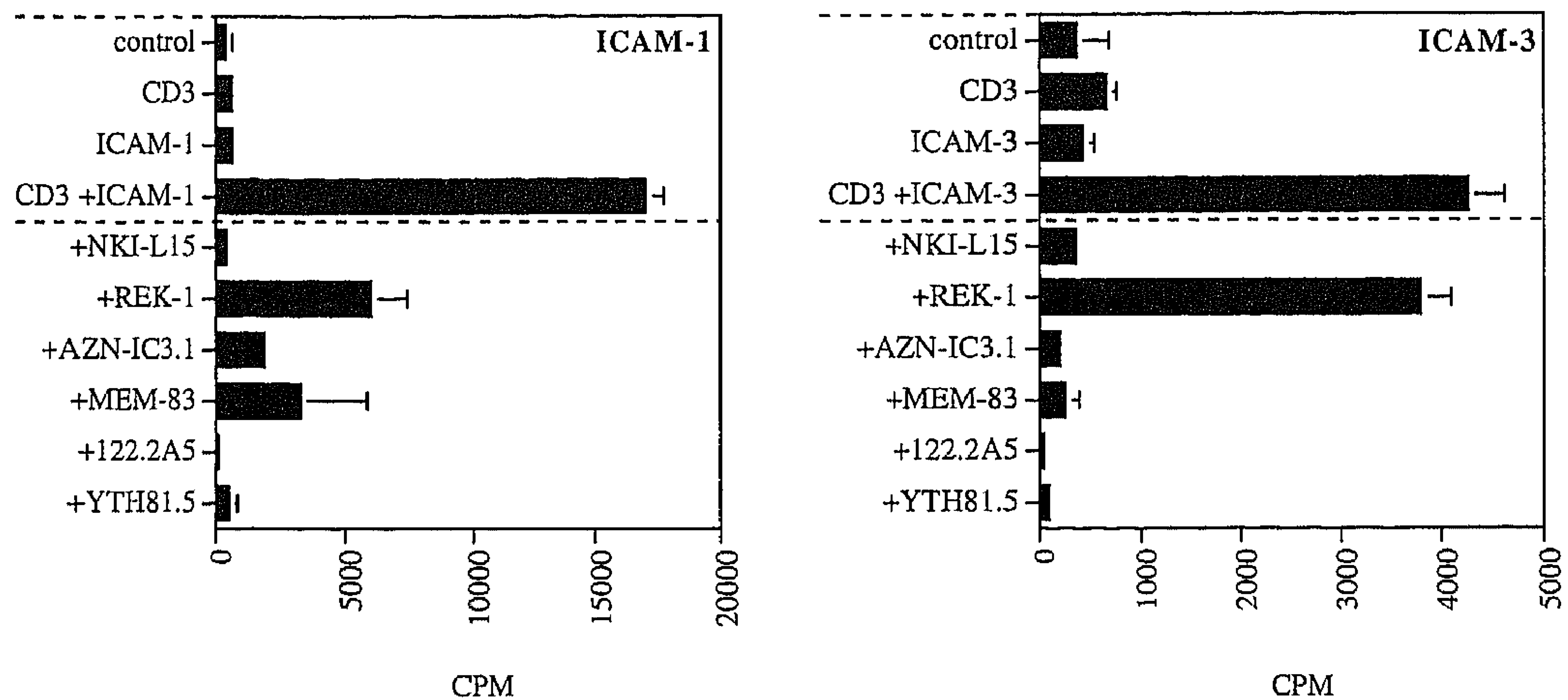

FIG. 2. **MEM-83, YTH81.5, and 122.2A5 inhibit ICAM-3-dependent costimulation of T cells.** Resting T cells were plated on ICAM-1Fc or ICAM-3Fc, together with suboptimal concentrations of coated anti-CD3 antibodies (30 ng/ml; 50 μl/well), in the presence or absence of mAbs MEM-83, YTH81.5, 122.2A5, NKI-L15 (CD11a), REK-1 (ICAM-1), or AZN-IC3.1 (ICAM-3), at 10 μg/ml. After 3 days of culture, ^{3}H incorporation was determined. Results are expressed as the mean counts/min of triplicate wells. Data are representative of three experiments.

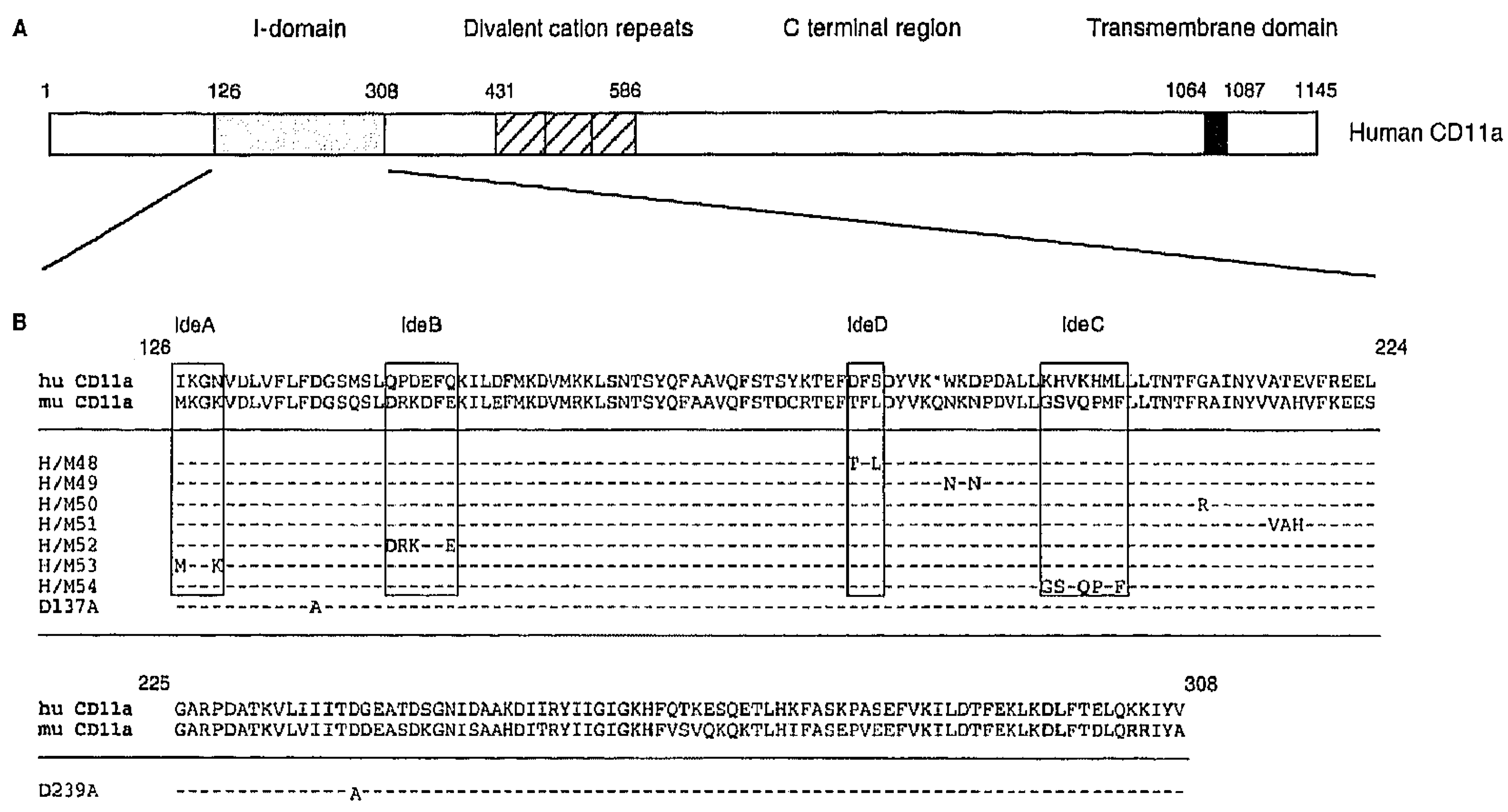

FIG. 3. **Amino acid sequences of murine and human CD11a I domains and human/murine substitution mutants.** *A*, schematic representation of CD11a with respective locations of the I domain and the putative metal binding (EF-hand) domains. *B*, in the human/murine chimeras (H/M48–54), one to five human residues were substituted for murine residues. In D137A and D239A mutants, aspartic acid residues were substituted for alanine.

antibodies that recognize functionally important epitopes within the CD11a I domain, we determined the ability of several anti-CD11a mAbs to selectively inhibit the binding of LFA-1 to ICAM-3 (Fig. 1). The capacity of the LFA-1-positive T cell line HSB and transfected 293 cells (293-LFA-1) to bind purified ICAM-1 (ICAM-1Fc) or ICAM-3 (ICAM-3Fc) coated onto tissue culture plates, in the presence of the anti-CD11a mAbs MEM-83, YTH81.5, 122.2A5, or NKI-L15 was determined. Activation of LFA-1 by an activating anti-CD18 mAb (MEM-48), induced adhesion of both HSB and 293-LFA-1 cells to ICAM-1Fc and ICAM-3Fc proteins. The anti-CD11a mAb NKI-L15 blocked binding to ICAM-1 as well as ICAM-3 (Fig. 1). In contrast, the anti-CD11a mAbs MEM-83, 122.2A5, and YTH81.5 blocked only adhesion to ICAM-3 and not to ICAM-1. Inhibition of LFA-1/ICAM-3 adhesion by these antibodies was also observed when LFA-1 on the HSB cells was activated with phorbol 12-myristate 13-acetate or Mn^{2+} (data not shown).

MEM-83, YTH81.5, and 122.2A5 Inhibit ICAM-3-dependent Costimulation of T Cells—We next determined whether MEM-83, YTH81.5, and 122.2A5 interfered with ICAM-3- or ICAM-1-dependent costimulation of T cells. Resting peripheral blood lymphocytes obtained from healthy donors were plated on ICAM-1Fc or ICAM-3Fc proteins together with suboptimal concentrations of coated anti-CD3 antibodies, in the presence of MEM-83, YTH81.5, or 122.2A5, or in the presence of blocking antibodies directed against LFA-1, ICAM-1, or ICAM-3. Fig. 2 demonstrates that although MEM-83, YTH81.5, and 122.2A5 selectively inhibited LFA-1/ICAM-3 adhesion, they interfered with both ICAM-1- and ICAM-3-dependent T cell proliferation. ICAM-1- and ICAM-3-dependent proliferation was prevented by blocking antibodies against LFA-1 and by antibodies directed against ICAM-1 or ICAM-3, respectively, indicating that proliferation was LFA-1- and ICAM-specific. Interestingly, proliferation on ICAM-1Fc was also prevented by blocking antibodies directed against ICAM-3, suggesting that proliferation on ICAM-1 involves LFA-1/ICAM-3 interactions between proliferating cells. These data may indicate that MEM-83, YTH81.5, and 122.2A5 inhibit proliferation on ICAM-1 Fc, by preventing these LFA-1/ICAM-3 interactions.

Mapping of the Epitope Recognized by MEM-83, YTH81.5, and 122.2A5 within the CD11a I Domain—MEM-83, YTH81.5, and 122.2A5 have previously been shown to bind to the CD11a

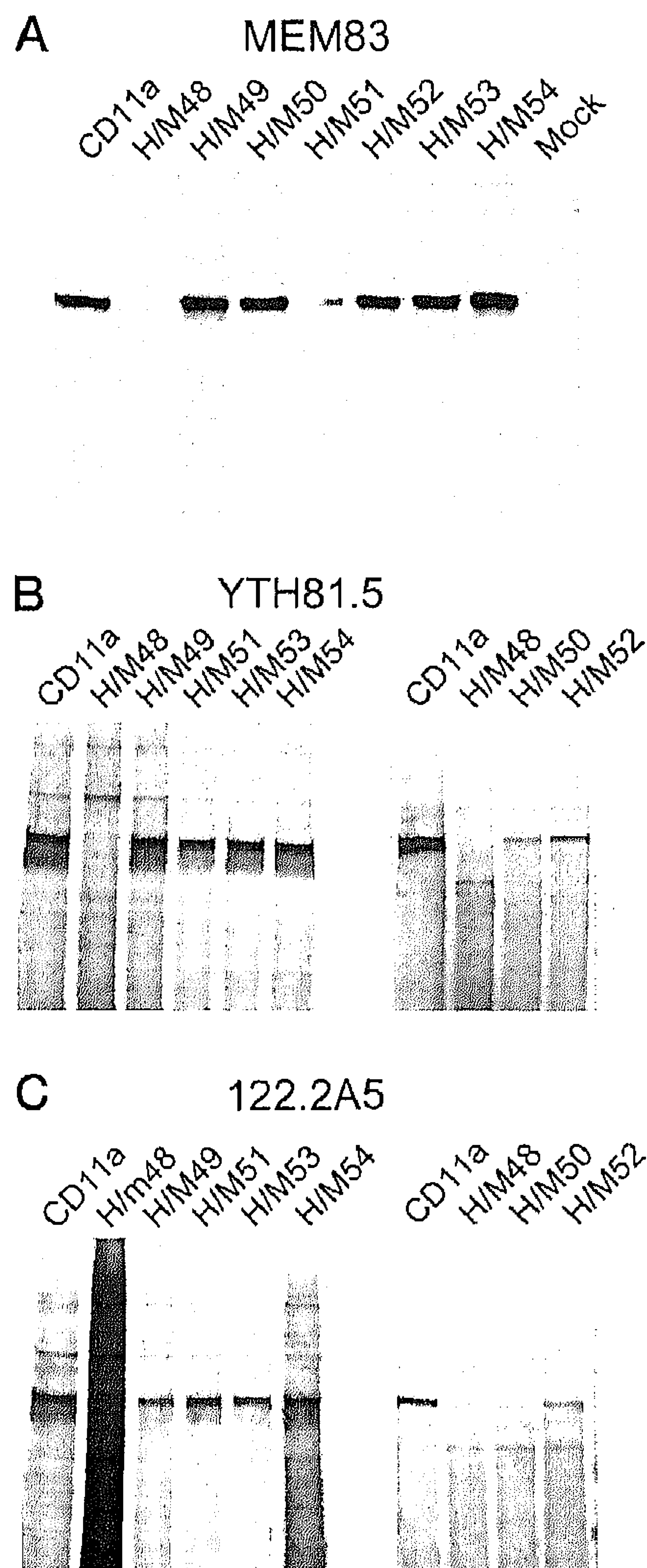

FIG. 4. **Immunoprecipitation of human CD11a variants with ICAM-3 blocking anti-CD11a monoclonal antibodies MEM-83, YTH81.5, and 122.2A5.** The human kidney adenocarcinoma cell line 293 was transfected with plasmids directing the expression of full-length human CD11a (CD11a), or human CD11a I domain variants in which human CD11a I domain residues were replaced by corresponding murine residues (H/M48–H/M54). A diagram listing the mutations introduced in each variant is provided in Fig. 3. ICAM-3 blocking anti-CD11a mAbs (MEM-83, YTH81.5, 122.2A5) were added to detergent lysates of transfected cells metabolically labeled with [^{35}S]methionine and subjected to immunoprecipitation. MEM-83 is shown in *panel A*, YTH81.5 in *panel B*, and 122.2A5 in *panel C*.

I domain (24, 39). To localize residues within in the CD11a I domain important for the binding of these mAbs, a panel of human/mouse CD11a chimeras (H/M48–54) was used in which small clusters of amino acids from murine CD11a replaced the corresponding residues of the human CD11a I domain (37, 40). A diagram of the chimeric gene products is provided in Fig. 3.

To identify residues within the CD11a I domain critical for binding of ICAM-3 blocking CD11a antibodies, we determined the ability of MEM-83, 122.2A5 and YTH81.5 to immunoprecipitate chimeric CD11a proteins from transiently transfected 293 cells. The MEM-83, 122.2A5, and YTH81.5 antibodies all readily immunoprecipitated H/M53, H/M52, and H/M54 proteins (Fig. 4), showing that these antibodies did not bind to the previously identified IdeA, IdeB, or IdeC epitopes within the CD11a I domain (40). In contrast, the ICAM-3 blocking antibodies were unable to immunoprecipitate the H/M48 protein, suggesting that antibody binding depended on Asp^{182} and Ser^{184} residues, replaced by Thr and Leu, respectively, in the H/M48 chimera. The H/M48 mutation did not simply inhibit

TABLE I
LFA-1 epitope expression on human/mouse I domain mutants

Summary of reactivity of anti-CD11a mAbs on 293 transfectants, as determined by immunofluorescence flow cytometry. 293-cells were transfected with CD18 alone (293-mock), with CD18 and CD11a (CD11a), or with human/mouse chimeric CD11a constructs and CD18 (H/M48-H/M54). + represents positive staining of 30–60% of the transfected cells, – represents no significant staining (0–9% of transfected cells, not higher than the negative control).

Cells	mAb				
	MHM.23	NKI-L16	MEM-83	YTH81.5	122.2A5
293-mock	–	–	–	–	–
CD11a	+	+	+	+	+
H/M48	+	+	–	–	–
H/M49	+	+	+	+	+
H/M50	+	+	+	+	+
H/M51	+	+	+	+	+
H/M52	+	+	+	+	+
H/M53	+	+	+	+	+
H/M54	+	+	+	+	+

antibody binding by disrupting the overall conformation of CD11a, since monoclonal antibodies recognizing the IdeA, IdeB, or IdeC epitopes were still able to bind the H/M48 protein (40). Thus, H/M48 appeared to define a new epitope recognized by several independently isolated monoclonal antibodies.

Since we observed some reduced immunoprecipitation of the H/M50 chimera by 122.2A5, we investigated the ability of human CD18 to form a heterodimeric complex with chimeric CD11a, to verify the conformational integrity of the H/M chimeras. In these experiments full-length chimeric CD11a variants were co-transfected with wild type human CD18 into 293 cells and transfectants were assayed for antibody binding by fluorescence-activated cell sorter analysis. Co-expression of CD18 with all CD11a variants was detected on the cell surface (Table I), suggesting that the CD11a I domain mutations preserved the structural elements required for heterodimer formation and export to the cell surface. Another indication for the conformational integrity of the H/M variants was provided by binding of a murine antibody to human CD18 (MHM.23), that recognizes an epitope critically dependent on α/β association of CD11a and CD18 (44, 49). The observation that MHM.23 bound all of the I domain variants examined (Table I) provided further data that the I domain variants used for epitope mapping studies did not interfere with structural features required for heterodimer formation. Similarly, the binding of the NKI-L16 mAb (CD11a) to the conformation-sensitive L16 epitope (23) located outside the CD11a I domain (37, 50), provided additional evidence that mutations in the CD11a I domain do not change the overall conformation of CD11a. In contrast to the immunoprecipitation studies, mAb 122.2A5 readily bound the H/M50 chimera when complexed with CD18 (Table I), suggesting that residues mutated in H/M50 do not directly contribute to the 122.2A5 epitope, but rather affect antibody binding by inducing subtle conformational changes in CD11a, which are not apparent in the CD11a/CD18 heterodimer. Together, these studies demonstrate that antibodies that selectively inhibit the LFA-1/ICAM-3 interaction, recognize a novel epitope within the CD11a I domain, termed IdeD, dependent on residues Asp^{182}–Ser^{184}.

Spatial Relationship between CD11a I Domain Residues Involved in LFA-1 Antibody and Ligand Binding—Recently, the crystal structure of the I domain of the LFA-1 α chain (CD11a) has been elucidated (41). The CD11a I domain comprises four parallel β strands (β1-β4) and one short anti-parallel β strand ($\beta 2'$), surrounded by seven α helices (α1-α7). A single cation binding pocket or metal ion-dependent adhesion site (MIDAS) (36), consisting of five cation coordinating residues, is located

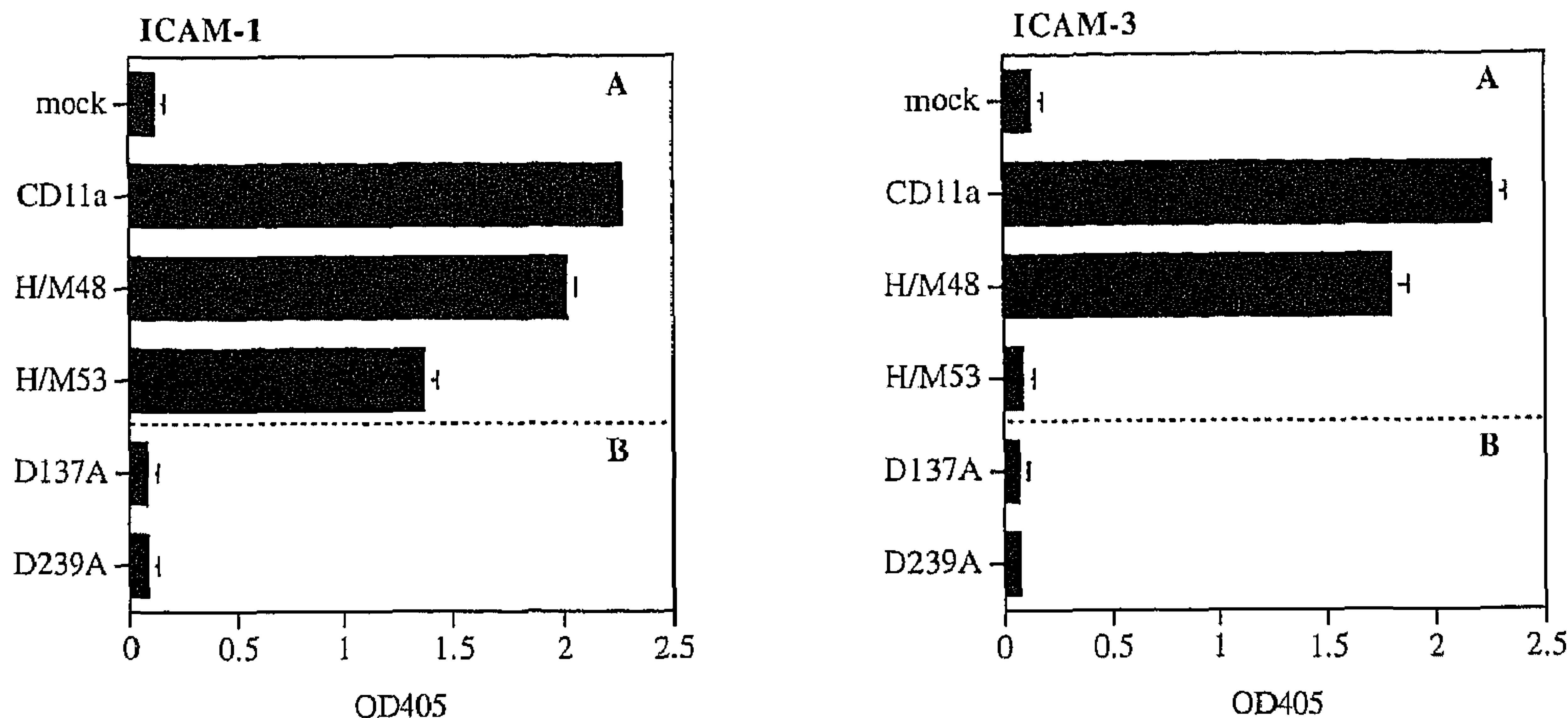

FIG. 5. **Adhesion of CD11a I domain mutants to ICAM-1 and ICAM-3.** The capacity of 293 cells transfected with chimeric CD11a and wild type CD18 to bind ICAM-1Fc or ICAM-3Fc was determined, in the presence of the activating CD18 mAb KIM185 (5–10 μg/ml). *A*, 293-cells were transfected with CD18 alone (293-mock), with CD18 and CD11a (CD11a), or with human/mouse chimeric CD11a constructs and CD18 (H/M48 and H/M53). *B*, the capacity of 293 cells cotransfected with CD18 and CD11a point mutants D137A or D239A, to bind to ICAM-1Fc or ICAM-3Fc was determined in the same experiment. Results are expressed as the mean OD_{405} from triplicate wells. One representative experiment out of three is shown.

at the top of the β sheet on the surface of the I domain (41). Residues important for LFA-1-ligand and antibody binding (Fig. 6) were aligned with the CD11a I domain crystal structure, to analyze their spatial organization. Interestingly, two of the epitopes (IdeB and IdeC) recognized by mAbs that block LFA-1 binding to ICAM-1, -2, and -3[2] (39, 40) are located in helical domains adjacent to the cation binding site. This cation binding site controls both ICAM-1 and ICAM-3 adhesion, since mutation of the cation coordinating residues Asp[137] and Asp[239] into alanine (D137A, D239A), abolished LFA-1 binding to both ICAM-1 and ICAM-3 (1, Fig. 5). The third epitope (IdeA), recognized by mAbs that block ICAM-1, -2, and -3 binding[2] (39, 40), is spatially distinct from the IdeB and IdeC epitopes and is located at the N terminus of the first β strand of the CD11a I domain (β1). In Fig. 6, only the fourth residue of the IdeA epitope is depicted, since the first three residues were not included in the CD11a I domain crystal. Recently, we showed that replacement of Ile[126] and Asn[129] by Met and Lys, respectively, selectively destroyed antibody binding to the IdeA epitope and the ability of the H/M53 chimera to bind to ICAM-3, while preserving its ability to bind ICAM-1 (Ref. 1; Fig. 5). Thus, residues critical for ICAM-3 binding appear to coincide with the IdeA epitope located at the very beginning of the first β strand of the CD11a I domain (β1). Finally, residues Asp[182] and Ser[184] in the α2 helix define the IdeD epitope, recognized by antibodies that selectively inhibit LFA-1 binding to ICAM-3. Disruption of the IdeD epitope (H/M48) did not result in reduced binding to ICAM-3 (Ref. 1; Fig. 5), indicating that the IdeD epitope does not contain residues directly involved in ICAM-3 binding. However, although Asp[182] and Ser[184] are located a considerable distance away from the IdeA epitope in primary structure, when placed on the CD11a I domain crystal, the IdeD epitope appears to be in close proximity of the IdeA epitope and residues critical for ICAM-3 binding, at the N terminus of the first β strand of the CD11a I domain (β1). Thus, regions important for ICAM-3 binding and binding of mAbs that block ICAM-3 binding to CD11a appear to be located in similar parts of the molecule.

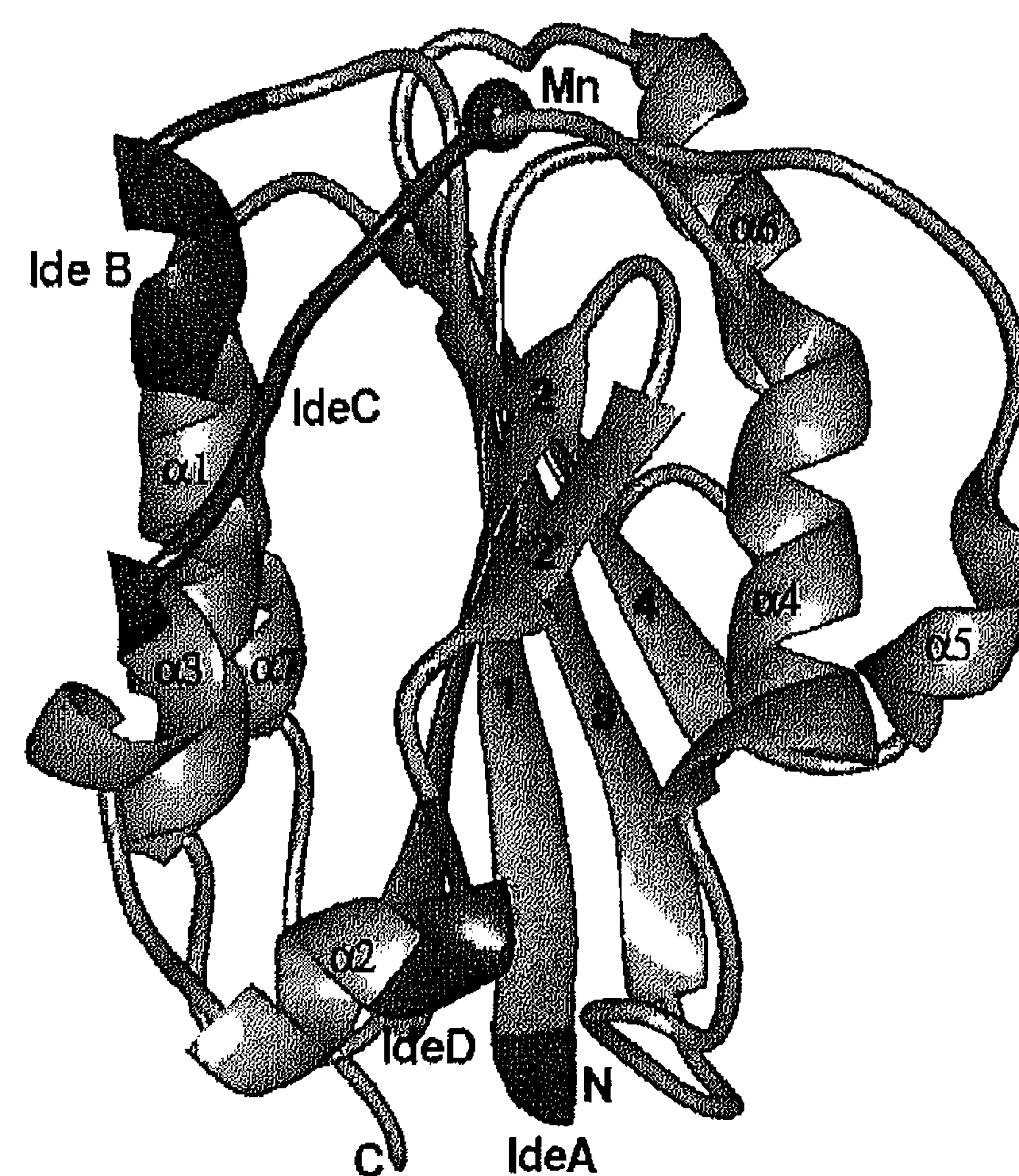

FIG. 6. **Ribbon representation of CD11a I domain based on coordinates provided by Leahy *et al.* (41).** Epitopes recognized by antibodies to CD11a (IdeA, IdeB, IdeC, and IdeD) have been aligned with the CD11a sequence and are shown as *black ribbon* or *tube*. Mn^{2+} ion (*black sphere*), and N and C termini have also been labeled. Ribbon diagram was drawn using the MIDAS program (University of California, San Francisco). Positions corresponding to the IdeB epitope (residues 143–148), the IdeC epitope (residues 198–204), and the IdeD epitope (Asp[182] and Ser[184]) are indicated. Of the IdeA epitope (Ile[126] and Asn[129]) only the fourth residue (Asn[129]) is indicated, since the first three residues were not included in the CD11a I domain crystal.

DISCUSSION

We have demonstrated that the CD11a I domain-specific antibodies MEM-83, YTH81.5 and 122.2A5 that selectively inhibit the interaction of LFA-1 with ICAM-3 (27, 39), bind to a novel epitope (IdeD) in the I domain of CD11a. This is distinct from the previously identified IdeA, IdeB and IdeC epitopes, recognized by antibodies that block LFA-1 binding to ICAM-1, -2 and -3[2] (39, 40). Site-directed mutagenesis demonstrated that the IdeD epitope comprises amino acids Asp[182] and Ser[184], although mutation of these residues failed to inhibit the binding of LFA-1 to ICAM-3. Thus antibody binding to the IdeD epitope appears to interfere with ICAM-3 binding by steric hindrance rather than by competitive binding to the ligand binding site. Placement of the IdeD epitope on the crystal structure of the CD11a I domain (41) suggested that the IdeD epitope was located in close proximity to residues recently identified as being critical for ICAM-3 binding to LFA-1 (1).

Interestingly, residues critical for ICAM-3 binding (Ile[126] and Asn[129]) and the IdeD epitope are both unique to human CD11a and are not found in CD11b or CD11c, suggesting that these represent a structural feature (*i.e.* all or part of a ligand binding domain) unique to LFA-1. These data are consistent

with earlier observations that MEM-83, YTH81.5, and 122.2A5 failed to cross-react with CD11b or CD11c (39). Although MEM-83, YTH81.5, and 122.2A5 all bind the IdeD epitope, they show some functional differences, since MEM-83 can activate LFA-1-mediated adhesion to ICAM-1 (24, 27), whereas 122.2A5 and YTH81.5 cannot (39). This suggests that MEM-83 can induce or stabilize an active ICAM-1 binding conformation of LFA-1 (24). Surprisingly, we observed that MEM-83, YTH81.5 and 122.2A5, as well as blocking antibodies directed against ICAM-3, inhibited ICAM-1-induced T cell proliferation. These data suggest that although T cell costimulation in this system is dependent on engagement of LFA-1 by coated ICAM-1, optimal proliferation may require LFA-1/ICAM-3 interactions between proliferating cells, which are prevented by IdeD-specific anti-CD11a antibodies. We previously reported that MEM-83 (27), as well as YTH81.5 and 122.2A5 (data not shown), are potent inhibitors of the LFA-1/ICAM-2 interaction, suggesting that residues critical for ICAM-2 and ICAM-3 binding may be located in close proximity. It will therefore be important to determine whether the sequence that has been shown to be essential for ICAM-3 binding, is also involved in LFA-1 binding to ICAM-2.

In addition to ligand-specific sequences, the LFA-1 I domain contains conserved sequences required for adhesion of LFA-1 to all ligands. Interestingly, when aligned with the CD11a I domain structure (41), two of the recently identified CD11a I domain epitopes (IdeB and IdeC) recognized by antibodies that block ICAM-1, ICAM-2, and ICAM-3 binding[2] (39, 40) were located in close proximity to the divalent cation binding pocket, or MIDAS motif (36). Residues within this motif (Asp^{137}, Asp^{239}, and Thr^{206}) have been implicated in cation binding (34) and/or ligand binding of the I domain containing integrins Mac-1, $\alpha_2\beta_1$, $\alpha_1\beta_1$, and LFA-1 (Refs. 1, 33–37, and 51; Fig. 5). In the CD11a I domain crystal structure, a Mn^{2+} ion is coordinated by five cation coordinating residues. A critical acidic glutamate residue (E) within the integrin binding motif I/L-E-T-P/S-L in the first Ig-like domains of ICAM-1, -2, or -3 (47, 52) may provide the sixth cation coordinating residue *in vivo*, implying a role for metal ions in the stabilization of LFA-1/ligand interactions (36, 53). In addition, residues in proximity of the divalent cation binding pocket (Met^{140}, Glu^{146}, Thr^{243}, and Ser^{245}) were shown to be critical for binding of LFA-1 to ICAM-1 (50), underlining the importance of this I domain region in LFA-1/ligand interactions. It is tempting to speculate that antibodies recognizing the IdeB and IdeC epitopes interfere with LFA-1 function by inhibiting actual ligand binding residues in this area, or by altering the conformation of such residues. Alternatively, these antibodies may affect adhesion by altering the conformation of residues involved in cation coordination, resulting in destabilization of the cation binding site.

Our data identify two distinct regions within the CD11a I domain that contain residues critical for ICAM-3 binding: the region involved in cation binding and the region defined by the IdeA epitope at the other side of the I domain. These regions may both contain actual contact sites for ICAM-3, implying that ICAM-3 contacts a relatively large binding face on LFA-1. However, it is also possible that mutations introduced in either one of these regions induce subtle conformational changes in the CD11a I domain, which reduce binding to ICAM-3. As yet we cannot distinguish between these possibilities.

In conclusion, our data indicate that distinct regions of the CD11a I domain contain epitopes recognized by antibodies that either selectively inhibit binding of LFA-1 to ICAM-3, or inhibit both ICAM-1 and ICAM-3 adhesion of LFA-1. These antibodies may inhibit LFA-1 function by either interfering with ligand-specific sequences, or conserved domains within the CD11a domain, that are required for binding of LFA-1 to both ICAM-1 and ICAM-3. The challenge of future research will be to understand how during integrin activation subtle conformational changes within the α and β subunits lead to exposure of these functionally important domains and subsequent ligand binding. The ability of antibodies to selectively inhibit LFA-1-ligand binding might find utility in the development of immune response and inflammatory response modulators. Moreover, these results suggest that the capacity of anti-CD11a antbodies to interfere with leukocyte function (*e.g.* antigen presentation, cytotoxic killing, and B cell activation), historically attributed to disruption of LFA-1/ICAM-1 interactions, should be re-examined to evaluate the possible role of ICAM-2 and ICAM-3.

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