

Critical Amino Acids in the Lymphocyte Function–associated Antigen-1 I Domain Mediate Intercellular Adhesion Molecule 3 Binding and Immune Function

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Summary

We have identified amino acid residues within the evolutionarily conserved I domain of the α -chain (CD11a) of the leukocyte integrin leukocyte function–associated antigen (LFA) 1 that are critical for intercellular adhesion molecule (ICAM) 3 (CD50) binding. ICAM-3, a ligand of LFA-1, is thought to mediate intercellular adhesion essential for the initiation of immune responses. Using a panel of human/murine I domain chimeras and point mutants, we observed that the Ile-Lys-Gly-Asn motif, located in the NH₂-terminal part of the CD11a I domain, is required for ICAM-3 but not ICAM-1 binding. These findings demonstrate that the I domain of CD11a contains distinct functional subdomains for ligand specific binding. An aspartic acid located at position 137, which is essential to ICAM-1/LFA-1 interactions (Edwards, C.P., M. Champe, T. Gonzalez, M.E. Wessinger, S.A. Spencer, L.G. Presta, P.W. Berman, and S.C. Bodary. 1995. *J. Biol. Chem.* 270:12635–12640), was also critical for ICAM-3 binding, whereas Ser at position 139 did not effect ICAM-1 or ICAM-3 binding. A synthetic peptide containing the Ile-Lys-Gly-Asn motif inhibited ICAM-3–dependent adhesion and proliferation of T cells at micromolar concentrations, suggesting that this peptide interferes with immune recognition. These observations underscore the importance of ICAM-3 in leukocyte function, and may lead to development of a new category of immunosuppressive agents.

The integrin leukocyte function–associated antigen (LFA) 1 (CD11a/CD18) is a leukocyte-specific adhesion receptor that modulates adhesive interactions and signaling functions in the immune system (1–3). LFA-1 mediates cell–cell adhesion upon binding to its ligands intercellular adhesion molecule (ICAM) 1 (CD54), ICAM-2 (CD102), or ICAM-3 (CD50) (4–8). Several studies (9–13) have demonstrated that activation of LFA-1 is required for adhesion, and that this can be induced in vitro by engagement of the TCR–CD3 complex. Alternatively, LFA-1 can be activated by exposure to divalent cations (Mn²⁺) or treatment with activating mAbs to CD11a or CD18.

Although LFA-1 binding sites have been located in the NH₂-terminal Ig domains of ICAM-1 and -3 (14–16), precise determination of the ligand-binding sites in LFA-1 is still lacking. It has been postulated that the 200–amino acid inserted or “I” domains, which are found in the α chains of integrins (LFA-1 [CD11a, α L], MAC-1 [CD11b, α M],

p150,95 [CD11c, α X], VLA-1 [α 1], VLA-2 [α 2], and α E β 7) and are homologous to the type A domains of von Willebrand factor, cartilage matrix-binding protein, and complement factor B (17, 18), are essential to ligand binding (19–23). The observations that most blocking mAbs to CD11a map to the I domain (22–24) and that recombinant I domains both inhibit integrin-mediated adhesion and possess ligand-binding activity (19–21) underscore the role of the I domain in ligand binding.

In this study a panel of human/murine CD11a I domain mutants were used to identify amino acids that were essential for the binding of LFA-1 to ICAM-3. We observed that residues located in the NH₂-terminal portion of the I domain of CD11a are critical for ICAM-3 binding, but not for ICAM-1 binding.

Materials and Methods

Antibodies. Function-blocking mAbs directed against human CD11a NKI-L15 (11), human CD18 (MHM23) (25), and murine CD11a (M17) (26) were used. mAbs Rek-1 (anti-ICAM-1;

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CD54) (6) and AZN-IC3.1 (anti-ICAM-3; CD50) (Binnerts, M.E., S.J. van Vliet, Y. van Kooyk, and C.G. Figdor, manuscript in preparation) were used to inhibit LFA-1/ICAM-1 or LFA-1/ICAM-3 interactions, respectively. For cell adhesion studies, the activating antibody KIM185, directed against CD18 (12), was used. The mAb T3b, directed against CD3 (27), was used for T cell costimulation assays.

Generation of CD11a I Domain Mutants. The generation of human/mouse (H/M) chimeras, the I domain point mutants, and mu3, has been described previously (24, 28). mu1 and mu2 were generated by overlap extension PCR. Briefly, using full-length human CD11a cDNA (pRK LFA α m) and mu3 cDNA as templates, the NH₂- and COOH-terminal halves of the mu1 and mu2 I domains were generated by PCR (mu1: for residues 125–234, mu3 was used, and for residues 235–308, pRK LFA α m was used; mu2: for residues 125–234, pRK LFA α m was used, and for residues 235–308, mu3 was used.) The BglII at residue 297 was removed from the mu1 I domain. The two halves of mu1 and mu2 were joined by overlapping extension PCR and cloned into the CD11a at the appropriate I domain restriction sites (NarI, BglII: mu1; NarI, PflM1: mu2).

Expression of CD11a I Domain Mutants in 293 Cells. Mutated 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 (29). Transfection efficiencies ranged from 20 to 70%. After 3 d, transfectants were harvested by EDTA (5 mM) treatment and were assayed for adhesion to ICAM-1 and ICAM-3. Staining of the transfectants with various anti-LFA-1 mAbs revealed that the mutations did not affect the overall conformation of the LFA-1 heterodimer (28) (data not shown).

Adhesion to ICAM-1 and ICAM-3. 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, ICAM-3Fc, respectively) were isolated from supernatants of L cell cultures stably transfected with pICAM-1Fc and pICAM-3Fc, respectively (7, 16). Culture supernatant was purified by protein A column affinity chromatography and eluted with 3.5 M MgCl₂ and 10% glycerol. 96-well plates (Maxisorb; Nunc, Roskilde, Denmark) were precoated with 4 μ g/ml goat anti-human Fc (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 2 h at 37°C and blocked with 1% BSA (Boehringer Mannheim, Mannheim, Germany) (1 h at room temperature). ICAM-1Fc or ICAM-3Fc proteins were coated overnight at 4°C or at 37°C for 2 h, at a concentration of 200 ng/well. Transfected 293 cells (200,000 cells/well) were added in adhesion buffer (0.14 M NaCl, 0.02 M HEPES, 0.2% glucose, 1 mM MgCl₂, and 1 mM CaCl₂), with the anti-CD18-activating mAb KIM185 (12) and allowed to adhere for 1.5 h at 37°C. Nonadherent cells were removed by washing three times with PBS, and cell attachment was measured using the PNAG (P-nitrophenyl-N-acetyl- β -D-glucosaminide) method of Landegren (30). The mean OD₄₀₅ of triplicate wells was determined and corrected for the expression levels of CD11a/CD18 on the distinct transfectants. For comparison 50,000 cells gave an average OD of 1. For adhesion of the human T cell line HSB (obtained from American Type Culture Collection, Rockville, MD), cells (40,000/well) were labeled with ⁵¹Cr for 45 min at 37°C. Cells were incubated with different concentrations of synthetic peptides followed by activation with KIM185. Subsequently, cells were incubated on ICAM-1Fc- or ICAM-3Fc-coated plates for 30 min at 37°C in the presence of peptides and KIM185. 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.

Proliferation Assay. 96-well plates were coated with suboptimal concentrations of anti-CD3 antibodies (T3b, 10 ng/well, 1 h at 37°C), followed by goat anti-human Fc (400 ng/well, 1 h at 37°C), 1% BSA (100 μ l/well, 30 min at 37°C) and ICAM-1Fc or ICAM-3Fc proteins (100 ng/well, 1 h at 37°C). Resting PBL obtained by centrifugal elutriation from normal donors as described previously (31) were added (100,000 cells/well) and cultured for 3 d. On day 3, cells were pulsed for 16 h with [³H]thymidine (1.52 TBq/mmol, 0.5 μ Ci/well; Amersham Corp., Arlington Heights, IL), and uptake was quantified to measure ICAM-1- or ICAM-3-dependent proliferation. To determine whether induced proliferation was LFA-1 and ICAM-1 specific, cells were cultured in the presence of function-blocking antibodies at a concentration of 10 μ g/ml.

Results and Discussion

Despite the high sequence homology between human and mouse LFA-1, murine LFA-1 does not bind human ICAM-1 (32). Exploiting this species specificity, we determined that substitution of murine I domain sequences into human CD11a abolished the ability of LFA-1 to bind ICAM-1 (28). In this study, we adopted a similar strategy to determine the role of I domain sequences in LFA-1/ICAM-3 interactions. A panel of H/M CD11a I domain mutants was used, some of which correspond to epitopes recognized by CD11a blocking mAbs (24), to identify amino acids that were essential for the binding of LFA-1 to ICAM-3 (Fig. 1). The capacity of 293 cells transfected with cDNAs encoding chimeric CD11a and human CD18 to bind purified human ICAM-1Fc and ICAM-3Fc is shown in Fig. 2A. Wild-type human CD11a/CD18 transfectants bound both ICAM-1 and ICAM-3. As had been previously shown with ICAM-1 (28), the mu3 chimera (which contains the complete murine I domain) did not bind human ICAM-3. FACS[®] analysis, using the M17, NKI-L16, and MHM23 mAbs (11, 25, 26), showed that the mu3 chimera contains the expected murine I domain epitopes as well as human CD11a and CD18 epitopes, suggesting that this heterodimer was correctly folded (28). Thus it appeared that the I domain of murine LFA-1 lacked the binding site(s) for ICAM-3 as well as ICAM-1. We also observed that mu1 (which contains the NH₂-terminal portion of the murine I domain) bound ICAM-1 but not ICAM-3, whereas mu2 (which contains the COOH-terminal portion of the murine I domain) bound to both ICAM-1 and ICAM-3. We conclude that the NH₂-terminal portion of the human I domain contains residues critical to ICAM-3 binding, and that ICAM-1 and ICAM-3 bind to distinct sites within the I domain of LFA-1. Precise mapping of residues involved in ICAM-1 binding is in progress.

To precisely locate the ICAM-3 binding site in the NH₂-terminal portion of the I domain, we tested the ability of seven H/M chimeras to bind ICAM-3. (Fig. 2) The overall conformation of these H/M LFA-1 chimeras was intact and did not affect ICAM-1 binding (24, 28). Of all H/M chimeras tested, only H/M53, in which the human

CD11a I domain mutants

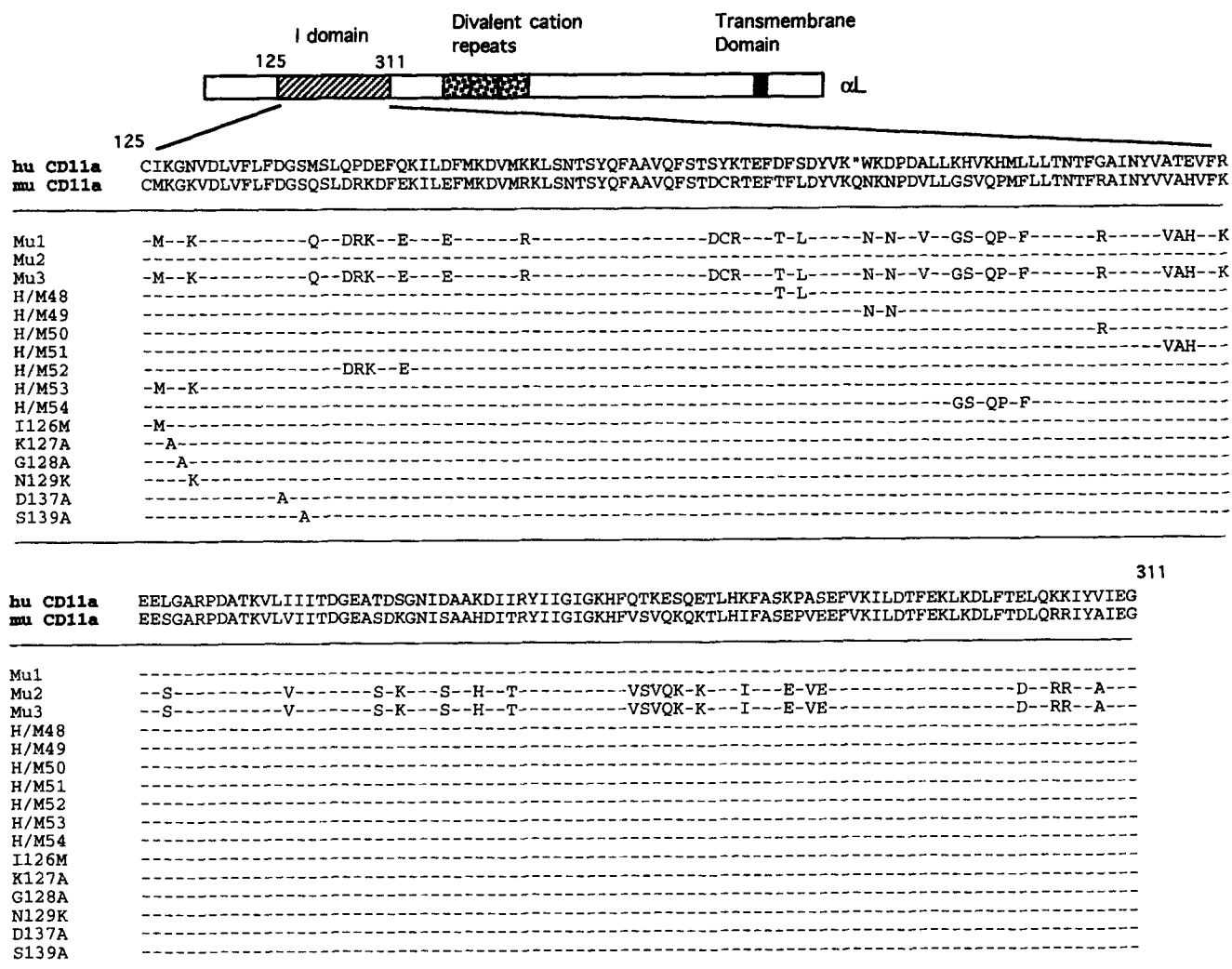


Figure 1. Amino acid sequences of the murine and human CD11a I domains and the H/M I domain chimeras and point mutants. Schematic representation of CD11a with the location of the I domain and the metal binding (EF hand) domains. Chimeras were generated in which the complete human I domain was replaced with the corresponding murine residues (amino acids 125–311; mu3), or in which the NH₂-terminal portion of the human I domain (amino acids 125–222; mu1) or the COOH-terminal portion of the human I domain (amino acids 223–311; mu2) were exchanged for the murine I domain residues. H/M chimeras (H/M48–54, I126M, K127A, G128A, N129K) contained from one to five murine residues substituted for the human I domain sequences (24). The Ala substitutions for conserved residues are shown for the constructs D137A and S139A. The human residues are represented by a dash, and where the sequence differs from the human sequence, the residue is shown. All chimeric proteins and H/M mutants were expressed in the human kidney cell line 293 as LFA-1 (CD11a/CD18) heterodimers.

Ile-126 and Asn-129 were replaced with murine residues Met and Lys, respectively, completely abrogated adhesion to ICAM-3 (Fig. 2 B). In contrast, the adhesion of this mutant to a wide range of concentrations of ICAM-1 showed identical binding as that of wild-type CD11a (data not shown). This indicates that the loss of binding of H/M53 to ICAM-3 was not due to the low affinity of ICAM-3, compared with ICAM-1, for LFA-1. Point mutations of Ile-126 and Asn-129 revealed that only the replacement of Asn with Lys at position 129 dramatically reduced the adhesion to ICAM-3. When Lys-127 and Gly-128, which are conserved between the human and mouse, were mutated to Ala, only the Lys-127 mutation led to reduced binding

to ICAM-3. These data demonstrate that residues critical for ICAM-3 binding are located in the NH₂-terminal portion of the I domain of CD11a within the Ile-Lys-Gly-Asn motif at positions 126–129, and that Lys-127 and Asn-129 are critical to ICAM-3 binding (Fig. 2 C).

It is interesting to note that the Ile-Lys-Gly-Asn sequence was previously found to be critical for the binding of several mAbs to murine and human CD11a that block binding to ICAM-1 (24). Although subsequent studies (28) demonstrated that these residues were not directly involved in ICAM-1 binding, the fact that an immediately adjacent sequence (residues 130–143) was highly conserved among the CD11 integrin I domains, as well as homologous domains

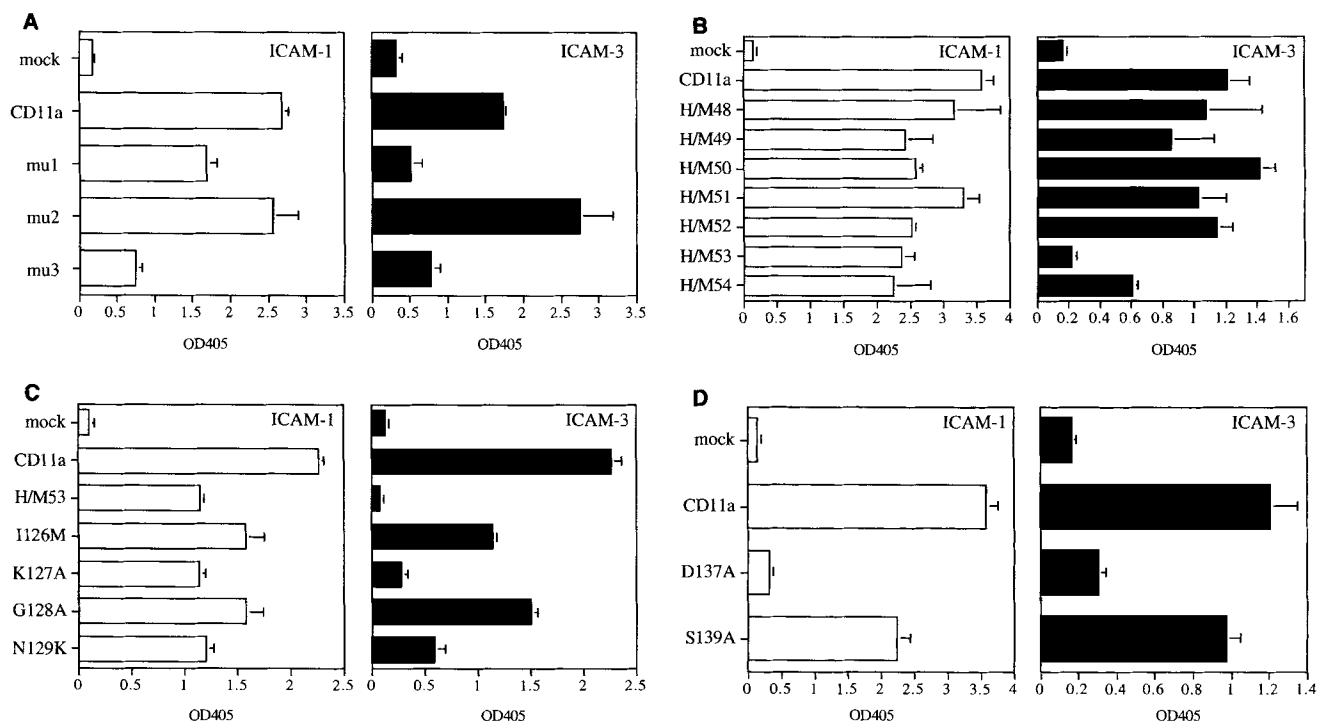


Figure 2. LFA-1-mediated adhesion of H/M CD11a I domain chimeras and point mutants to purified human ICAM-1 and ICAM-3. 293 cells, transfected with pAdv RNA (*mock*) or together with wild-type human CD18 and the indicated CD11a constructs, were tested for their capacity to bind ICAM-1 (*white bars*) and ICAM-3 (*black bars*) in the presence of 5–20 $\mu\text{g/ml}$ activating anti-CD18 mAb (KIM185) (12). (*A*) Adhesion of the mu1, mu2, and mu3 chimeric proteins to human ICAM-1 and ICAM-3. (*B*) Adhesion of the H/M I domain chimeras to human ICAM-1 and ICAM-3. (*C* and *D*) Adhesion of the I domain point mutants to human ICAM-1 and ICAM-3. One representative experiment out of three is shown.

of other proteins (e.g., cartilage matrix protein, von Willebrand factor, and factor B [33]), suggested that this was a functionally significant domain. Studies by Michishita et al. (19) showed that mutation of Asp-140GlySer to AlaGlyAla of the closely related integrin CD11b abolished binding to C3bi. Recent x-ray diffraction studies of the I domain of Mac-1 have shown Asp-140 and Ser-142 to be part of a novel cation binding site in which the acidic side chain coordinates directly to a Mg^{2+} ion (34). Studies of CD11a (28) have shown that the homologous Asp-137 in CD11a was critical to ICAM-1 binding. We found that mutation of Asp-137 to Ala abrogated binding to ICAM-3 (Fig. 2 *D*). In contrast, mutation of Ser-139 to Ala did not profoundly effect LFA-1 binding to ICAM-1 or ICAM-3, suggesting that Ser-139 of CD11a may not be involved in cation coordination. Since the residues corresponding to the Ile-Lys-Gly-Asn motif were absent from the Mac-1 I domain fragment used for the x-ray diffraction study (34), and since Mac-1 lacks a homologous sequence, it will be of interest to determine the structure and proximity of this motif to the CD11a cation binding site.

To obtain further insight into the role of the Ile-Lys-Gly-Asn-126 to 129 and the Asp-137 sequences in binding of ICAM-3 to LFA-1, a series of peptides that spanned this region of CD11a were synthesized (Fig. 3). Interestingly, we observed that low concentrations of one peptide efficiently inhibited the ability of the LFA-1-expressing T cell clone HSB to bind to ICAM-3, but not to ICAM-1 (pep-

tide 2; $\text{IC}_{50} = 25 \mu\text{g/ml}$; $10.8 \mu\text{M}$). In contrast, the two other peptides (peptides 1 and 3), which also contain the Ile-Lys-Gly-Asn motif, did not inhibit LFA-1-mediated adhesion at concentrations up to $100 \mu\text{g/ml}$. These data in-

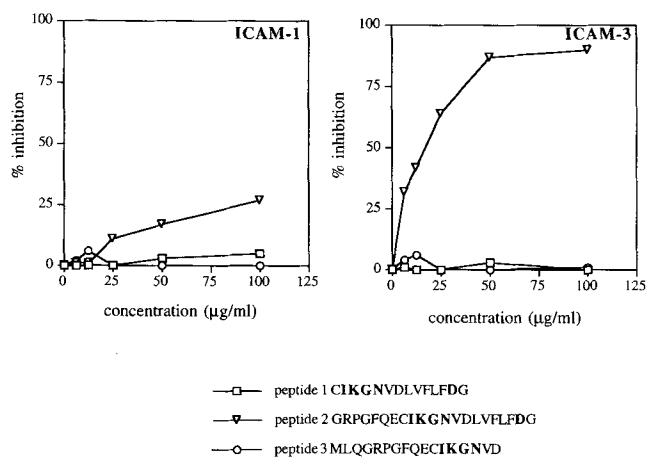


Figure 3. Inhibition of LFA-1/ICAM-3-mediated adhesion by low concentrations of synthetic I domain peptides. The LFA-1-expressing HSB T cell clone was preincubated with different concentrations of the indicated synthetic peptides (6.25–200 $\mu\text{g/ml}$) followed by activation with the anti-CD18 mAb KIM185 (10 $\mu\text{g/ml}$). The capacity of the peptides to block binding to ICAM-1 or ICAM-3 was measured. Results are expressed as the mean percentage of inhibition of cell binding from triplicate wells. Standard deviation was $<8\%$. One representative experiment out of three is shown.

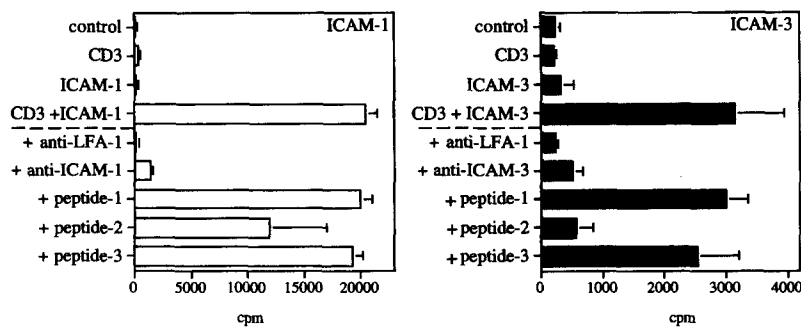


Figure 4. Inhibition of ICAM-3 costimulation of PBL with I domain peptides. Resting PBL (100,000 cells/well) were cultured in the presence of the synthetic peptides (100 μ g/ml; depicted in Fig. 3) on plates coated with ICAM-1Fc (white bars) and ICAM-3Fc (black bars) (100 ng/well) along with suboptimal concentrations of anti-CD3 mAb (1 ng/well) for 3 d at 37°C. One experiment out of three, in which triplicate determinations were carried out, is shown.

indicate that the sequences surrounding this motif are important for the inhibitory effect of peptide 2. Since ICAM-3 is thought to play a role in the initiation of the immune response, we investigated whether the Ile-Lys-Gly-Asn-containing peptides also affected immune function. We assayed whether the peptides would inhibit either the ICAM-1 or ICAM-3/CD3-induced costimulation of resting PBL proliferation (35). As illustrated in Fig. 4, peptide 2 inhibited ICAM-3-induced proliferation, but not ICAM-1-induced proliferation. Peptides 1 and 3 were inactive. The inhibitory activity of the peptides is likely to result from binding of the peptide to residues of ICAM-3 that are involved in LFA-1 binding. This is currently under investigation.

Comparison of the sequence homology of the I domains of the other CD18 integrins indicated that the Ile-Lys-Gly-Asn motif is unique to CD11a, suggesting that LFA-1 contains a unique binding site for ICAM-3. Indeed, no studies have reported Mac-1- or p150/95-mediated binding to

ICAM-3. It will be interesting to know whether the recently described fourth member of the CD18 integrins CD11d/CD18 contains the Ile-Lys-Gly-Asn sequence in the I domain, since there is evidence that this heterodimer binds to ICAM-3 with high affinity (36).

Collectively, these findings demonstrate for the first time that the I domain of CD11a contains distinct functional subdomains for ligand-specific binding: a conserved Asp-137 residue important for binding of ICAM-1 and -3, an Ile-Lys-Gly-Asn domain important for binding of ICAM-3, and distinct, yet-to-be-defined residues important for binding of ICAM-1 (37). Our finding that low concentrations of I domain peptides significantly inhibit ICAM-3-dependent immune function, without affecting ICAM-1-dependent function, may direct the development of a new class of antiinflammatory/immunosuppressive agents for the treatment of diseases such as arthritis or graft rejection after organ transplantation.

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