The I Domain Is a Major Recognition Site on the Leukocyte Integrin Mac-1 (CD11b/CD18) for Four Distinct Adhesion Ligands

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Abstract. Despite the identification and characterization of several distinct ligands for the leukocyte integrin (CD11/CD18) family of adhesion receptors, little is known about the structural regions on these molecules that mediate ligand recognition. In this report, we use α subunit chimeras of Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18), and an extended panel of newly generated and previously characterized mAbs specific to the α chain of Mac-1 to map the binding sites for four distinct ligands for Mac-1: iC3b, fibrinogen, ICAM-1, and the as-yet uncharacterized counter-receptor responsible for neutrophil homotypic adhesion. Epitopes of mAbs that blocked ligand binding were mapped with the chimeras and used to localize the ligand recognition sites because the data obtained from functional assays with the Mac-1/p150,95 chimeras were not easily interpreted. Results show that the I domain on the α chain of Mac-1 is an important recognition site for all four ligands, and that the NH2-terminal and perhaps divalent cation binding regions but not the COOH-terminal segment may contribute. The recognition sites in the I domain appear overlapping but not identical as individual Mac-1-ligand interactions are distinguished by the discrete patterns of inhibitory mAbs. Additionally, we find that the α subunit NH2-terminal region and divalent cation binding region, despite being separated by over 200 amino acids of the I domain, appear structurally apposed because three mAbs require the presence of both of these regions for antigenic reactivity, and chimeras that contain the NH2 terminus of p150,95 require the divalent cation binding region of p150,95 to associate firmly with the β subunit.

The leukocyte integrins comprise a group of three closely related cell surface receptors that coordinate adhesive interactions in the immune system (82). The members of this subfamily of integrins (37, 40) are lymphocyte function–associated antigen (LFA)-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18). These glycoproteins share a common CD18 β subunit (95,000 Mr) but have individual CD11 α subunits (175,000, 160,000, 150,000 Mr) that are structurally homologous (49). All three α subunits share two prominent features in the extracellular region of the molecule, a putative divalent cation binding region consisting of three tandem repeats of an EF-hand motif, and a 200–amino acid inserted or "I" domain (6, 15, 16, 43, 49, 70). The I domain is absent in all other known integrin α subunits except for the α1 and α2 subunits of the VLA subfamily of integrins (37, 41).

Mac-1, which is expressed primarily on myeloid and natural killer cells, has been shown in experiments with blocking mAbs to be responsible, in part, for myeloid cell adhesion to and transmigration across endothelium (5, 36, 81) or epithelium (64), neutrophil homotypic adhesion (4, 66) and chemotaxis (4), myeloid cell adhesion to serum coated substrates (4, 76), and the binding and phagocytosis of opsonized particles (9, 95). These Mac-1-dependent adhesive interactions occur through binding to several cell surface and soluble ligands including iC3b (9, 60, 95), ICAM-1 (25, 81), fibrinogen (35, 97), and factor X (I). p150,95, whose expression is restricted to myeloid cells, activated B cells, and some T cell subpopulations is implicated in the adhesion of monocytes and neutrophils to endothelium and other substrates (4, 44, 91). While the identity of ligands for p150,95 is less well characterized, recent reports suggest that it binds to fibrinogen (53, 69), complement (57, 61, 72), and at least one counter-receptor on the surface of endothelial cells (86).

Despite the cDNA cloning and the identification of an array of ligand interactions, the region(s) on the α and β subunits of the leukocyte integrins that mediate ligand binding have not been characterized. The platelet αMβ2 molecule (CD41/CD61) is the integrin family member that is best...
Materials and Methods

mAbs

The following murine mAbs against human antigens were used as ascites: TSI12 (anti-CD1a, IgG1) (75), LPM19c (anti-CD1b, IgG2a, a gift of Dr. K. Pulford, Oxford, UK) (92), TMG-65 (anti-CD1b, IgG1, a gift of Dr. E. Rao, Ortho Pharmaceuticals) (95), and TS1/22 (anti-CD1a, IgG1) (75), LPM19c (anti-CD1b, IgG2a, a gift of Dr. K. Pulford, Oxford, UK) (92), Mx41 (anti-CD1b, IgG1, a gift of Dr. J. Buyon, New York) (30), OKM10 (anti-CD1b, IgG2a, a gift of Dr. P. Rao, Ortho Pharmaceuticals, Raritan, NJ) (95), VIM21 (anti-CD1b, IgG1) (10), 14B6E.2 (anti-CD1b, IgM) and 5A4.C5 (anti-CD1b, IgGi), gifts of Dr. L. Ashman, Adelaide, Australia (92), CBRIp504G4l (anti-CD1c, IgG2a) (86), L29 (anti-CD1c, IgG2a) (92), BLY6 (anti-CD1c, IgG1) (92), and TS1/18 (anti-CD1c, IgG1) (75). The following mAbs were used as purified IgG: R15.7 (anti-CD1S, a gift of Dr. R. Rothlein, Boehringer-Ingelheim, Ridgefield, CT) (32), OK11 (anti-CD1b, IgG2a) (95), OKM9 (anti-CD1b, IgG1, a gift of Dr. P. Rao, Ortho Pharmaceuticals) (95), LM2/1 (anti-CD1b, IgG1) (58), and SHC3L (anti-CD1c, IgG2b) (77). M1/87 (anti-Forsmann antigen, IgM) (83) was used as a culture supernatant after treatment at 60°C for 1 h. X63 (nonbinding mAb, IgG1) was used as culture supernatant.

Generation of mAbs against human Mac-1 (CBRM1 series) was based on a previously published protocol (86). In brief, female BALB/c mice were immunized with purified Mac-1 (2 μg/immunization) (25) on day 31 (intraperitoneally) and day 3 (intraperitoneally and intravenously) before fusion with the nonsecreting murine myeloma P3X63Ag8.653 (CRL 1580; American Type Culture Collection, Rockville, MD). Mac-1 was pre pared for the first immunization by combining 10 μg of purified Mac-1 with trehalose dimycolate from Mycobacterium phlei, monophosphoryl lipid A from Salmonella minnesota R595, PBS, 1 mM MgCl2, 0.2% Tween 80, and squalene as described in the manufacturer's instructions (RIBI Immunocomb. Res. Inc., Hamilton, MT). The second immunization was performed in PBS. Mice were bled on day 8 before fusion and the titers of hybridomas by ELISA against purified Mac-1 was 1/5000. The protocol for fusion and hybridoma maintenance has been described (84).

Indirect immunofluorescence flow cytometry was used to identify hybridomas that produced mAbs that bound to peripheral blood neutrophils (25). Of the 500 hybridoma supernatants examined ~20% contained mAbs that recognized antigens on the neutrophil cell surface. These were tested subsequently for their ability to bind to peripheral blood lymphocytes and to Mac-1, or p150,95-transfected COS or CHO cells. Those mAbs that recognized the Mac-1 or p150,95 subunit of Mac-1 were subcloned and determined to be specific for the Mac-1 α or p150,95 subunit. mAbs were isotypes using an Immunopure mAb Isotyping Kit (Pierce, Rockford, IL).

Generation of Mac-1/p150,95 Chimeras

For production of chimeric Mac-1 and p150,95 α chains, oligonucleotide-directed mutagenesis (46, 67) was used to introduce restriction sites into homologous regions of the cDNA without altering the coding sequence of α4 (Mac-1) (16) and α5 (p150,95) (15) in pCDM8. The nucleotide sequence numbering was according to the published cDNA clones (15, 16).

Changes were introduced at the following nucleotide sequences to facilitate reciprocal exchanges: EcoRV (α4 bp 520-525, GACATT is changed to GATTAC; α5 bp 515-520, GACATT is changed to GATATC), BgIII (α4 bp 1064-1069, AGATCT is native; α5 bp 1059-1064, AGATCT is native), and AflII (α4 bp 1908-1913, GCTCAG is changed to GTTAAAG; α5 bp 1900-1905, GCTCAG is changed to GTTAAAG). The sites were selected to bracket the I domain and dative cation binding regions based on amino acid sequence homology patterns among members of the leukocyte integrin family (49). Genomic cloning of p150,95 (17) identified the exon–intron boundaries that distinguish these regions and confirmed that EcoRV and BgIII bracket the I domain: the EcoRV site is located 23 nucleotides into the first exon (exon 7) of the I domain and the BgIII site is 15 nucleotides before the end of the last exon (exon 10) of the I domain. The BgIII and AflII restriction sites define a region that contains exons 11-13 and the three dative cation binding repeats (exons 14-16): for simplicity this will be referred to as the dative cation binding region: the BgIII site is 15 nucleotides before the end of exon 10 and the AflII site is precisely at the end of the last exon (exon 10) of the dative cation binding repeats (17). Mac-1 α subunits that contained silent mutations were intact antigenically and functionally as they bound ic3b-E and appropriate α chain-specific mAbs after transfection into COS cells (data not shown).

For all chimeras, M represents a region of the Mac-1 α subunit, X represents a region of the p150,95 α subunit, and e, b, and a refer to the restriction sites EcoRV, BgIII, and AflII. M-e-X and X-e-M were constructed by ligating the EcoRV/NolI fragments of pCDMBα5 (4.7 kb) or pCDMBα4 (4.1 kb) with the NolI/EcoRV fragments (3.5 or 4.7 kb) of pCDMBα5 or pCDMBα4, M-b-X and X-b-M, or M-a-X and X-a-M, were constructed using the BgIII/NolI or the AflII/NolI fragments of the corresponding plasmids. M-b-X-a-M and X-b-M-a-X were constructed by ligating the 0.8-kb BgIII/AflII of α4 or α5 with the AflII/BgIII fragment of pCDMBα5 (6.7 kb) or pCDMBα4 (7.2 kb). M-e-X-b-M and X-e-M-a-X were constructed by ligating a SalI/EcoRV (4.5 kb) fragment of pCDMBα4 with a BgIII/SalI fragment (3.0 kb) of pCDMBα5 and an EcoRV/BgIII (0.5-kb) fragment of α5. X-e-M-b-X was constructed by ligating an EcoRV/BgIII fragment (0.5-kb) of α4 with the BgIII/EcoRV fragment (7 kb) of pCDMBα4. The construction of each chimera was confirmed by restriction digest analysis, hybridization with appropriate segments of cDNA, and by sequencing through the junctions (55).

Tissue Culture, Transfection, and Cell Preparation

Neutrophils were isolated from whole blood of healthy volunteers by dextran sedimentation. Ficoll gradient centrifugation, and hypotonic lysis as described (31, 59). Before experimentation, cells were stored at room temperature in HBSS, 10 mM Hepes pH 7.3, 1 mM MgCl2, in polypropylene tubes (Falcon 2097; Becton Dickinson, Lincoln Park, NJ).

COS cells were grown on 15-cm tissue culture-treated plates in RPMI 1640, 10% FCS (JRH Biosciences, Lenexa, KS) 2 mM glutamine, and 50 μg/ml gentamicin, and then cotransfected with COS cells. Chimeric α5 chain was expressed in COS cells and the chimera α5 chain and the chimera p150,95 were examined in the absence of β2 chain (45) cDNA in pCDM8 by the DEAE- dextran method as described (88). Surface expression was monitored 3 d after transfection by flow cytometry as described (25).

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CHO DG 44 cells which have a deletion of the dihydrofolate reductase gene were obtained from Dr. L. Chasin (Columbia University, NY) and maintained as described (71). Cells were cotransfected by electroporation at 380 V and 960 μF (14) with 25 μg of wild-type or chimeric c~ in pCDMS, 25 μg of wild-type β in pCDM8, and 5 μg of pDCHIP vector that contains a CHO dihydrofolate reductase minigene. Cells were selected and maintained as described (71) with 0.02 mM methotrexate. A homogeneous population of positively expressing cells was obtained by immunopanning (7). Cells were detached with HBBS, 5 mM EDTA, washed three times in α-MEM, 2.5% dilaizy FCS, incubated with mAb, and panned for 5 min at room temperature on 10-cm nontissue culture–treated plates (Lab-Tek 4026; Nunc Inc., Naperville, IL) that were coated with 50 μg/ml rabbit anti-mouse IgG (Zymed Laboratories, San Francisco, CA). Nonadherent cells were removed by eight changes of media, and adherent cells were recovered after treatment with trypsin-EDTA. Cells were cultured and repanned several days later. Expression was augmented by increasing the concentration of methotrexate (0.05–0.20 mM) in culture. As controls, CHO cells expressing a domain-deleted form of ICAM-1 (domain 3 deleted, P185) (67) were generated by cotransfection of the P185 cDNA in pCDM8 with pDCHIP followed by selection and immunopanning as described. For all transfecteds, surface expression was monitored by immunofluorescent flow cytometry.

The ICAM-1 + L cell stable transfecteds were maintained on 15-cm tissue culture plates in DME, 10% heated-inactivated FCS (low endotoxin serum [defined]; HyClone Labs., Logan, UT), 2 mM glutamine, and 50 μg/ml gentamycin as previously described (25). Before adhesion assays, cells were removed from tissue culture plates with trypsin-EDTA, and washed twice in PBS, 2 mM MgCl2, 0.5% heat-treated BSA (25).

Surface Labeling, Immunoprecipitation, and Gel Electrophoresis

Transfected COS or CHO cells (1 × 10⁶ cells) were detached with HBBS, 5 mM EDTA, washed four times with PBS, and resuspended in 2 ml. The cells were iodinated, lysed, and preclarified as described (26). Immunoprecipitations were performed as follows: Immune complexes were formed by incubating mAb (250 μl vol of neat supernatant, 1:100 dilution of ascites, 25 μg/ml purified mAb) with purified rabbit anti-mouse antibody (5 μl of 1 mg/ml, Zymed Laboratories) for 4 h at 4°C. Protein A-Sepharose (Pharmacia LKB, Piscataway, NJ) (25 of a 1:1 slurry) was added for over-night incubation. Eppendorf tubes were centrifuged, supernatants were aspirated, and iodinated lysate (50 μl) was added and incubated for 2 h at 4°C while shaking vigorously. The washing and elution of the immunoprecipitates have been described (26). Samples were loaded and subjected to SDS-PAGE (47) in the presence of 5% β-mercaptoethanol and autoriadographed (50).

iC3b-E Preparation and Binding Assay

IgM- and iC3b-coated erythrocytes (IgM-E, iC3b-E) were prepared as described previously (73) with the following modifications. In some experiments, sheep erythrocytes (Colorado Serum Co., Denver, CO) were washed once in PBS, and fluoresceinated for 2 h at 37°C with a 0.22-μm filtered solution of FITC (0.8 mg/ml in PBS after being dissolved in 30 ml DMSO). Erythrocytes were washed three times in HBBS, 10 mM Heps, pH 7.3, 2 mM MgCl₂, resuspended (6 × 10⁶ cells in 10 ml), and incubated with a 1:128 dilution of heat-inactivated anti-Forssman IgM mAb (MI/87) for 60 min at room temperature while shaking gently. IgM-coated erythrocytes (IgM-E) were washed three times, resuspended (1 ml) in HBBS, 10 mM Heps, 1 mM MgCl₂, 1 mM CaCl₂ supplemented with C5-deficient human serum (100 μl) (Sigma Chem. Co., St. Louis, MO), and incubated for 60 min at 37°C while rotating. IC3b-E and IgM-E were washed three times and resuspended (4 × 10⁶ cells/ml) in HBBS, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM Heps, pH 7.3.

Neutrophils and transfected CHO cells were assayed for binding to iC3b-E. Peripheral blood neutrophils (1.25 × 10⁶ cells/well in 50 μl) were seeded onto 96-well tissue culture–treated plates (Corning Glass Inc., Corning, NY) in HBBS, 10 mM Heps, pH 7.3, 1 mM MgCl₂, 1 mM CaCl₂ for 20 min at 37°C in a 5% CO₂ incubator; nonadherent cells were removed after two washes with the same buffer. Adherent neutrophils were preincubated with mAb (addition of 50 μl of diluted ascites or purified mAb in PBS, 1 mM MgCl₂, 0.5% BSA) for 20 min; each condition was performed in quintuplicate. Subsequently, iC3b- or IgM-sensitized fluorescein-labeled erythrocytes (0.25 × 10⁶/ml in 50 μl) and PMA (100 ng/ml final concentration) were added; the erythrocytes were centrifuged (300 rpm, 5 min, 4°C) on the neutrophils and the plates were incubated in a 37°C water bath for 15 min. Nonadherent erythrocytes were removed by flicking the plates six times after addition of PBS, 1 mM MgCl₂, 0.5% BSA (175 μl/well). Bound erythrocytes were quantitated in the 96-well plates using a Pansorbin fluorescence concentration analyzer (Baxter Healthcare Corp., Mundelein, IL). The percentage of inhibition was normalized as follows:

\[\%\text{Inhibition} = 100 \times \frac{\%\text{iC3b-E binding without mAb} - \%\text{iC3b-E binding with mAb}}{\%\text{iC3b-E binding without mAb}}\]

Transfected CHO cells (2 × 10⁶ cells/ml in 250 μl) were seeded onto 6-well tissue culture–treated plates in L15, 10 mM Heps, pH 7.3, 5% heat-inactivated FCS and adhered for >3 h at 37°C. Subsequently, iC3b-E or IgM-E (2 × 10⁶/ml, 50 μl) and PMA (100 ng/ml final concentration) were added and cells were bound for 60 min at 37°C. Nonadherent erythrocytes were removed after six washes and rosettes (>10 erythrocytes/CHO cell, >100 cells examined) were scored by light microscopy at 200× magnification.

Neutrophil Homotypic Adhesion Assays

Previously, two methods have been utilized to quantitate neutrophil aggregation: microscopic determination of the number of free neutrophils (those not found in cell conjugates) (24, 65) or aggregometry, which measures the increase in light transmission that occurs when cells form clusters (4, 12, 35). While both methods are reproducible, the former is labor intensive, and the latter indirectly measures the number and size of cell aggregates. We devised an alternate method which uses a Coulter counter; it is automated, reproducible, and scores mechanically the total number of cell particles, regardless of size, as single particles, small numbers of two- or three-cell aggregates significantly affect the particle count. Neutrophils (2 × 10⁶ cells/ml) were resuspended in HBBS, 10 mM Heps, 1 mM MgCl₂, 1 mM CaCl₂, 0.5% HSA (HMMCH) 0.325-m1 aliquots were preincubated with mAb (1:200 dilution of ascites, 20 μg/ml purified mAb) for 25 min at room temperature. These cells (0.3 ml) were then added to a preset polypropylene eppendorf tube (1.5 ml) that contained 0.1 ml of HMMCH. PMA was added (100 ng/ml final concentration) and the tubes were rocked gently on their sides for 15 min at 37°C on a Belco Rocker Platform (Belco Biotechnology, Vineland, NJ). Subsequently, 0.2-ml aliquot was transferred to a conical tube (3 ml, Sarstedt Inc., Germany) that contained HBBS, 10 mM Heps, 2% paraformaldehyde (0.1 ml), and the number of particles (single cells or cell aggregates) in a fixed volume was determined with a Model S-Plus counter (Coulter Corp., Hialeah, FL). The average of two readings was used as an individual datapoint. Each condition was repeated at least three times with different donors. The percent aggregation was determined according to the following equation:

\[\%\text{Aggregation} = 100 \times \frac{(\text{n particles in a sample stimulated with PMA}) - (\text{n particles in an unstimulated sample})}{\text{n particles in a sample stimulated with PMA}}\]

The percent inhibition was determined according to the following equation:

\[\%\text{Inhibition} = 100 \times \frac{(\text{aggregation with PMA} - \text{aggregation with mAb} + \text{PMA})}{\text{aggregation with PMA}}\]

Neutrophil aggregometry was performed as described (35). 0.5-ml aliquots of neutrophils (10⁷ cells/ml) were preincubated with mAbs for at least 15 min at room temperature. 0.4 ml aliquots of neutrophils were stirred (700 rpm) at 37°C in a siliconized cuvette in a standard platelet aggregometer (model DP-247F; Sienco Inc., Morrison, CO). A baseline was recorded for 3 min at which time PMA (100 ng/ml) was added and the change in light transmission was monitored on a strip chart recorder.

Adhesion of Neutrophils to Purified Fibrinogen

Purified fibrinogen (Sigma Chem. Co.) was resuspended in PBS (2.0 mg/ml) and spotted (25 μl) onto 6-cm bacterial petri dishes (Falcon 1007, Becton Dickinson) for 90 min at room temperature. Plates were blocked with the detergent Tween 20 as described (26). Neutrophils (4 × 10⁶ cells in 1 ml) were resuspended in HBBS, 10 mM Heps, pH 7.3, 1 mM MgCl₂ and preincubated with mAbs for 25 min at room temperature. Subsequently, cells were added to the dishes in the presence or absence of formyl-methionine-leucine-phenylalanine (FMLP) (10⁻⁷ M, final volume of 3 ml), and al-
lowed to adhere for 3 min and 45 s. Nonadherent cells were removed by 12 washes with a Pasteur pipette after gentle swirling with the same buffer supplemented with 0.5% BSA. Binding was quantitated by scoring the number of adherent cells in at least four different fields using an ocular grid at 100x magnification. The percent inhibition by mAb was determined upon comparison with the media control.

**Binding of ICAM-1** L Cells to Purified Mac-1

The binding of ICAM-1+L cells to Mac-1 is a modification of previously described protocol (25). Briefly, Mac-1 that was purified from leukocyte lysates as described was diluted (30 μl) to 6-cm petri dishes, and blocked with 0.5% heat-treated BSA as described (25). 30 min before the binding assay, the plates were preincubated at room temperature with mAb (1:200 dilution of ascites or 20 μg/ml of purified mAb) in PBS, 2 mM MgCl₂, 0.5% heat-treated BSA (PMBSA, 2 ml). ICAM-1+L cells were removed from tissue culture plates with trypsin-EDTA (GIBCO BRL, Gaithersburg, MD), washed twice, and resuspended in PMBSA (0.5-1.0 × 10⁶ cells/ml). Some aliquots were preincubated for 25 min at room temperature with control or anti-ICAM-1 mAbs. The binding assay was performed as described (26). The percent inhibition by mAb was determined upon comparison with the media control.

**Binding of CHO Cell Chimeras to Purified ICAM-1**

The binding of transfected CHO cells to ICAM-1 is a modification of a previously described protocol for COS cell transfectants (25). Briefly, ICAM-1 that was purified from the erythroleukemic cell line K562 by RR1-Sepharose immunoadfinity chromatography (56) was diluted and adsorbed (25 μl) to 6-cm petri dishes. After a 90-min incubation, non-specific binding sites were blocked with 0.5% heat-treated BSA. CHO cell transfectants, after removal from tissue culture plates with HBSS, 10 mM Hepes, pH 7.3, 5 mM EDTA, were washed twice and resuspended (8 × 10⁵ cells/ml) in HBSS, 10 mM Hepes, pH 7.3, 1 mM MgCl₂, 0.5% heat-treated BSA, and bound to ICAM-1-coated Petri dishes for 90 min at room temperature. Nonadherent cells were removed after five washes with a Pasteur pipette and the number of adherent cells was determined by light microscopy at 100x using an ocular grid.

**Results**

**Generation and Expression of Mac-1/pl50,95 Chimeras**

Naturally occurring restriction sites and sites introduced by silent mutations were used to divide the Mac-1 and pl50,95 α subunits into four structurally discrete regions (Fig. 1). COS cells transiently transfected with the chimeric α subunits and the common CD18 β subunit were subjected to immunofluorescence flow cytometry and immunoprecipitation with a panel of previously characterized mAbs to the α and β subunits of Mac-1 and pl50,95. Most of the chimeras were expressed on the cell surface, immunoprecipitation studies revealed an abnormal pattern of immunoprecipitation, as mAbs to the α subunit precipitated only the X-e-M subunit and mAbs to the β subunit precipitated neither the α nor β chains (data not shown).

Figure 1. Schematic representation of the leukocyte integrin α chain and Mac-1/pl50,95 chimeras. Restriction sites (ε, EcoRV; b, BglII; a, AffII) that facilitated reciprocal exchanges are indicated with arrows or in the name of each chimera.

Since a subset of chimeras was not expressed in COS cells, stable expression in CHO cells was used to map definitively the structural epitopes of mAbs and to perform functional analyses. Chimeric α and wild-type β subunits were co-transfected with the pDHCP plasmid into CHO DG 44 cells (71) that lack functional dihydrofolate reductase. After drug and antibody selection, flow cytometric analyses confirmed that all of the chimeric molecules were expressed on the surface of CHO cells and the levels of the α and β chains were equivalent for each chimera (Fig. 3). In one case (M-b- X-a-M), the level of β subunit on the surface exceeded the level of α subunit (Fig. 3). Despite expressing all molecules on the cell surface, immunoprecipitation studies revealed an aberrant association between the α and β subunits in three of the chimeras. Immunoprecipitation of surface-labeled X-e-M, X-b-M, and X-b-M-a-X chimeras with the β subunit mAb (TS1/18) were either weak or absent (Fig. 4, B, E, and J; lanes 3). These three chimeras apparently lost the TSI/18 epitope on the β subunit after detergent solubilization. Previously we have shown that TSI/18 immunoprecipitates LFA-1, Mac-1, and pl50,95 αβ complexes but not the unassociated α and β subunits (29, 85). Despite the loss of the TSI/18 epitope, the β subunit remained partially associated with these

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| mAb          | M-e-X | X-e-M | M-b-X | X-b-M | M-a-X | X-a-M | M-e-X-b-M | X-e-M-b-X | M-b-X-a-M | X-b-M-a-X |
|-------------|-------|-------|-------|-------|-------|-------|-----------|-----------|-----------|-----------|
| LPM19c      | -     | +     | +     | -     | +     | -     | -         | +         | +         | -         |
| 14B6E.2     | -     | +     | +     | -     | +     | -     | -         | +         | +         | -         |
| 5A4.C5      | -     | +     | +     | -     | +     | -     | -         | +         | +         | -         |
| Mn41        | -     | +     | +     | -     | +     | -     | -         | +         | +         | -         |
| TMG65       | -     | +     | +     | -     | +     | -     | -         | +         | +         | -         |
| VIM12       | -     | ±     | -     | -     | +     | -     | +         | +         | +         | -         |
| OKM1        | -     | +     | +     | -     | +     | -     | -         | +         | +         | -         |
| OKM9        | -     | +     | +     | -     | +     | -     | -         | +         | +         | -         |
| OKM10       | -     | +     | +     | -     | +     | -     | -         | +         | +         | -         |
| SHCL3       | -     | -     | -     | -     | +     | -     | -         | +         | +         | -         |
| L29         | +     | -     | +     | -     | +     | -     | -         | +         | +         | -         |
| BLY6        | +     | -     | +     | -     | +     | -     | -         | +         | +         | -         |

Summary of mAb reactivity as determined by immunofluorescent flow cytometry on COS cells transfected with wild-type or chimeric Mac-1 and p150,95 molecules. (+) Positive staining; (-) staining was not significantly different from the negative control (X63); and (±) staining was marginally greater than the negative control.

Figure 2. SDS-PAGE of Mac-1, p150,95, or Mac-1/p150,95 chimeras immunoprecipitated from 125I-COS cell detergent lysates. COS cells were cotransfected with the β subunit and (A) Mac-1, (B) p150,95, (C) M-e-X-b-M, or (D) X-e-M-b-X cDNA, surface labeled with 125I, and immunoprecipitated with X63 (lanes 1, negative control), TS1/18 (lanes 2, anti-CD18), LM2/1 (lanes 3, anti-CD11b), Mn41 (lanes 4, anti-CD11b), VIM12 (lanes 5, anti-CD11b), OKM1 (lanes 6, anti-CD11b), OKM10 (lanes 7, anti-CD11b), SHCL3 (lanes 8, anti-CD11c), BLY6 (lanes 9, anti-CD11c), and L29 (lanes 10, anti-CD11c) as described in Materials and Methods. Material was boiled in SDS sample buffer with 5% β-mercaptoethanol, electrophoresed on a 7% polyacrylamide gel, and autoradiographed. Molecular weights of the protein standards are indicated in the center.

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Figure 3. Flow cytometry profiles of CHO cells expressing Mac-1, p150,95 and Mac-1/p150,95 chimeras. CHO cell transfecants (indicated on right) were immunostained with either a negative control (X63), a mAb to the I domain (LM2/1) or COOH-terminal (OKM1) region of the Mac-1 α subunit, a mAb to the COOH-terminal region of the p150,95 α subunit (CBRpl50/4G1), or a mAb to the CD18 β subunit (T51/18). Cells were subjected to immunofluorescent flow cytometry. Each histogram shows the negative control (X63, light line) and the mAb indicated at the top of the column (dark line).

Epitope Mapping of mAbs to Mac-1

Because an improper association of α and β subunits in a subset of chimeras could affect functional assays and our subsequent analyses, we used mAbs to generate a structure–function map of the ligand binding domains on Mac-1. mAb epitopes were localized on the Mac-1 α subunit by their ability to bind to a particular subset of chimeras and the results correlated with the ability of the mAb to block distinct adhesive functions of the native Mac-1 molecule. To supplement the 10 mAbs that had been previously characterized, 24 new mAbs against the α chain of Mac-1 were generated (Fig. 5) by immunization with purified Mac-1 and screening by immunofluorescent flow cytometry on neutrophils, lymphocytes, and transfected cells.

To map the structural epitopes of the 34 mAbs against the Mac-1 α subunit, the CHO cells expressing wild-type and chimeric Mac-1 and p150,95 were tested for their differential ability to bind mAbs (Fig. 5). Of the 34 mAbs examined, 19 localized to the I domain, 11 to the COOH-terminal region, and one (CBRM1/20) to the region containing the divalent cation binding repeats. Two mAbs (CBRM1/32 and OKM10α) localized to a discontinuous epitope that required the presence of both the NH2-terminal and divalent cation binding regions. (The OKM10α that we received from Ortho Pharmaceuticals is distinct from the OKM10α. The new OKM10 does not block iC3b-E binding to Mac-1 as was described [4, 95] and as we have verified for OKM10α, and the two mAbs do not localize to the same region of Mac-1 [Fig. 5]). We were unable to localize the structural epitope of two mAbs (OKM10α and CBRM1/28). Three mAbs against the p150,95 α subunit also were mapped with the CHO cell chimeras: CBRpl50/4G1 mapped to the COOH-terminal region, BLY6 localized to the I domain, and SHCL3 mapped to sites both in the NH2-terminal and divalent cation binding regions.

Inhibitory Activity of mAbs

iC3b-E Binding to Mac-1. The ability to inhibit individual Mac-1-dependent adhesive functions was investigated with the extended panel of mAbs. To test which mAbs block iC3b binding to Mac-1, a quantitative assay was developed in which FITC-labeled iC3b erythrocytes were incubated with adherent neutrophils in the presence of mAbs to Mac-1 (Fig. 6). mAbs to the I domain inhibited iC3b-E binding to neutrophils with a mean of 72.6 ± 24.9%, whereas mAbs to the COOH-terminal region blocked with a mean of 33.6 ± 166%. 11 of 19 mAbs that mapped to the I domain blocked strongly (>85%), whereas no mAb that localized to the COOH-terminal region showed >60% inhibition. The one mAb (CBRM1/20) which mapped directly to the divalent cation binding region had little inhibitory effect. The two mAbs (OKM10α and CBRM1/32) that mapped to sites in both the NH2-terminal and divalent cation regions blocked 81 and 65% of the binding (Fig. 6).

Neutrophil Homotypic Adhesion. Several groups have demonstrated that activated neutrophils bind homotypically, and that this aggregation response is blocked by mAbs to the α or β chains of Mac-1 [4, 8, 12, 65]. The ligand on neutrophils for Mac-1 that mediates homotypic aggregation remains uncharacterized. To determine the regions of Mac-1 that were important for neutrophil homotypic adhesion, we tested the panel of mAbs for their ability to inhibit this interaction in a newly developed Coulter counter assay (see Materials and Methods). This assay, which counts the number of particles, is more stringent than the classical aggregometry assay; mAbs that inhibit in this assay by 20–30% such as CBRM1/2 and Mn41 (Fig. 6) show 56 and 67% inhibition in the light scattering aggregometry assay (Fig. 7). mAbs to the I domain blocked the aggregation assays with a mean of 25.9 ± 16.1%, whereas mAbs to the COOH-terminal region blocked with a mean of 1.8 ± 1.1%. Two mAbs (LPM19c and CBRM1/29) to the I domain blocked neutrophil homotypic adhesion in the Coulter counter assay strongly by 81 and 51%. The CBRM1/32 mAb to the NH2-terminal and divalent cation binding regions blocked 21%, and the CBRM1/20 mAb to the divalent cation binding region inhibited 7%.

Neutrophil Adhesion to Purified Fibrinogen. When neutrophils are stimulated with fMLP (10−7 M) they bind to fibrinogen in an almost exclusively Mac-1-dependent manner. mAbs to the I domain of Mac-1 inhibited binding to fibrinogen strongly (Fig. 6) with an average of 77.6 ± 13.2%, whereas mAbs to the COOH-terminal region re-
Figure 4. SDS-PAGE of Mac-1, p150,95, or Mac-1/p150,95 chimera immunoprecipitated from 125I-CHO cell detergent lysates. CHO cell transfectants (A) Mac-1, (B) X-e-M, (C) M-e-X, (D) X-a-M, (E) X-b-M, (F) X-e-M-b-X, (G) M-b-X-a-M, (H) p150,95, (I) M-b-X, (J) X-b-M-a-X, (K) M-a-X, and (L) M-e-X-b-M were surface labeled with 125I, and immunoprecipitated with a negative control mAb (X63, lanes 1); a mAb to the I domain of Mac-1 (LM2/1, lanes 4, 2, 12, and K2; a mAb to the COOH-terminal region of Mac-1 (OKM1, lanes D2, E2, G2, and J2); a mAb to the COOH-terminal region of p150,95 (CBRPI50/4G1, lanes C2, H2, and J2); and a mAb to the CD18 β subunit (TS1/18, lanes 3) as described in Materials and Methods. Material was boiled in SDS sample buffer with 5% 2-mercaptoethanol, electrophoresed on a 5% polyacrylamide gel, and autoradiographed. Molecular weights of protein standards are indicated to the left.
of stimulation (data not shown). Two chimeras (M-a-X, M-b-X) which contained the I domain of Mac-1 bound to ICAM-1 more weakly than anticipated. Unexpectedly, the CHO cells that expressed wild-type p150,95 also bound to ICAM-1. Despite the low level of binding, it was reproducible, inhibited by mAbs to p150,95, and not observed with control CHO cells transfected with ICAM-1 (Fig. 8 A, and data not shown). Although we are uncertain of the physiologic role of this p150,95-ICAM-1 interaction, p150,95 acts as a counter-receptor for an uncharacterized adhesion molecule on stimulated endothelial cells (86), and L cell transfectants that express ICAM-1 bind weakly to purified p150,95 (Stacker, S. A., and T. A. Springer, unpublished observations). Chimeras in which the I domains of Mac-1 and p150,95 were exchanged (M-e-X-b-M, X-e-M-b-X) showed no significant difference in adhesion to ICAM-1. Although both wild-type Mac-1 and p150,95 transfectants adhered to ICAM-1, three chimeras (X-e-M, X-b-M, X-b-M-a-X) did not bind to ICAM-1; these three chimeras, however, were expressed improperly on COS and CHO cells (see above). Thus, despite expressing the chimeras at relatively high levels on CHO cells, no readily interpretable pattern of binding to ICAM-1 was observed.

We tested the CHO cell transfectants for their ability to bind iC3b-E. Almost all of the CHO cells expressing wild-type Mac-1 rosetted with iC3b-E (Fig. 8 B). Although the binding occurred in the absence of stimulation, the average number of iC3b-coated particles per cell increased after phorbol ester treatment (data not shown; Diamond, M. S., D. M. Mosser, and T. A. Springer, unpublished observations). The pattern of binding of iC3b-E to the Mac-1/p150,95 chimeras again did not localize the binding region definitively (Fig. 8 B). The three chimeras (X-e-X-M, X-b-M, X-b-M-a-X) that were not expressed properly in COS and CHO cells and did not bind to ICAM-1 in CHO cells, failed to rosette with iC3b-E. Four chimeras that contained the I domain of Mac-1 and p150,95 again did not localize the binding region (Fig. 8 A).

**Figure 5.** Summary of mAb reactivity as determined by immunofluorescent flow cytometry with CHO cells transfected with wild-type or chimeric Mac-1 and p150,95 molecules. (+++) 100% of cells stained the mAb with a pattern similar to that shown in Fig. 3. (+) 100% of cells stained positively with the mAb but with a significantly lower fluorescence intensity. (-) The mAb stained a subpopulation of cells positively. (--) The mAb staining was not significantly different from the negative control (X63).

**Discussion**

Using chimeras of Mac-1 and p150,95, we have localized the epitopes for 34 mAbs to Mac-1 and correlated this with their binding to ICAM-1.
| mAb     | Epitope | IC3b-E Binding | Neutrophil Aggregation | Fibrinogen Binding | ICAM-1 Binding |
|---------|---------|----------------|------------------------|-------------------|----------------|
| LPM10c  | 1 domain | 94.1 ± 5.9 | 81.2 ± 12.3 | 97.7 ± 1.8 | 100.0 ± 0.1 |
| OKM9    | 1 domain | 133.6 ± 9.7 | 8.4 ± 1.3 | 70.0 ± 5.6 | 49.9 ± 13.2 |
| LGM21   | 1 domain | 18.1 ± 8.7 | 8.7 ± 3.4 | 47.6 ± 9.9 | 11.8 ± 20.4 |
| TMC-65  | 1 domain | 87.1 ± 14.7 | 35.7 ± 5.9 | 91.8 ± 0.8 | 95.1 ± 3.9 |
| Mac1    | 1 domain | 91.5 ± 10.6 | 30.4 ± 4.7 | 94.0 ± 0.3 | 93.1 ± 4.0 |
| 144DE7.2| 1 domain | 85.0 ± 10.7 | 28.8 ± 8.9 | 78.8 ± 10.0 | 99.7 ± 0.2 |
| 5A4.C3  | 1 domain | 75.9 ± 10.0 | 21.8 ± 9.7 | 77.0 ± 6.5 | 100.0 ± 0.1 |
| CBRMI/1 | 1 domain | 63.1 ± 10.3 | 20.7 ± 5.2 | 96.3 ± 2.4 | 88.7 ± 2.3 |
| CBRMI/4 | 1 domain | 83.3 ± 7.2 | 19.3 ± 4.4 | 86.7 ± 2.0 | 93.1 ± 3.5 |
| CBRMI/13| 1 domain | 36.8 ± 5.0 | 15.3 ± 2.1 | 45.9 ± 13.3 | 32.7 ± 3.1 |
| CBRMI/12| 1 domain | 88.6 ± 8.4 | 21.2 ± 1.6 | 86.8 ± 0.9 | 99.4 ± 0.4 |
| CBRMI/22| 1 domain | 71.2 ± 11.6 | 18.0 ± 2.0 | 71.9 ± 2.9 | 99.3 ± 0.5 |
| CBRMI/24| 1 domain | 93.6 ± 10.4 | 21.4 ± 1.1 | 90.6 ± 1.6 | 83.6 ± 3.6 |
| CBRMI/27| 1 domain | 45.0 ± 12.6 | 21.7 ± 8.2 | 84.2 ± 3.5 | 45.3 ± 13.3 |
| CBRMI/29| 1 domain | 99.1 ± 12.7 | 23.6 ± 5.9 | 98.1 ± 0.2 | 99.1 ± 0.2 |
| CBRMI/31| 1 domain | 45.6 ± 12.0 | 30.9 ± 10.4 | 81.9 ± 3.3 | 77.5 ± 13.2 |
| CBRMI/33| 1 domain | 94.7 ± 6.3 | 21.5 ± 6.1 | 71.7 ± 1.9 | 99.0 ± 1.2 |
| CBRMI/34| 1 domain | 90.8 ± 3.5 | 29.5 ± 5.7 | 88.6 ± 4.3 | 99.3 ± 0.3 |

| OKM1    | C-terminal | 38.1 ± 9.3 | 2.2 ± 1.5 | -31.9 ± 17.5 | 41.6 ± 28.3 |
| VO412   | C-terminal | 13.2 ± 9.8 | 1.2 ± 0.6 | -7.3 ± 9.6 | 4.9 ± 2.7 |
| CBRMI/9 | C-terminal | 41.4 ± 2.9 | 0.0 ± 0.7 | -2.8 ± 7.6 | 3.4 ± 3.1 |
| CBRMI/9 | C-terminal | 72.7 ± 2.9 | 2.8 ± 1.0 | 58.0 ± 5.5 | 42.5 ± 4.3 |
| CBRMI/16| C-terminal | 58.3 ± 11.7 | 1.2 ± 1.9 | -4.3 ± 4.1 | 37.9 ± 15.9 |
| CBRMI/7 | C-terminal | 50.2 ± 7.1 | 0.6 ± 1.5 | -3.4 ± 4.4 | 21.9 ± 12.4 |
| CBRMI/8 | C-terminal | 49.5 ± 12.8 | 13.3 ± 2.0 | -3.6 ± 3.2 | 33.8 ± 16.3 |
| CBRMI/23| C-terminal | 20.5 ± 11.3 | 1.1 ± 1.1 | 18.0 ± 3.8 | 12.2 ± 17.5 |
| CBRMI/25| C-terminal | 10.5 ± 13.1 | 2.7 ± 1.5 | 14.0 ± 5.6 | 32.5 ± 10.6 |
| CBRMI/26| C-terminal | 93.3 ± 14.5 | 3.2 ± 1.4 | 29.6 ± 9.5 | 47.8 ± 18.3 |
| CBRMI/50| C-terminal | 42.5 ± 9.2 | 3.4 ± 4.2 | -5.7 ± 5.3 | 50.0 ± 14.2 |

| OKM10oA| NH2/pitation | 80.9 ± 21.6 | ND | ND | ND |
| CBRMI/52| NH2/pitation | 64.8 ± 3.3 | 21.2 ± 7.3 | 84.9 ± 3.8 | 96.8 ± 1.1 |
| CBRMI/20| pitation | 26.7 ± 12.4 | 6.6 ± 0.9 | -12.9 ± 9.8 | 5.4 ± 5.6 |
| OKM10oG| pitation | 5.7 ± 11.3 | 9.4 ± 1.2 | 9.8 ± 11.0 | 25.3 ± 14.8 |
| CBRMI/28| pitation | 25.7 ± 11.5 | 0.7 ± 0.2 | 8.8 ± 2.4 | 2.4 ± 3.4 |

| TSI/22| LFA-1 | 0.0 ± 10.9 | 0.4 ± 0.4 | ND | 2.7 ± 7.0 |
| p150.95| p150.95 | ND | ND | 5.4 ± 5.0 | ND |
| TSI/19| CD18 | 14.4 ± 13.3 | 75.7 ± 12.2 | 46.5 ± 6.5 | 91.0 ± 3.5 |
| R15.7 | CD18 | 46.6 ± 15.1 | 53.0 ± 2.9 | 89.3 ± 1.8 | 68.3 ± 11.5 |
| R1.5 | CD94 | 6.5 ± 14.1 | 75.7 ± 12.2 | 46.5 ± 6.5 | 91.0 ± 3.5 |

**Figure 6.** Summary of the functional effects of mAbs on Mac-1 interaction with ligands. The assays for the interaction with the four ligands are described in Materials and Methods. The inhibition data with OKM10oG was obtained from IC3b binding to transfected COS cells instead of neutrophils. The data is expressed as the percent inhibition by each mAb and is the average of at least three independent experiments. SEMs are indicated after the ± sign. ND, not determined.

**Figure 7.** Aggregometry profiles of neutrophils activated with PMA. Neutrophils were preincubated with the indicated mAbs for 25 min at room temperature, equilibrated at 37°C and activated with PMA (100 ng/ml, t = 0 min). Individual representative tracings are shown for experiments that were performed at least three times. I/L, represents the relative light transmission.

**Figure 8.** CHO cell transfectant binding to ICAM-1 (A) and IC3b-E (B). (A) Mac-1, p150,95, Mac-1/p150,95 chimeras, and ICAM-1-transfected CHO cells were detached, resuspended in 1 ml, stimulated with PMA (100 ng/ml), and bound to immunoaffinity-purified ICAM-1 adsorbed to plastic for 90 min at room temperature. Unbound cells were removed by five washes with a transfer pipette. Bound cells were quantitated by visually scoring the number of cells in five microscopic fields (40× magnification). Background binding to plates lacking ICAM-1 was determined for each transfectant and subtracted. The data is the average of three experiments and is normalized to the binding of wildtype Mac-1. Bars indicate the standard error of the means. (B) Transfected CHO cells were detached, replated on tissue culture-treated well plates (2 × 105 cells/ml, 0.5 ml/well), and adhered for >3 h at 37°C at 10% CO2. Erythrocytes (iC3b-E, 50 μl of 2 × 105 cells/ml) were added and incubated with transfectants for 60 min at 37°C. Nonadherent erythrocytes were removed after eight washes with a transfer pipette and rosettes (>10 erythrocytes/CHO cell, >100 cells examined) were scored by light microscopy at 200× magnification. The data is the average of three experiments. Bars indicate the standard error of the means.
ability to block distinct adhesive functions of the native Mac-1 molecule. This approach was used because interpretation of functional assays with the Mac-1/p150,95 chimeras was not straightforward. We find that the 200- amino acid I domain on the \( \alpha \) subunit of Mac-1 is a major site of ligand recognition for iC3b, ICAM-1, fibrinogen, and the undefined counter-receptor for neutrophil aggregation because several different mAbs that map to the I domain abolish binding of these ligands to Mac-1. Only one mAb (CBRM1/32) that maps outside the I domain, to an epitope shared by the NH2-terminal and divalent cation binding regions, blocks binding to iC3b, ICAM-1, and fibrinogen. None of the mAbs that localize to the COOH-terminal region strongly blocks any of the adhesive interactions examined.

In each ligand binding assay, the mAbs that abrogate binding completely are found to map to the I domain. mAbs that localize to the I domain reduce adhesion 73% for iC3b-E, 78% for fibrinogen, 82% for ICAM-1, and 26% for neutrophil homotypic adhesion as determined by the Coulter counter assay which corresponds to 63% inhibition by aggregrometry. Two thirds of the 19 mAbs that map to the I domain block Mac-1 interaction with iC3b, fibrinogen, and ICAM-1 by >70%, and neutrophil homotypic adhesion is inhibited most effectively by mAbs that map to the I domain.

While the majority of mAbs to the I domain are strongly inhibitory in at least one of the assays, not all of the mAbs block interactions with multiple ligands. Only five mAbs (LPM19c, TMG-65, Mn41, CBRM1/29, CBRM1/34) eliminate binding of all Mac-1 ligands. Many reduce binding to fibrinogen, ICAM-1, and iC3b but inhibit only partially neutrophil homotypic adhesion. Two (OKM9 and CBRM1/27) that block adhesion to fibrinogen strongly, inhibit binding to the other three ligands weakly. Furthermore, two (CBRM1/1, CBRM1/2) that block adhesion to fibrinogen and ICAM-1, only mildly decrease binding to iC3b. The differences in patterns of mAb inhibition suggest the existence of several immunoreactive and functional subdomains within the I domain. For each ligand, some or all of these subdomains may contribute to the architecture of the binding site. Alternatively, the I domain may contain a core sequence that facilitates central recognition of all four ligands, and distinct peripheral sites that contribute to ligand specificity. Thus, we speculate that the ligand recognition sites within the I domain are not identical, but are overlapping.

The 11 mAbs that localize to the 493-amino acid COOH-terminal region show little capacity to block any of the ligand interactions with Mac-1. The average inhibition ranged from 18% for neutrophil homotypic adhesion to 33.6% for binding to iC3b. Thus, we suggest that the COOH-terminal region of Mac-1 does not contain recognition sites that mediate a direct interaction with any of the four ligands examined.

Because we generated only one mAb that maps to the 362-amino acid region that contained the 294-amino acid divalent cation binding repeats and the 68 amino acids immediately NH2-terminal to them, little can be concluded about the role of this part of the molecule in ligand recognition. The apparent lack of immunogenicity may be attributed, in part, to the 84% amino acid identity between the corresponding regions of human and murine Mac-1. The one mAb that localizes strictly to this region, CBRM1/20, does not lessen adhesion in any of the assays by >30%. We cannot exclude, however, recognition sites within the divalent cation binding region that are not blocked by CBRM1/20. Indeed, a site within the divalent cation binding repeats of the platelet integrin \( \alpha_{IIb}\beta_{3} \), recognizes fibrinogen (19, 20). Future studies with additional mAbs to this region may clarify whether the strong sequence conservation among divalent cation binding modules maintains receptor conformation or sustains a direct interaction with ligand.

Two mAbs to Mac-1 (OKM10old and CBRM1/32) and one mAb to p150,95 (SHCL3) bind to discontinuous epitopes that require the presence of both the NH2-terminal and divalent cation binding regions of Mac-1 or p150,95. SHCL3 differs from CBRM1/32 and OKM10old as it binds to chimeras that contain only the NH2-terminus of p150,95; however, it binds more strongly when both the NH2-terminus and divalent cation binding region of p150,95 are present. Importantly, these are the only mAbs that map outside the I domain and block ligand interaction strongly. CBRM1/32 inhibits by 70–95% binding to iC3b, fibrinogen, and ICAM-1, and OKM10old blocks binding to iC3b (Fig. 6 and references 4, 95) and fibrinogen (97). SHCL3 blocks binding of p150,95 to stimulated endothelial cells (86) but not to fibrinogen (69). The mapping of three mAbs to discontinuous regions of the \( \alpha \) chain provides immunological evidence for associations between the NH2-terminal and the divalent cation binding regions, which are separated by the 200 amino acids of the "inserted" or I domain. Previous electron microscopy studies on the \( \alpha_{IIb}\beta_{3} \) and \( \alpha_{5}\beta_{1} \) integrins indicate that the NH2-terminal portions of the \( \alpha \) and \( \beta \) subunits form a globular head that is linked by two stalks to the membrane-spanning domain (13, 41, 62). The antigenic relationship between the NH2-terminal and the divalent cation binding regions suggests that the insertion in evolution of the I domain into a subset of integrin \( \alpha \) subunits does not spatially separate these domains. This contrasts with domains that are linearly arranged as "beads on a string," such as immunoglobulin domains, the A domains of von Willebrand factor, and epidermal growth factor, fibronectin type III, and short consensus repeats (11, 21, 27, 33, 34, 42, 54, 68, 78, 79, 90, 94).

Despite screening on the basis of antigenic reactivity on peripheral blood neutrophils, the majority of new mAbs to Mac-1 localize to the I domain. This is unanticipated because the I domain composes only 18% of the \( \alpha \) subunit extracellular domain. The \( \alpha \) subunits of human and murine Mac-1 are 74% identical at the amino acid level (6, 16, 70), with the divalent cation binding region having the greatest identity (84%) and the COOH-terminal region the least identity (74%) among extracellular domains. It is unclear why the I domain is immunodominant; it is intermediate in homology (81% identity), similar in size to the NH2-terminal and divalent cation binding regions, and smaller than the COOH-terminal region.

Although the studies with mAbs show that the I domain is an important ligand recognition site, no discernible pattern is observed when the Mac-1 and p150,95 chimeras are tested in functional assays. Attempts to generate chimeras with Mac-1 and LFA-1 which have less amino acid identity and ligand overlap were unsuccessful as these molecules were not expressed on the surface of COS cells (48). Several factors may contribute to the failure to locate directly the binding sites for iC3b and ICAM-1 with the chimeric molecules. Increasing evidence suggests that Mac-1 and p150,95 bind a
similar repertoire of ligands including fibrinogen (53, 69) and iC3b (57, 61, 72). However, in our experiments, iC3b-E do not rosette with transfected CHO cells expressing p150,95 (Fig. 8 B). Possibly, in transfected cells, p150,95 lacks the proper conformation to bind iC3b. On leukocytes, to recognize ligands, Mac-1 and LFA-1 convert from a low avidity to high avidity state (12, 28, 51, 93). Conformational changes appear to be transmitted by signals from within the cell through the cytoplasmic domains of integrin subunits (38, 39, 63). Conformational changes that enhance or inhibit ligand binding may also arise when chimeric α subunits are constructed or when nonnative αβ subunit associations occur. In favor of this, a hybrid pl50,95 molecule comprised of a human αε subunit and chicken βε subunit expressed in COS cells rosettes strongly with iC3b-E although the wild-type human βε molecule does not (Billsland, C., and T. A. Springer, unpublished observations). Our experiments also suggest that at least in vitro, pl50,95 binds weakly to ICAM-1 (Fig. 8 A). The I domain of pl50,95 in the context of a wild-type pl50,95 frame binds to ICAM-1 poorly, but in the context of a Mac-1 frame (M-e-X-b-M) in which other ligand binding sites may be present or the conformation is altered, the binding to ICAM-1 is enhanced.

The interpretation of the functional assays also is complicated by the improper expression or association with the β subunit by three of the chimeric α subunits (X-e-M, X-b-M, X-b-M-a-X) in CHO cells. In immunoprecipitates with the β subunit mAb (TS1/18), these three chimeras are weak or lacking, although the β subunit is still associated with the α subunit as it coprecipitates with the α subunit-specific mAbs. TS1/18 has previously been shown to precipitate native αβ complexes, but not the unassociated β subunit (85). In the present study, TS1/18 does not recognize normally the αβ complex of X-e-M, X-b-M, and X-b-M-a-X after detergent solubilization, although it does react with these chimeras on intact CHO cells. In addition, the amount of β subunit precipitated by anti-α subunit mAbs is reduced in these three chimeras relative to the other α subunits. Solubilization may dissociate an inherently weak interaction between the X-e-M, X-b-M, and X-b-M-a-X chimeric α subunits and the β subunit. In contrast, the parental α subunits, αεαε and αεεα, and other chimeric α subunits associate stably with the β subunit. The common feature of these unstable αβ subunit chimeras is the presence of the NH2-terminal region of pl50,95 and the divergent cation binding region of Mac-1. These findings suggest that these two regions interact, and that in the above combinations there are noncomplementary amino acid residues that prevent proper folding of the chimeric α subunit and association with the β subunit. In light of this, it is not surprising that X-e-M, X-b-M, and X-b-M-a-X do not bind to iC3b or ICAM-1 when expressed in CHO or COS cells. Two other chimeras (M-a-X and M-b-X), which contain the I domain of Mac-1 and bind to ICAM-1 and iC3b poorly, show evidence of subtle structural abnormalities based on changes in antigenic reactivity. M-a-X and M-b-X show a reduction or loss of binding of two mAbs that map to the I domain: CBRM1/2 (Fig. 5) and CBRM1/5 (24). It is noteworthy that CBRM1/5 recognizes a subpopulation of functionally active Mac-1 molecules on neutrophils and monocytes (23), and is expressed on other chimeras (X-e-M-b-X, M-b-X-a-M) that contain the I domain of Mac-1 and bind ligand (data not shown). The molecular packing of the α subunits of M-a-X and M-b-X may be disrupted sufficiently so that these chimeras cannot attain a conformation that sustains ligand binding.

In summary, the studies presented here suggest that the I domain on Mac-1 is a major recognition site for both cellular and soluble ligands. Previously, because of its homology to ligand binding regions in von Willebrand factor, cartilage matrix protein, and complement proteins, the evolutionarily inserted I domain has been speculated to be important in ligand recognition (49, 70). This is the first study to demonstrate a role for the I domain on integrin α subunits. Future amino acid substitution studies are required to delineate the amino acids in the I domain of Mac-1 that contact individual ligands. It will be interesting to examine whether the I domain is similarly important for the other integrins that contain it, LFA-1, pl50,95, VLA-1, and VLA-2.

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