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S A Stacker; ... et. al

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LEUKOCYTE INTEGRIN P150,95 (CD11c/CD18) FUNCTIONS AS AN ADHESION MOLECULE BINDING TO A COUNTER-RECEPTOR ON STIMULATED ENDOTHELIUM[1]

STEVEN A. STACKER AND TIMOTHY A. SPRINGER[2]

From the Department of Pathology and Center for Blood Research, Harvard Medical School, 800 Huntington Avenue, Boston MA 02115

p150,95 is a member of the β_2 family of integrins, which includes both LFA-1 and Mac-1. These molecules are known to play a role in the adhesion of lymphocytes, granulocytes, and monocytes to various cell types including vascular endothelium. p150,95 is presumed to have an adhesive function because of its structural relationship to the other β_2 integrins and the ability of anti-p150,95 mAb to inhibit some myeloid cell interactions with tumor cells, endothelial cells, and other substrates. In an endeavor to demonstrate directly that p150,95 can act as an adhesion molecule, we raised a mAb (CBRp150/4G1) to the α subunit of p150,95, which allows for the purification of functional intact p150,95 heterodimers. The antibody was selected by using a high pH elution ELISA. The assay was designed to select for antibodies directed to the α-chain of p150,95, which could be readily dissociated from p150,95 under conditions of high pH and 2 mM $MgCl_2$. p150,95 purified under these conditions with CBRp150/4G1-Sepharose could be immunoprecipitated by using antibodies to the α- and β-chains of p150,95 indicating that the structural integrity of the heterodimer was preserved during purification and elution. Elution in the absence of divalent cations yielded primarily dissociated α and β subunits. Other antibodies previously made to p150,95 α-chain such as SHCL3 were greatly reduced in their efficiency of yielding intact heterodimer under these conditions. Mapping of the epitopes by using chimeric molecules of p150,95/Mac-1 revealed that antibodies that react with the divalent cation sites of p150,95 are inferior for the purification of intact p150,95. The adhesive capacity of p150,95 was demonstrated by the specific binding of 18-h rIL-1β or LPS-stimulated endothelial cells to purified p150,95 absorbed to plastic microtiter plates. These results indicate that p150,95 can function independently as an adhesion molecule and that it can interact with a counter-receptor on stimulated endothelium.

The integrins comprise a family of structurally related cell-surface receptors that participate in a range of cell-cell and cell-matrix interactions (1). LFA-1,[3] Mac-1, and p150,95 constitute a subfamily of the integrins commonly called the *leukocyte integrins*, which are involved in immune cell adherence (reviewed in Reference 2). These molecules are composed of a common 95-kDa β subunit (β_2, CD18) combined noncovalently with a specific α subunit (LFA-1: 180 kDa, αL, CD11a; Mac-1: 160 kDa, αM, CD11b; p150,95: 150 kDa, αX, CD11c) (3). Their importance in leukocyte adhesion is illustrated by a clinical condition called leukocyte-adhesion deficiency in which the β subunit gene is defective resulting in deficient expression of all three leukocyte integrins (4). The major features of this disease are recurrent, persistent soft tissue infections that often result in premature death because of deficiencies in neutrophil and monocyte adhesion-related functions (4).

LFA-1 functions in a broad range of Ag-dependent and Ag-independent interactions of leukocytes (2) via at least two known ligands, ICAM-1 (5) and ICAM-2 (6). Mac-1 is involved in a number of myeloid cell adhesive functions including binding iC3b-coated target cells (7, 8), neutrophil aggregation (9), neutrophil and monocyte chemotaxis (9, 10), and binding to endothelium (11). The known counter-receptors for Mac-1 are iC3b (7), ICAM-1 (12),[4] fibrinogen (13, 14), and factor X (15).

p150,95 was originally defined after immunoprecipitation with an anti-β-chain antiserum revealed a third α subunit distinct in size from the α subunits of LFA-1 and Mac-1 (3). mAbs have since been developed that recognize the α-chain of p150,95 (16, 17). Subsequent cDNA cloning has shown that the p150,95 α subunit is a transmembrane protein with a large extracellular domain containing two distinctive features: a divalent cation-binding region and an inserted or "I" domain that is common to a subgroup of the integrins. p150,95 is most homologous to the Mac-1 α subunit with 63% amino acid identity, whereas it is only 37% identical to the LFA-1 α subunit (18–20). p150,95 is restricted in distribution to macrophages, monocytes, granulocytes, and a small population of T and B lymphocytes, and it is expressed to a high

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[2] Address correspondence and reprint requests to Dr. T. A. Springer, Dept. of Pathology and Center for Blood Research, Harvard Medical School, 800 Huntington Avenue, Boston, MA 02115.

[3] Abbreviations used in this paper: LFA-1, lymphocyte function-associated Ag-1; ELAM-1, endothelial leukocyte adhesion molecule-1; FBS, fetal bovine serum; HSA, human serum albumin; ICAM-1, intercellular adhesion molecule-1; Mac-1, macrophage Ag-1; OG, octyl-β-D-glucopyranoside; TEA, triethylamine; VCAM-1, vascular adhesion molecule-1; RT, room temperature; TSM, 20 mM Tris HCl (pH 8.0), 150 mM NaCl, and 2 mM $MgCl_2$; HUVEC, human umbilical vein endothelial cells.

[4] Diamond, M. S., D. E. Staunton, A. R. de Fougerolles, S. A. Stacker, J. Garcia-Aguilar, M. L. Hibbs, and T. A. Springer. ICAM-1 (CD54)—A counter-receptor for Mac-1 (CD11b/CD18). *J. Cell Biol. In press.*

degree on hairy cell leukemia (16, 21, 22). The function of p150,95 on these cell types is not clear but like Mac-1 it appears to be involved in binding iC3b-opsonized particles (23) and adhesion of monocytes and granulocytes to endothelium (22, 24, 25) and other substrates (9). Both p150,95 and Mac-1 are stored in intracellular vesicles in neutrophils that are brought to the surface upon stimulation with chemoattractants (reviewed in Reference 26).

The contribution of p150,95 to cell-cell interactions has often been obscured by the activity of other adhesion receptors present on the same cell. A means of overcoming some of these problems is to purify p150,95 and examine its cell-binding capabilities without the contribution of other cell-surface receptors. In this study we have developed a protocol to obtain purified p150,95 in a functional form by mAb affinity chromatography. Our studies have shown that p150,95 can function as an adhesion molecule independent of other cell-surface receptors and, like LFA-1 and Mac-1, interacts with a counter-receptor on stimulated endothelial cells.

MATERIALS AND METHODS

mAb. SHCL3 (IgG2b), BLY6 (IgG1), L29 (IgG1), and F9083 (IgG1) have previously been shown to recognize the α subunit of the p150,95 heterodimer (CD11c) and were obtained as part of the CD11/CD18 workshop (IVth International Workshop and Conference on Human Leukocyte Differentiation Antigens (27)). TS1/18 (IgG1, anti-CD18), LM2/1 (IgG1, anti-CD11b), and TS2/4 (IgG1, anti-CD11a) have been described (3, 21). mAb were purified from ascities by protein A affinity chromatography or used as expended hybridoma culture supernatant. Purified SHCL3, TS1/18, bovine γ-globulin (Armour Pharmaceutical Company, Kankakee, IL) and CBRp150/4G1 were covalently coupled to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) at a concentration of 2.0 to 2.5 mg of antibody/ml of beads.

Cell culture The murine myeloma P3X63Ag8.653 (28) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 5 mM L-glutamine and 50 μg/ml gentamicin (supplemented Dulbecco's modified Eagle's medium) at 37°C in a humidified atmosphere containing 10% CO_2. Hybridomas were grown in supplemented DMEM at all stages after removal from hypoxanthine-aminopterin-thymidine selection medium. HUVEC (passages 2 to 4) were maintained on fibronectin-coated dishes in M199 medium, 20% FBS, 5 mM L-glutamine, 50 μg/ml gentamicin, 100 μg/ml endothelial cell growth supplement (Biomedical Technologies Inc., Stoughton, MA), and 100 μg/ml heparin at 37°C in a humidified atmosphere containing 5% CO_2. For stimulation, 5 U/ml of human rIL-1β (Boehringer Mannheim, Mannheim, FRG) or 5 ng/ml of LPS (Sigma Chemical Co., St Louis, MO) were added to the medium 18 to 24 h before harvesting. Stimulation of HUVEC was monitored by analyzing stimulated and unstimulated cells for ICAM-1 by flow cytometry. All other human cell lines were maintained in RPMI 1640 medium plus supplements in 5% CO_2.

Production of mAb. p150,95 purified on a SHCL3-Sepharose affinity column was used to immunize female BALB/c mice (1 to 5 μg/immunization) on days 43 (i.p.), 29 (i.p.), and 3 (i.p. and i.v.) before fusion with the murine myeloma P3X63Ag8.653. p150,95 was prepared for the first two i.p. immunizations by combining 200 μg of p150,95 with trehalose dimycolate from *Mycobacterium phlei*, monophosphoryl lipid A from *Salmonella minnesota* R595, PBS/0.2% Tween 80 and squalene as detailed in the manufacturer's instructions (RIBI Immunochem Research, Hamilton, MT). The final immunization was performed by using purified p150,95 diluted 1/1 with PBS. Mice were test bled on day 19 before fusion and the titer of anti-p150,95 antibodies in their serum as judged by binding to purified p150,95 was determined to be 1/5,000 to 1/10,000. The protocol for fusion and subsequent selection of antibody secreting hybridoma is as previously described (29). mAb selected for further analysis were cloned twice by limiting dilution.

High pH elution ELISA. Immunopurified p150,95 was absorbed onto microtiter plates (96-well flat bottom No. 76-232-05; Flow Laboratories, McLean, VA) by adding 3 μl of purified p150,95 to 45 μl of TSM in a well and thereby diluting the OG below its critical micelle concentration of 0.73%. After incubation at RT for 2 h or at 4°C overnight, the plates were washed twice with TSM and nonspecific binding sites were blocked by addition of 0.5% HSA/TSM (HSA; Alpha Therapeutics Corporation, Los Angeles, CA) at RT for 30 min. Control antibodies to p150,95 or hybridoma supernatants were added and incubated at 4°C for 30 min. Wells were washed three times with TSM, then exposed to 200 μl of 50 mM TEA (pH 11.5) (at 4°C), 150 mM NaCl, 2 mM $MgCl_2$ for 5 min at 4°C. During this period of time control wells were incubated with 200 μl of TSM. After incubation, all wells were washed three more times with TSM before addition of 50 μl of diluted rabbit anti-mouse horseradish peroxidase-conjugated Ig (Zymed Laboratories, San Francisco, CA) for 30 min at 4°C. After six additional washes with TSM the assay was developed by using a 2.2, azino-di [3 ethylbenthio-zoline] sulfonate substrate (Zymed) in 0.1 M citrate buffer (pH 4.0)/0.05% H_2O_2 for 30 min at RT. Plates were read at an absorbance of 414 nm in a multiwell plate reader (Flow Laboratories MCC/340, McLean, VA).

Affinity chromatography. p150,95 was obtained from Triton X-100 lysates of hairy cell leukemic spleens (spleen tissue was generously supplied by Dr. Susan Gregory, University of Chicago, Chicago, IL). Spleen (30 g) was diced, sieved, and lysed at 0.05 *g* or approximately 5×10^7 cells/ml of lysis buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% TX-100, 0.02% NaN_3, 0.2 mM diisopropylfluorophosphate, 0.2 mM PMSF, 0.22 trypsin-inhibitory units/ml aprotinin, and 5 mM iodoacetamide) for 1 h at 4°C. The nuclei and insoluble debris were removed by centrifugation at 10,000 rpm in a GSA rotor (Beckman Instruments, Irvine, CA) for 2 h at 4°C and filtration through a Whatman No. 1 filter. The lysate was precleared by incubation for 2 h at 4°C with 5 ml of packed bovine Ig-Sepharose and then used for either large scale purifications or immunoprecipitation analysis. For immunopurification the lysate was loaded onto a 6-ml column of CBRp150/4G1-Sepharose (2.5 mg/ml) at a flow rate of 0.3 ml/min. The column was then washed sequentially with 5 volumes of lysis buffer, 5 volumes of 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% TX-100, and 5 volumes of 100 mM glycine (pH 10.0), 150 mM NaCl, 2 mM $MgCl_2$, 1% OG. After washing, the column was eluted with 50 mM TEA (pH 11.0), 400 mM NaCl, 2 mM $MgCl_2$, and 1% OG at a flow rate of 0.1 ml/min. Fractions were immediately neutralized with 0.16 vol of 1 M Tris-HCl (pH 6.7)/1% OG, snap frozen in liquid N_2, and stored at −70°C until use. LFA-1 was purified from SKW3 lysates on TS2/4-Sepharose by using the protocol of Dustin and Springer (30). Mac-1 was purified from granulocyte lysates by affinity chromatography on LM2/1-Sepharose (see footnote 4).

Immunofluorescence flow cytometry. Cells were washed with PBS/2% FBS and 50 μl of a 1×10^6 cells/ml suspension added to 50 μl of either mAb-containing hybridoma supernatant or ascities diluted to 20 μg/ml of specific antibody for 30 min at 4°C. Cells were washed, incubated with 50 μl of FITC-labeled goat anti-mouse Ig (Zymed) for 30 min at 4°C, rewashed, and fixed with 1% paraformaldehyde/PBS. Samples were analyzed by using an Epics V flow cytometer.

cDNA and transfection. p150,95, Mac-1, LFA-1, and β subunit cDNA were subcloned into the transient expression vector CDM8 (31, 32) (M. L. Hibbs, unpublished observations). The p150,95/Mac-1 chimeric cDNA were constructed in CDM8 as described by Aguilar and Springer.[5] cDNA in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA was transfected into COS cells by using DEAE-dextran as previously described (33). Cell-surface expression of transfectants was quantitated by flow cytometry.

SDS-PAGE. Immunoprecipitates and fractions from affinity purifications were diluted 1/1 with 2 × SDS-PAGE sample buffer, boiled for 2 min, and loaded onto 6% vertical slab polyacrylamide gels as previously described (34). Proteins were visualized by silver staining (35).

Immunoprecipitations. To evaluate mAb for their ability to purify functional p150,95, 50 μl of packed beads (SHCL3-Sepharose or CBRp150/4G1-Sepharose) were incubated with 1 ml of hairy cell spleen lysate for 1 h at 4°C, washed with lysis buffer, and treated with 50 mM TEA (pH 11.0), 400 mM NaCl, and either 2 mM $MgCl_2$, 2 mM $CaCl_2$, or no divalent cations for 20 min at 4°C. After centrifugation at 10,000 rpm for 0.5 min in a microfuge the eluted material was removed and neutralized with 0.16 vol of 1 M Tris-HCl, pH 6.7. Equal volumes of the eluted material were then incubated with 50 μl of either SHCL3-Sepharose or TS1/18-Sepharose for 1 h at 4°C. After washing, the samples were eluted by boiling in SDS sample buffer and analyzed by SDS-PAGE.

Site-number determinations. Protein A-purified CBRp150/4G1 or TS1/18 (100 μg) was labeled with 200 μCi of ^{125}I (New England Nuclear, Boston, MA) to a specific activity of about 0.3 to 0.45 μCi/μg by using Iodogen (Pierce Chemical Co., Rockford, IL) as previously described (36). Saturating amounts of [^{125}I]CBRp150/4G1 or [^{125}I] TS1/18 were added to wells coated with purified p150,95 or LFA-1 and incubated for 2 h at 4°C (36). After washing rapidly three times

[5] Garcia-Aguilar, J., and T. A. Springer. *Submitted for publication.*

with PBS/1% HSA the bound antibody was eluted with 1% SDS and subjected to gamma counting. Site numbers were calculated assuming bivalent binding of the mAb and a surface area of 3.85×10^8 μm^2 in each microtiter well.

Adhesion assay. HUVEC were removed from tissue culture plates by incubation with a solution of HBSS/10 mM EDTA followed by two washes in assay buffer (PBS/5% FBS/2 mM $MgCl_2$). HUVEC were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear) for 1 h at 37°C then washed three times with assay buffer. Purified p150,95 or LFA-1 was absorbed to microtiter wells as already described. p150,95-coated plates were pretreated with 50 μl of mAb (either a 1/400 dilution of ascities or neat tissue culture supernatant) for 30 min at 4°C and then 5×10^4 cells were added to each well. The cells were centrifuged onto the solid phase p150,95 or LFA-1 at $5 \times g$ for 3 min and then incubated at 37°C for 20 min. The plates were washed with assay buffer by three complete aspirations through a 26-gauge needle. Bound cells were eluted with 100 μl of 0.1 M NaOH/0.1% TX-100 and quantitated by gamma counting. Alternatively, assays were performed on 60-mm bacteriologic Petri dishes (Falcon 1007; Becton Dickinson Labware, Lincoln Park, NJ). Purified p150,95 or LFA-1 was diluted in assay buffer and 20 μl were absorbed to a demarcated area of the dish for 2 h at RT after which the plates were blocked with a solution of 1% HSA in assay buffer for 30 min at RT. HUVEC in assay buffer (3×10^5) were added to the Petri dish, allowed to settle for 5 min at 4°C, then incubated at 37°C for 45 min. After incubation the dishes were washed by addition of 3 ml of assay buffer, gentle tipping, and aspiration with a Pasteur pipette. This was repeated once and the cells were then fixed with a solution of 1% paraformaldehyde in PBS. Binding was quantitated by averaging the number of cells bound per high powered field as judged by two independent observers. Pictures were taken of fixed cells by using a Nikon Diaphot-TMD inverted microscope (Nippon Kogaku, Tokyo, Japan) and phase-contrast optics.

Statistical analysis. Data is presented as the mean ± 1 SD unless otherwise indicated. Comparisons between two means were performed by using the two-tailed Student *t*-test.

RESULTS

mAb production and selection. In order to examine the functional properties of p150,95 independent of other adhesion mechanisms, we have developed a protocol to purify intact p150,95 by mAb affinity chromatography. Initial attempts at purifying p150,95 α/β heterodimers using mAb SHCL3 were unsuccessful because p150,95 could not be eluted from the SHCL3 column under conditions required to yield functional material (presence of 2 mM $MgCl_2$). We therefore made mAb to p150,95 purified from hairy cell spleen lysates and selected those mAb that could be eluted from p150,95 under conditions of high pH and in the presence of $MgCl_2$. mAb already described to the p150,95 α-chain when evaluated by the high pH elution ELISA have vastly different abilities to be removed from p150,95 under conditions of high pH and the presence of divalent cations (Fig. 1*A*). Consistent with our findings that p150,95 could not be eluted successfully from SHCL3-Sepharose, mAb SHCL3 was found to be resistant to removal from p150,95 in the high pH elution ELISA in the presence of 2 mM $MgCl_2$. Only 13% of total SHCL3 bound was removed from the solid phase p150,95. mAb BLY6 and F9083 were more readily removed from p150,95 in the presence of Mg^{2+} giving an 89% and 52% reduction, respectively. Elution in the absence of divalent cations showed that all mAb could be removed to a larger degree, however, these conditions caused dissociation of the α and β subunits and were therefore of no practical use. The high pH elution ELISA is therefore useful for selecting mAb that can be eluted from p150,95 in the presence of Mg^{2+}. Reduction in binding activity seen after exposure to high pH was not caused by removal of solid phase p150,95 because treatment of the plates with high pH buffers for a period of 5 min did not cause significant removal of p150,95 as indicated by

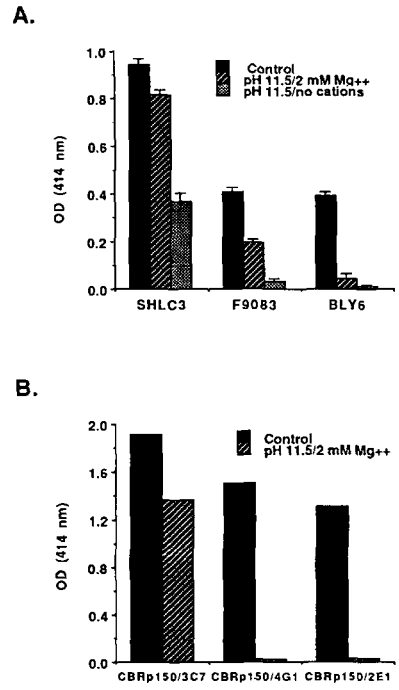


Figure 1. Analysis of CD11c mAb by the high pH elution ELISA. mAb SHCL3, BLY6, and F9083 (*A*) or CBRp150/3C7, CBRp150/4G1, and CBRp150/2E1 (*B*) were bound to purified p150,95 and subject to treatment with high pH buffers containing either 2 mM $MgCl_2$ or no divalent cations as described in *Materials and Methods*. mAb remaining bound after treatment was quantitated by using a rabbit anti-mouse horseradish peroxidase-conjugated second antibody and developed by using a 2,2, azino-di[3 ethylbenthiozoline] sulfonate substrate system.

the retention of activity seen with the SHCL3 mAb after treatment (Fig. 1*A*). Having evaluated the conditions required to select mAb useful for purification of p150,95, hybridoma supernatants were then initially screened on immunopurified p150,95 and Mac-1 to eliminate mAb directed to the common β subunit. Those mAb that reacted with purified p150,95 and not with Mac-1 (74 of 396 wells screened) as judged by a binding enzyme immuno assay on purified p150,95 and Mac-1 (not shown) were subjected to further selection by the high pH elution ELISA. mAb were also further selected for their ability to inhibit 18 to 24-h stimulated HUVEC binding to purified p150,95 (not shown). mAb CBRp150/4G1 and CBRp150/2E1 were effectively removed from p150,95 in the presence of Mg^{2+} compared with mAb SHCL3 (Fig. 1*A* and *B*). Only 2% of mAb CBRp150/4G1 and 4% of mAb CBRp150/2E1 remained attached to the plastic bound p150,95 after treatment (Fig. 1*B*) compared with an average of 72% for SHCL3 (Fig. 1*A*). In contrast, mAb such as CBRp150/3C7 showed similar properties to SHCL3 with 71% being resistant to removal in the high pH elution ELISA (Fig. 1*B*). Overall only about 10% of mAb made to CD11c were as resistant as SHCL3 to removal in the presence of Mg^{2+}. mAb CBRp150/4G1 was chosen for further evaluation because of its ability to be eluted successfully in the high pH elution ELISA.

Immunoprecipitation and flow cytometry were performed to confirm that CBRp150/4G1 was recognizing the α subunit of p150,95 (Fig. 2). CBRp150/4G1 precipitates a heterodimer from hairy cell spleen lysates corresponding to the α- and β-chains of p150,95 (Fig. 2*A*, *lane 2*), which is identical to that precipitated by mAb SHCL3 (Fig. 2*A*, *lane 3*). Flow cytometric analysis of COS cells

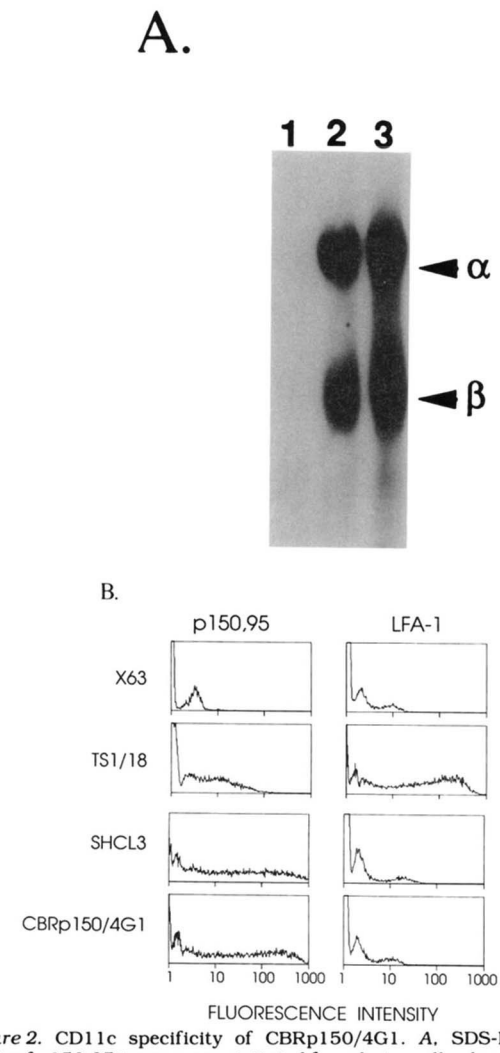


Figure 2. CD11c specificity of CBRp150/4G1. *A*, SDS-PAGE (6%) analysis of p150,95 immunoprecipitated from hairy cell spleen lysates by using either bovine Ig-Sepharose (*lane 1*), CBRp150/4G1-Sepharose (*lane 2*), or SHCL3-Sepharose (*lane 3*). Material eluted from the beads was run under reducing conditions and developed by silver staining. *B*, flow cytometry of COS cells transfected with either p150,95 or LFA-1 cDNA. Transfectants were stained with mAb X63 (nonbinding control), TS1/18 (anti-β subunit), SHCL3 (anti-p150,95 α), and CBRp150/4G1 (anti-p150,95 α) as described in *Materials and Methods*.

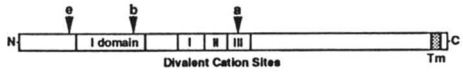
transfected with LFA-1, Mac-1, and p150,95 cDNA shows that CBRp150/4G1 reacts exclusively with p150,95 transfectants and not with LFA-1 or Mac-1 transfectants, which share the common β-chain (Figs. 2*B* and 3). These two pieces of evidence demonstrate the CD11c specificity of mAb CBRp150/4G1.

Epitope mapping. To examine the epitopes on the p150,95 α-chain that mAb CBRp150/4G1 recognize, immunofluorescence flow cytometry was performed on a series of p150,95/Mac-1 chimeric molecules expressed in COS cells (Fig. 3) (see footnote 5). p150,95/Mac-1 α subunit chimeric molecules have been constructed by exchanging a segment or segments of the α subunit cDNA. As expected, CBRp150/4G1 was reactive with p150,95 transfected COS cells but unreactive with those expressing Mac-1, confirming its CD11c specificity. mAb CBRp150/4G1 was unreactive with three of the chimeric molecules, M-b-X-a-M, M-e-X-b-M, and X-a-M, but strongly reactive with X-e-M-b-X, M-a-X, and M-e-X (Fig. 3). This maps the CBRp150/4G1 epitope to a segment between the third divalent cation binding domain and the C terminus. In contrast, mAb SHCL3 only reacts with chimerics X-e-M-b-X and X-a-M (Aguilar et al., unpublished observations). This implies that a region N-terminal to the last divalent cation binding site but excluding the "I" domain is involved in SHCL3 binding.

Immunoprecipitation and purification. CBRp150/4G1 (IgG2a) was selected by using the high pH elution ELISA for its ability to be eluted from p150,95 under conditions anticipated to be useful for the purification of intact p150,95. To test this, small scale purifications of p150,95 from hairy cell spleen lysates were performed (Fig. 4*A*). p150,95 immunoprecipitated by CBRp150/4G1-Sepharose was eluted under conditions of high pH with or without divalent cations, neutralized, then reprecipitated by antibodies directed to the α- and β-chains of p150,95 (Fig. 4*A*). SDS-PAGE of material remaining on the beads (Fig. 4*A*, *lanes 1* to *3*) showed that most of it had been eluted. The α and β subunits were coimmunoprecipitated from the neutralized eluate with anti-α or anti-β mAb, showing that the α/β heterodimer remained intact after elution in the presence of Mg^{2+} (Fig. 4*A*, *lanes 4* and *8*) or Ca^{2+} (Fig. 4*A*, *lanes 5* and *9*). With no divalent cations present, the α- and β-chains were largely dissociated after high pH treatment of CBRp150/4G1-Sepharose; only small amounts of intact heterodimer was immunoprecipitated (Fig. 4*A*, *lanes 6* and *10*). In contrast, attempts to elute p150,95 from SHCL3-Sepharose under the same conditions were unsuccessful; the majority of the p150,95 remained bound to the beads after elution in the presence of Mg^{2+} or Ca^{2+} (Fig. 4*B*, *lanes 1* and *2*). Reprecipitation of p150,95 eluted from the SHCL3-Sepharose showed that only a small amount of heterodimer could be detected (Fig. 4*B*, *lanes 3* and *4*). p150,95 could only be removed from SHCL3-Sepharose in the absence of divalent cations and, as was the case with CBRp150/4G1, this resulted in dissociation of the subunits. Other elution conditions of pH 10.0 to 12.0, 150 to 500 mM NaCl and 0 to 2.0 mM $MgCl_2$ were also evaluated, however, these all gave reduced yields of intact α/β heterodimer (data not shown). Optimal conditions for elution from CBRp150/4G1 were therefore found to be 50 mM TEA (pH 11.0), 400 mM NaCl, and 2 mM $MgCl_2$.

Large scale purification of p150,95 was performed by using hairy cell spleen lysates that express high levels of p150,95 as previously reported (16, 19). Fractions eluted from the CBRp150/4G1 column were analyzed by reducing SDS-PAGE, which showed that the predominant species isolated was the p150,95 α/β heterodimer (Fig. 5). Reprecipitation with SHCL3-Sepharose confirmed that these bands corresponded to p150,95 α and β subunits and that these had remained intact during elution with 50 mM TEA, 400 mM NaCl, and 2 mM $MgCl_2$ (data not shown) (Fig. 4*A*). Comparision to stained m.w. standards indicated that a yield of approximately 200 to 400 μg of p150,95 had been obtained from 30 g of hairy cell spleen.

Functional analysis. To determine whether p150,95 could mediate cellular adhesion, purified material was absorbed to plastic microtiter wells at concentrations similar to those that have been shown to allow binding of cells to purified, plastic-bound LFA-1 and Mac-1 (6) (see footnote 4). Previous reports have suggested that p150,95 may play a role in the attachment of granulocytes to endothelium stimulated for 18 h with rIL-1β (37). Therefore, we assessed the effect of stimulating endothelial cells with inflammatory mediators on their ability to



Chimerics	X63	TS1/18	CBRp150/4G1
	Fluorescence Intensity		
αM	8	36	9
αX	11	55	68
M-b-X-a-M	10	48	10
M-e-X-b-M	13	38	12
X-e-M-b-X	8	41	81
X-a-M	11	62	12
M-a-X	14	42	51
M-e-X	11	41	66

Figure 3. Schematic representation and flow cytometric analysis of p150,95/Mac-1 chimeric molecules. mAb used for flow cytometric analysis were X63 (nonbinding control), TS1/18 (anti-β-chain) and CBRp150/4G1 (anti-p150,95 α-chain). Chimeric molecules were constructed by the ligation of fragments from either p150,95 cDNA or Mac-1 cDNA. Those parts of the chimers from p150,95 are represented by *closed bars*, those from Mac-1 are represented by *open bars*. See footnote 5 for a more comprehensive description of the chimeric molecules. Values represent the mean channel number of fluorescence intensity.

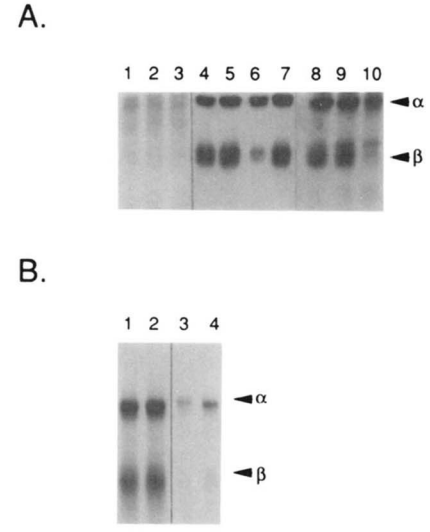


Figure 4. Small scale purification of p150,95 analyzed by SDS-PAGE. p150,95 was immunoprecipitated by using either CBRp150/4G1-Sepharose (*A*) or SHCL3-Sepharose (*B*), eluted with 50 mM TEA, 150 mM NaCl (pH 11.0) containing either 2 mM $MgCl_2$ 2 mM $CaCl_2$, or no divalent cations, neutralized, and reprecipitated with either SHCL3-Sepharose or TS1/18-Sepharose. Elutions and reprecipitations were performed in the presence of either 2 mM $MgCl_2$ (*A*, *lanes 1*, *4*, and *8* and *B*, *lanes 1* and *3*); 2 mM $CaCl_2$ (*A*, *lanes 2*, *5*, and *9* and *B*, *lanes 2* and *4*); or no divalent cations (*A*, *lanes 3*, *6*, and *10*). Material remaining on the beads after the elution step is shown in *A* (*lanes 1* to *3*) and *B* (*lanes 1* and *2*). Neutralized eluate reprecipitated by SHCL3 anti-α-chain mAb is shown in *A*, (*lanes 4* to *6*) and *B* (*lanes 3* and *4*). Neutralized eluate reprecipitated by TS1/18 anti-β-chain mAb is shown in *A* (*lanes 8* to *10*). Figure 4*A*, *lane 7* represents the amount of p150,95 initially immunoprecipitated by the CBRp150/4G1-Sepharose beads. Material was eluted with SDS sample buffer and analyzed by 6% SDS-PAGE and silver staining. Only the relevent portions of the gels are shown.

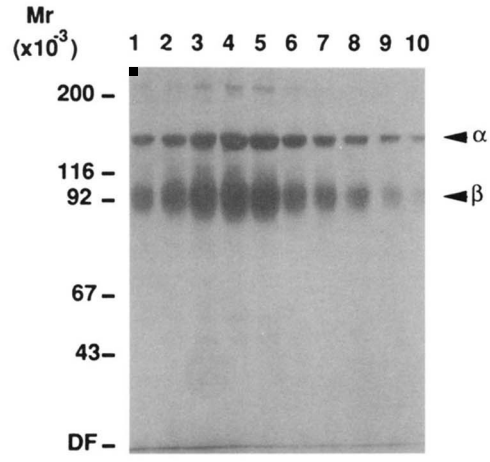


Figure 5. Large scale purification of p150,95 on CBRp150/4G1-Sepharose. SDS-PAGE analysis of 1-ml fractions eluted from a CBRp150/4G1-Sepharose column. A total of 12.5 μl of each fraction was diluted with an equal volume of 2 × SDS-PAGE sample buffer, boiled, loaded, and silver stained as described in *Materials and Methods*.

bind purified p150,95.

Unstimulated endothelial cells bound to p150,95 at a level of 13% of input cells (Fig. 6*A*). However, stimulation of endothelial cells with either rIL-1β or LPS for a period of 18 h caused a significant increase in the level of binding observed to p150,95 at a site density of 1200 sites/μm^2 (Fig. 6*A*). Both the binding of unstimulated and stimulated endothelial cells to p150,95 was also significantly different from the binding seen to wells coated with HSA alone.

The interaction of stimulated HUVEC with purified p150,95 could be specifically inhibited by mAb to the p150,95 α-chain. Specific binding to purified p150,95 of HUVEC stimulated with rIL-1β was reduced 96% by mAb CBRp150/4G1 and 89% by mAb SHCL3 (Fig. 6*B* and 7). The binding could also be reduced by 95% with 10 mM EDTA showing the divalent cation-dependent nature of the p150,95-HUVEC interaction.

To determine the amount of p150,95 required to mediate adhesion of stimulated HUVEC, binding assays were performed by using a range of p150,95 site densities. At a density of greater than 900 to 1000 sites/μm^2, p150,95 mediated binding of rIL-1β-stimulated HUVEC; below this level the binding was reduced to that seen on HSA-coated wells alone (Fig. 6*C*). Saturation of p150,95 binding appeared at levels > 1200 sites/μm^2 with 50% of maximal binding occuring at 1100 sites/μm^2. The binding of stimulated HUVEC was therefore highly dependent on the density of p150,95. In contrast, binding to LFA-1 was seen at much lower densities, with about one-half as much LFA-1 required to mediate the same percentage binding of HUVEC. This implies a difference in the avidity of p150,95 and LFA-1 for their respective ligands on HUVEC. The degree of washing had a marked effect on the percentage of HUVEC binding to p150,95. Binding assays shown in Figure 6 were generated by washing with a 26-gauge needle; however, the percentage of bound

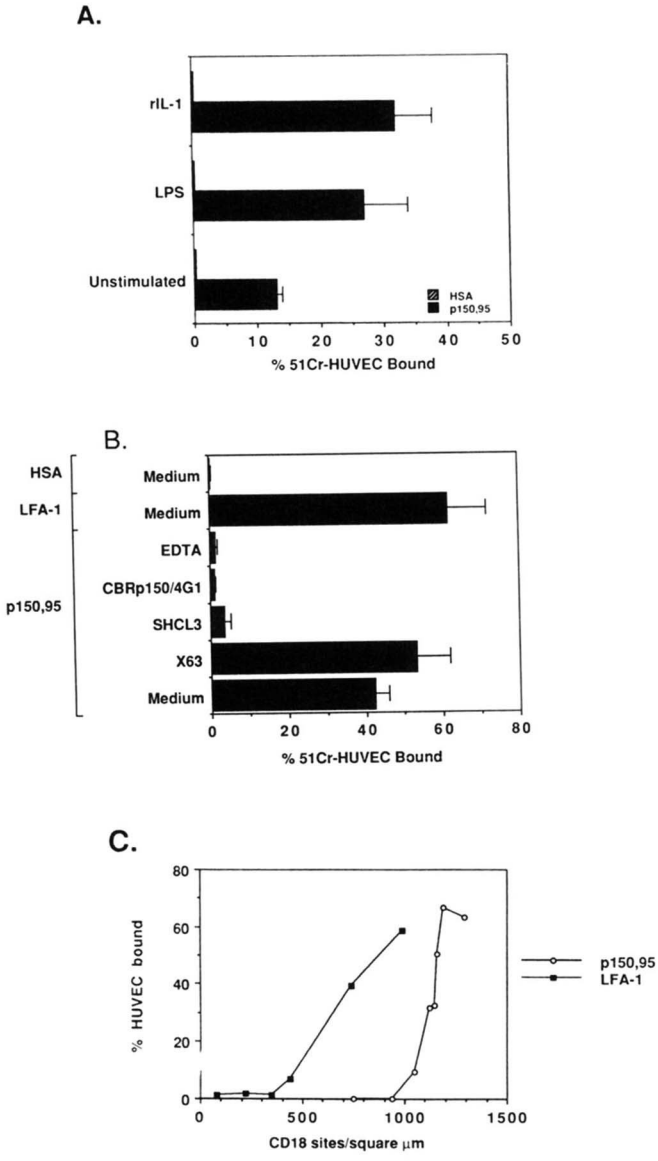


Figure 6. Binding of rIL-1β-stimulated HUVEC to purified p150,95. *A*, adhesion of unstimulated or stimulated [^{51}Cr]HUVEC to purified p150,95. HUVEC were incubated with medium or medium containing either 5 U/ml of rIL-1β or 5 ng/ml of LPS for 18 to 24 h, washed, and added to plates coated with p150,95 (site number of 1200 sites/μm^2). After incubation, plates were washed three times and bound cells eluted for γ-counting. Data is representative of two experiments. *B*, adhesion of rIL-1β-stimulated [^{51}Cr]HUVEC to purified p150,95. Stimulated HUVEC were washed and added to HSA, p150,95 or LFA-1-coated plates pretreated with mAb X63, CBRp150/4G1, or SHCL3. Plates were pretreated with mAb for 30 min at 4°C. After incubation plates were washed three times and bound cells eluted for gamma counting. Data is representative of four experiments. *C*, adhesion of stimulated [^{51}Cr]HUVEC to various densities of purified p150,95 and LFA-1. HUVEC were stimulated for 18 to 24 h with 5 U/ml of rIL-1β, washed, and added to plates coated with varying concentrations of p150,95 and LFA-1. Site densities were determined by using saturating amounts of [^{125}I]CBRp150/4G1 or [^{125}I]TS1/18. *Error bars* are not shown but were less than 12% in all cases. Data is representative of two experiments.

cells could be further reduced or increased depending on the stringency of washing (data not shown). Using a more gentle washing protocol, binding of stimulated HUVEC to p150,95 was seen at site densities of 500 sites/μm^2. Nevertheless, the relative ability of p150,95 and LFA-1 to bind cells remained the same under these washing conditions.

DISCUSSION

Studies using mAb specific for the p150,95 α subunit (CD11c) to inhibit cell binding have shown that they can

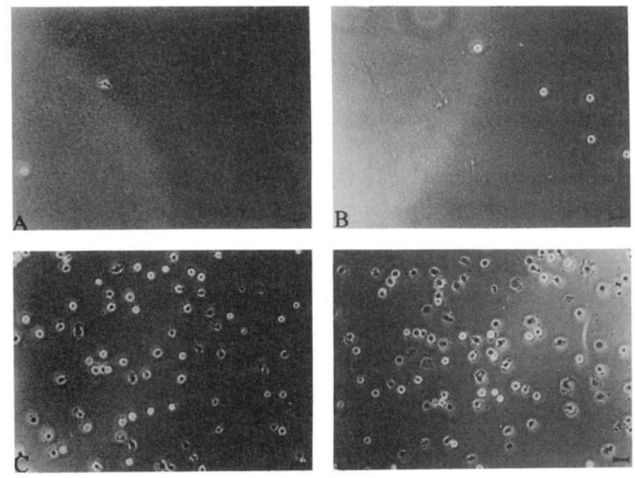


Figure 7. Photomicrographs of rIL-1β-stimulated HUVEC binding to HSA (*A*), p150,95 (*B*) and (*C*), or LFA-1 (*D*) coated wells in the presence of no antibody (*A*), CBRp150/4G1 (*B*) and (*D*), or X63 (*C*). *Bars* represent 20 μm.

modulate the adhesion of monocytes and granulocytes to endothelium, tumor cells, and adhesive substrates (9, 24, 25, 37). Nevertheless, the exact mechanisms whereby these mAb exert their effects in these systems is not clear. Inhibition of cell binding by anti-p150,95-specific mAb could also be explained by negative signal transduction. This theory would require p150,95 to function as an essential membrane protein which, when bound by a mAb, reduces adherence functions of cells while not being directly involved in adhesion. Identification of potential counter-receptors for p150,95 would confirm the direct involvement of p150,95 in these interactions; however, a number of factors have hindered attempts to define such molecules. p150,95 is expressed highly on only very few cell types and cell lines (e.g., monocytes and tissue macrophages) and these cells are difficult to obtain in large quantities for in vitro assays. In addition, p150,95 is frequently co-expressed on many of these cell types with Mac-1 and less often with LFA-1, molecules that contribute to the adhesion of leukocytes to similar or identical target cells and substrates. Therefore, assessing the real contribution of p150,95 to leukocyte adhesion has been hindered somewhat by the activity of other members of the leukocyte integrins. In this study we examined whether p150,95 can act directly as an adhesion-receptor independent of LFA-1, Mac-1, and other adhesion receptors by purifying p150,95 in a functional form by mAb affinity chromatography and assessing its cell-binding capability. We have demonstrated that p150,95 has a counter-receptor that is present on vascular endothelium and that can be stimulated by inflammatory mediators.

The purification protocol developed here to isolate functional p150,95 relies on the selection of a mAb to CD11c that allows for the elution of p150,95 under conditions of high pH and the presence of 2 mM Mg^{2+}. An essential structural feature of the p150,95 α subunit is the three divalent cation binding sites located in the extracellular domain that are common to all the leukocyte integrins (18). Leukocyte integrin activity is dependent on the presence of divalent cations; EDTA inhibits all known integrin functions. Magnesium is thought to be the essential functional cation for p150,95 as Mg^{2+} and not Ca^{2+} is required for the p150,95-dependent binding of mono-

cytes to endothelium (25). Presumably the divalent cations act in some way to form the binding site of the active molecule (18). High pH was used for the elution of functional p150,95 as residues within the divalent cation binding site that appear critical for chelating the Mg^{2+} ions are acidic in nature (Asp and Glu) and would thus remain ionized and available to bind Mg^{2+} ions under these conditions (18). Elution of p150,95 from SHCL3 was not possible at concentrations of 0.5 to 2.0 mM $MgCl_2$ indicating that when Mg^{2+} is present pH 11.5 cannot disrupt this association. In contrast, a mAb selected by using the high pH elution ELISA, CBRp150/4G1, was reactive to a region C-terminal to the divalent cation-binding sites and could be dissociated under conditions deemed optimal to yield functional p150,95. mAb LM2/1, used to purify functional Mac-1 by affinity chromatography, also mapped outside the divalent cation-binding sites but within the "I" domain of αM (see footnote 4) (J. Garcia-Aguilar and T. A. Springer, unpublished observations). Elution of p150,95 in the absence of divalent cations led to extensive dissociation of the α and β subunits indicating that Mg^{2+} stabilized the association of the two chains during the high pH elution. This study underlines the importance of selecting an appropriate mAb for the functional purification of integrins. Clearly the principle of the high pH elution ELISA described here could be applied to other members of the integrin family.

Purified integrins absorbed to plastic surfaces function in binding ligand positive cells. LFA-1 and Mac-1 purified by mAb affinity chromatography have both been shown to mediate binding to ICAM-1-expressing cells under these conditions indicating that the binding sites remain essentially intact after absorption to plastic (6, 30) (see footnote 4). This approach was used to analyze the capacity of purified p150,95 to mediate cell binding as it effectively eliminates the contributions of other cell-surface proteins, a problem with previous studies examining p150,95 function. Stimulated HUVEC bound to purified p150,95 and this binding could be neutralized by using anti-CD11c mAb. The binding was site number dependent, and minimal binding was seen at levels below a threshold of 1000 sites/μm^2. These levels are higher than that required by LFA-1 and Mac-1 to mediate binding to stimulated HUVEC in a similar assay and may indicate that the p150,95 interaction with its ligand on HUVEC is of a lower avidity than either LFA-1 or Mac-1. It may also indicate that p150,95 has to be at a high cell-surface density or that the receptors have to be aggregated to mediate binding.

Previous studies by Luscinskas et al. (37) have shown that mAb L29 (anti-CD11c) inhibits p150,95-dependent binding of neutrophils to stimulated endothelium whereas SHCL3 does not. Others have shown that SHCL3 can inhibit the interaction of human monocytes with unstimulated endothelial cells (25). Our study showed that both SHCL3 and CBRp150/4G1, which maps to the same region of the p150,95 α subunit as L29 (J. Garcia-Aguilar and T. A. Springer, unpublished observations), and SHCL3 inhibit p150,95-dependent binding of HUVEC to purified p150,95. This could indicate that multiple regions of the p150,95 α subunit are involved in the interaction of p150,95 to its ligand on HUVEC. Disparities in mAb inhibition seen in the various studies may be because of differences between cell-cell and cell-plastic-bound protein interactions as previously described (17) or, alternatively, differences between stimulated and unstimulated endothelial cells or presentation of p150,95 on the different cell types.

A number of molecules have been described on the endothelial cell surface that are involved in mediating cell adhesion and whose expression is regulated by inflammatory cytokines. ICAM-1 (38) and VCAM-1, (39), are both upregulated on HUVEC by rIL-1β with maximal expression 18 h after stimulation. ELAM-1 (40) is upregulated by rIL-β; however, its expression is maximal after 4 h and declines by 18 to 24 h. ICAM-1 and VCAM-1, but not ELAM-1, are good candidates as counter-receptors for p150,95 as their expression correlates with the binding to purified p150,95 seen with endothelial cells stimulated for 18 to 24 h. Given the high degree of structural and functional homology between Mac-1/CR3 and p150,95, it is likely that p150,95 not only binds an ICAM-1-like ligand on the endothelial cell surface but other ligands as well.

The results presented in this study show that affinity-purified p150,95 can function as an adhesion molecule independent of other cellular adhesion mechanisms. This implies that previous data examining the adhesion of p150,95 bearing cells to tumor cells and endothelium most likely reflected the inhibition of receptor-ligand interactions rather than the transmission of negative signals to the cell. The affinity of p150,95 for its counter-receptor on HUVEC appears to be lower than that of LFA-1 and Mac-1 for ICAM-1 and it is therefore possible that other higher affinity counter-receptors may exist on other tissues for p150,95. This would be consistent with the finding that all integrins described to date appear to have at least one high affinity ligand.

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