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Biosynthesis and Function of Membrane Bound and Secreted Forms of Recombinant CD11b/CD18 (Mac-1)

Phillip W. Berman, Gerald R. Nakamura, Lavon Riddle, Henry Chiu, Karen Fisher, Mark Champe, Alane M. Gray, Pamela Ward, and Sherman Fong

Departments of Immunology (P.W.B., G.R.N., H.C., M.C., A.M.G., P.W., S.F.), Process Sciences (L.R.), and Cardiovascular Biology (K.F.), Genentech, Inc., South San Francisco, California 94080

Abstract Full-length (membrane bound) and truncated (secreted) forms of the beta 2 integrin heterodimer, CD11b/CD18 (Mac-1), were expressed in a human kidney cell line (293) that normally does not express leukocyte adhesion molecules (Leu-CAMs). The biosynthesis of recombinant Mac-1 in 293 cells differed from that reported for leukocytes in that heterodimer formation was not required for CD11b to be exported to the cell surface. A stable cell line was constructed that constitutively secreted the recombinant, truncated Mac-1 heterodimer into growth conditioned cell culture medium. A novel monoclonal antibody that enabled an immunoaffinity method for the selective purification of recombinant Mac-1 heterodimers was identified. Sufficient protein was purified to allow the first measurement of the 50% inhibitory concentration (IC_{50}) for CD11b/CD18 and for the direct comparison of the inhibitory activity of recombinant soluble Mac-1 with that of various CD18 and CD11b specific monoclonal antibodies. Purified recombinant soluble Mac-1 inhibited the binding of neutrophils, activated by opsonized zymosan or fMet-Leu-Phe peptide, to human umbilical vein endothelial cells. Similarly, the recombinant integrin was effective in inhibiting the binding of unactivated neutrophils to tumor necrosis factor (TNF-alpha) activated endothelial cells. The availability of an abundant source of purified, biologically active Mac-1 will enable direct physical and chemical investigations into the relationship between the structure and function of this leukocyte adhesion molecule.

Key words: integrin, CD11b/CD18, Mac-1, Leu-CAM, neutrophil

The cell surface adhesion molecule, CD11b/CD18 (Mac-1), occurs on granulocytes and monocytes, is the receptor for the C3bi component of complement, and mediates cell-to-cell and cell-to-subtratum adherence [Sanchez-Madrid et al., 1983; Wright et al., 1983; Kishimoto et al., 1989; Arnaout, 1990]. Mac-1 plays a central role in inflammation, and individuals with a genetic deficiency of CD18 (leukocyte adhesion deficiency) are severely deficient in granulocyte function and their inflammatory response [Arnaout et al., 1984; Springer et al., 1984; Anderson and Springer, 1987]. Accordingly, it has been suggested that inhibitors of Mac-1 might have clinically important anti-inflammatory applications [Arfors et al., 1987; Simpson et al., 1988]. Because granulocytes typically express both Mac-1 and the closely related leukocyte adhesion molecules (Leu-CAMs), LFA-1 (CD11a/CD18), and p150,95 (CD11c/CD18), it has previously been difficult to study the ligand binding properties and signal transduction mechanisms of Mac-1 in isolation. Like other members of the integrin family [Ruoslahti and Pierschbacher, 1986; Hynes, 1987], Mac-1 is promiscuous and binds multiple ligands [Wright et al., 1983; Arnaout, 1990; Altieri and Edgington, 1988b; Altieri et al., 1990; Diamond et al., 1990], some of which (e.g., ICAM-1), also bind to LFA-1. Moreover, granulocytes possess other adherence mechanisms (e.g., Lec-CAMs) whose adhesive properties overlap, to some extent, those of the beta 2 integrins [Luscinskas et al., 1989; Jutila et al., 1989]. The development of systems to study beta 2 integrins individually, in the absence of interactions mediated by other adhesion molecules, should help clarify the specificity and function of these molecules.

In the present study we describe the biosynthesis of membrane bound and secreted variants of Mac-1 in the 293 (embryonic human kidney adenocarcinoma) cell line [Graham et al., 1977] that normally does not express members of the

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Address reprint requests to Phillip W. Berman, Ph.D., Department of Immunobiology, Genentech, Inc., 460 Pt. San Bruno Blvd., South San Francisco, CA 94080.

beta 2 integrin family [McLean et al., 1990]. These studies demonstrated that the biosynthesis and export of CD11b in 293 cells differed from that reported for leukocytes [Ho and Springer, 1983] in that heterodimer formation between CD11b and CD18 was not required for export to the cell surface. In addition, we describe the construction of a stable cell line that constitutively secreted a truncated form of the Mac-1 heterodimer that lacked the transmembrane domains and cytoplasmic tails of both CD11b and CD18. Because Mac-1 is known to mediate the attachment of granulocytes to plastic [Dana et al., 1986; Anderson et al., 1986], the isolation of biologically active, noncovalently associated Mac-1 heterodimers represented a particularly challenging purification problem. In this study we report the identification of unique monoclonal antibody that specifically recognized mature Mac-1 heterodimers and allowed for the recovery of intact, functional CD11b/CD18 complexes from growth conditioned cell culture medium. The resulting recombinant secreted Mac-1 (rsMac-1) was evaluated in several functional assays. It was found that purified rsMac-1 was able to block the binding of activated neutrophils to unactivated endothelial cells and the binding of unactivated neutrophils to cytokine activated endothelial cells. These studies demonstrated that rsMac-1 is an effective inhibitor of granulocyte:endothelial cell interactions with a 50% inhibitory concentration (IC_{50}) in the 10^{-7} M range. The availability of a convenient source of purified rsMac-1 will enable in vitro assays to evaluate potential inhibitors inhibitors of Mac-1 and direct physical and biochemical studies to define the relationship between the structure and function of Mac-1.

MATERIALS AND METHODS

Cloning and Mutagenesis of the cDNAs Encoding CD11b and CD18

Standard techniques [Ausubel et al., 1989] were employed to clone cDNAs encoding CD18 and CD11b. CD18 was cloned from a λgt-10 cDNA library prepared from oligo-dT primed U937 cell mRNA. CD11b was cloned from a λgt-10 cDNA library prepared from random primed U937 and peripheral blood mononuclear cell mRNA. Phage containing the 5′ and 3′ fragments of the genes were identified with the use of synthetic DNA probes synthesized on the basis of published sequence data for CD18 [Kishimoto et al., 1987] and CD11b [Arnaout et al., 1988] and subcloned into the bacteriophage M13 [Messing, 1983] for oligonucleotide sequence analysis [Sanger et al., 1977]. Throughout this paper the length of proteins is specified with respect to the initiator methione as residue 1. Full-length CD18 cDNA was contained on a 2.7 kb SalI to DraI fragment and encoded a protein 769 amino acids in length. Full-length CD11b cDNA was contained on a 4.0 kb ClaI to XbaI fragment and encoded a protein of 1,153 amino acids. A truncated form of the CD18 gene (rsCD18), which lacked sequences corresponding to the transmembrane domain and cytoplasmic tail, was prepared by insertion of a stop codon (TAA) at residue 2100 and encoded a protein of 700 amino acids. A truncated form CD11b (rsCD11b) that similarly lacked the transmembrane domain and cytoplasmic tail was prepared by insertion of a stop codon (TAA) at residue 3324 and encoded a protein of 1,108 amino acids (Fig. 1).

Expression of CD11b and CD18 in 293 Cells

Chimeric transcription units for the expression of full-length or truncated CD11b or CD18 were created by cloning the integrin genes into the pRK 5 and pRK 7 expression plasmids [Eaton et al., 1986] that provided a CMV promoter and SV-40 polyadenylation sites (Fig. 1). The plasmids encoding full-length CD11b and CD18 were transfected into the 293 human kidney adenocarcinoma cell line [Graham et al., 1977] using a $CaPO_4$ precipitated plasmid DNA [Graham and Van der Eb, 1973]. Three days after transfection, the cells were metabolically labeled with ^{35}S-methionine and detergent solubilized cell lysates or growth conditioned cell culture medium was immunoprecipitated as described previously [Berman et al., 1988]. Cell surface labeling with [$Na^{125}I$] was accomplished using a lactoperoxidase catalyzed labeling technique similar to that described by Hynes [1973]. A continuous cell line expressing recombinant soluble Mac-1 (rsMac-1) was constructed by co-transfection of a plasmid containing cDNAs for both rsCD11b and rsCD18 along with another plasmid containing a murine cDNA encoding the selectable marker, dihydrofolate reductase [Simonson and Levinson, 1983] into 293 cells.

Immunoprecipitation and Endoglycosidase Digestions

The CD18 specific monoclonal antibodies (MAbs), MHM.23 [Hildreth and August, 1985], H52 [Hildreth et al., 1983], and PLM-2 [Hildreth et al., 1989], and the CD11b specific MAb,

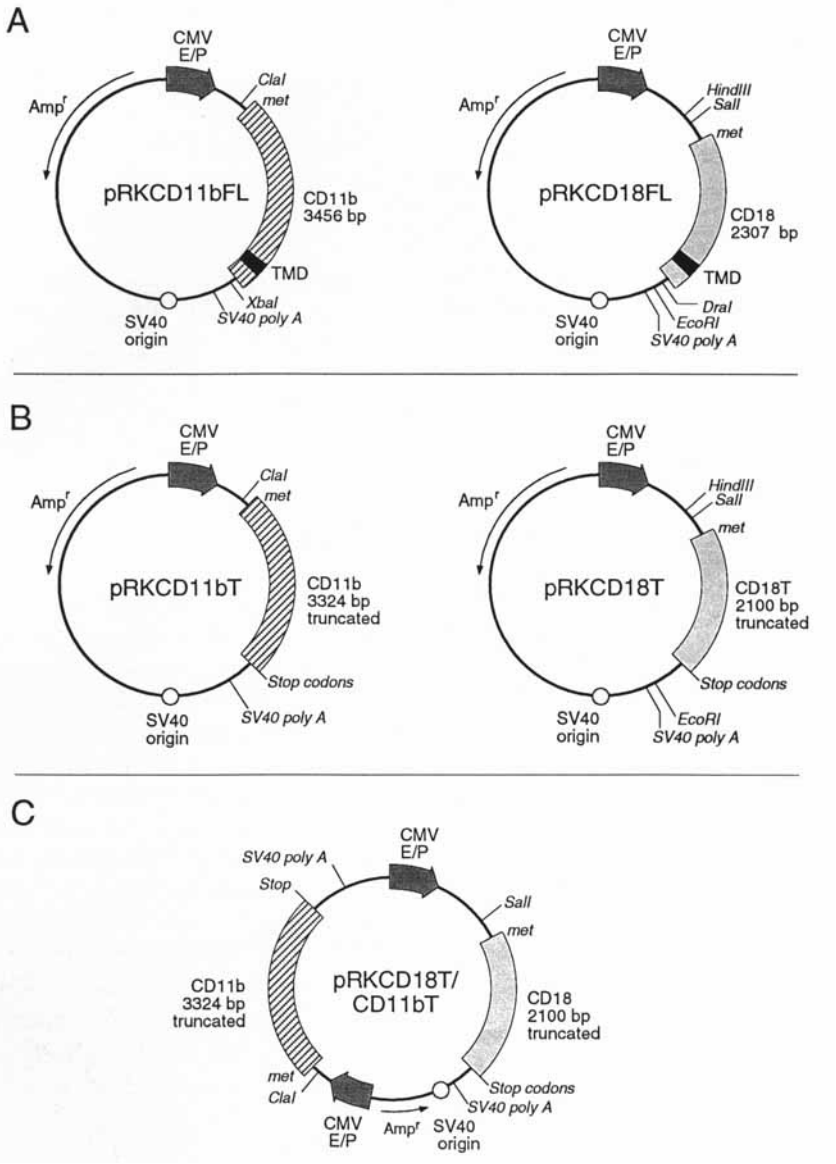

Fig. 1. Schematic diagrams of plasmids containing full-length and truncated CD11b and CD18 genes for expression in 293 cells. **A:** Plasmids for the transient expression of full-length CD11b and CD18. **B:** Plasmids for the transient expression of truncated CD11b and CD18. **C:** Plasmid for expression of rsMac-1 in a stable cell line.

H5A4 [Hildreth and August, 1985], were kindly provided by Dr. James Hildreth (Johns Hopkins University Medical School, Baltimore, MD). For studies of Mac-1 function, $F(ab')_2$ fragments of the CD11b specific MAb, 904 [Dana et al., 1986], were employed (Coulter Pharmaceuticals, Hialeah, FL). Radioimmunoprecipitation of CD11b or CD18 from metabolically labeled cells was carried out as described previously [Berman et al., 1988]. Carbohydrate analysis of metabolically labeled, immunoprecipitated proteins was performed using the enzymes endo-β-N-acetylglucosaminidase H (EndoH) [Tarentino et al., 1974] and piptid:N-glycosidase F (N-glycanase) [Tarentino et al., 1985] according to a protocol similar to that described previously [Berman et al., 1988]. The immunoprecipitated proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 7% gels according to the method of Laemmli [1970] and visualized by autoradiography.

Purification of rsMac-1

An immunoaffinity column to purify rsMac-1 was prepared by coupling the CD18 specific monoclonal antibody, MHM.23, to glycerol coated controlled pore glass matrix (Electro-Nucleonics Corp., Fairfield, NJ) using a modifi-

cation of the method described by Roy et al. [1984]. Antibodies were coupled at a concentration of 8 mg per milliliter of resin, and the columns were repeatedly washed by cycling at high and low pH. The columns were equilibrated in 0.02 M Hepes buffer containing 0.15 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (pH 7.0). Clarified, filter sterilized cell culture supernatants were then directly applied to the affinity column. After extensive washing with equilibration buffer, the rsMac-1 was eluted with 0.02 M Tris buffer containing 0.5 M NaCl, pH 9.0, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The eluate was neutralized by the addition of 1.2 N HCl and concentrated by ultrafiltration using a Centricon 30 ultrafiltration membrane. Protein concentration was determined spectrophotometrically using a calculated extinction coefficient of 0.65. The final material was dialyzed to a concentration of 0.15 mg/ml in 0.02 M Tris buffer containing 0.5 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4. The resulting preparation was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized by silver staining [Morrisey, 1981]. Final yields of rsMac-1 ranged from 0.1–0.5 mg per liter of cell culture supernatant.

Isolation, Purification, and Labeling of Human Neutrophils

Heparinized (10 U/ml) venous blood was obtained from healthy volunteers and mixed with an equal volume of 3% (w/v) Dextran T-500 (Pharmacia, Piscataway, NJ) in saline and sedimented at room temperature for 30 min. The peripheral blood leukocytes located in the top layer were placed in a 50 ml conical polypropylene centrifuge tube and underlayed with approximately 15 ml of Lymphocyte Separation Medium (Organon Teknika, Durham, NC). The tubes were centrifuged for 40 min at 22°C at 400*g*, and neutrophils were isolated from the pellet of the density gradient separation by hypotonic lysis of the erythrocytes. The cell pellet was resuspended in 10 ml of 0.2% (w/v) NaCl and vortexed for 20 seconds followed by the addition of an equal volume 1.6% NaCl. The cells were again pelleted, the lysis procedure repeated, and the cells washed twice and resuspended at 10×10^6 cells/ml in Hanks balanced salt solution (HBSS) without Ca^{++} and Mg^{++}. Neutrophils were then labeled with 1 ml of 2′,7′-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein, acetoxymethyl ester (BCECF) purchased from Molecular Probes (Eugene, OR) for 20–30 min at 37°C as described by Gimbrone et al. [1989].

Adherence of Neutrophils to Endothelial Cell Monolayers

Neutrophil adhesion to human umbilical vein endothelial cells (HUVEC) was quantified by an adhesion assay similar to that described by Gimbrone et al. [1989]. HUVEC and media were purchased from Cell Systems (Kirkland, WA). HUVEC were plated in 96 well microtiter plates at 3×10^4 cells/well and allowed to attach overnight. Media was removed and purified antibodies or rsMac-1 was added to quadruplicate cultures in 25 μl (4× the final concentration). BCECF labeled neutrophils were then added (2×10^5 cells/well in 50 μl). Neutrophils were activated by the addition of 25 μl of either formyl methionyl-leucyl-phenylalanyl peptide (fMLP) or opsonized zymosan (OpZy). Tumor necrosis factor-α (TNF-α) activation of HUVEC was accomplished by addition of TNF-α to the HUVEC at time of plating. To assess binding, BCECF labeled neutrophils (activated or unactivated) were incubated with the HUVEC for 10 min at 37°C and the plates were then sealed, inverted, and centrifuged at 400*g* for 5 min. The supernatants and unattached cells were aspirated and the cells attached to the HUVEC monolayer were solubilized with 0.1% SDS in 50 mM Tris (pH 8.5). The fluorescence intensity was measured at 485/535 nm in a Pandex Fluorescence Concentration Analyzer (Mundelein, IL).

RESULTS

Expression of Membrane Bound Mac-1

cDNAs corresponding to the coding regions of CD11b and CD18 were identified in λgt-10 libraries and subcloned into the bacteriophage, M13, for oligonucleotide sequence analysis. The sequence obtained for CD11b agreed with that published by Larson et al. [1989] and differed from that published by Arnaout et al. [1988] in that a glutamine residue occurred at residue 510. The sequence obtained for CD18 agreed perfectly with that of Kishimoto et al. [1987]. In order to produce secreted variants, the sequences encoding the transmembrane domains and cytoplasmic tails of the CD11b and CD18 cDNAs were deleted by in vitro mutagenesis where stop codons were inserted at positions immediately adjacent to the first residue of the

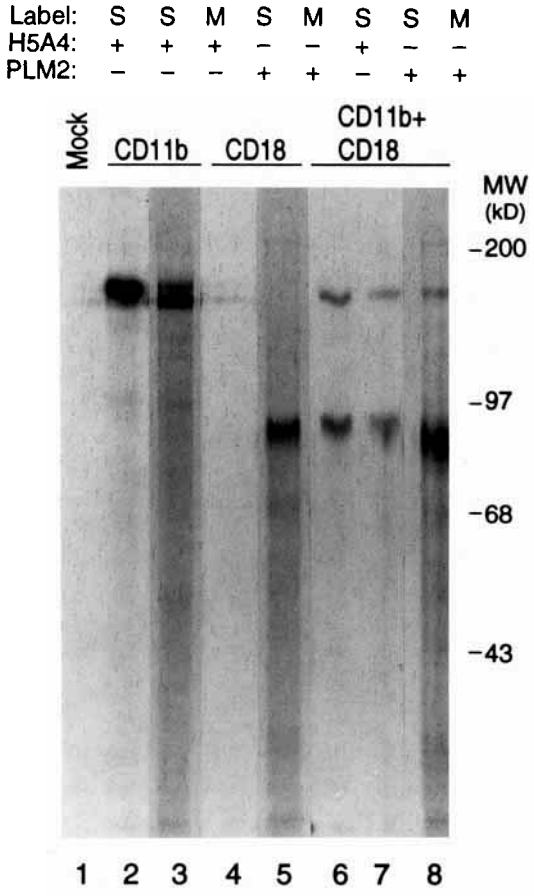

Fig. 2. Immunoprecipitation of CD11b, CD18, or Mac-1 heterodimers from transfected 293 cells Cells were transfected with plasmids for the expression of CD11b alone (CD11b) or CD18 alone (CD18) or cotransfected with both CD11b and CD18 expression plasmids (CD11b + CD18) using a $CaPO_4$ precipitate transfection technique Three days after transfection, the cells were surface labeled (S) with $Na^{125}I$ using a lactoperoxidase catalyzed technique, or metabolically labeled (M) with ^{35}S-methionine MAbs to CD11b (H5A4) or CD18 (PLM2) were used to immunoprecipitate the radiolabeled integrins from cell lysates The immunoprecipitated proteins were resolved by SDS-PAGE using 7% gels and visualized by autoradiography The mobilities of molecular weight markers are indicated in the margin

transmembrane domains (nucleotide 3324 of CD11b and nucleotide 2100 of CD18). The resulting mutants encoded proteins of 1,108 and 700 amino acids, respectively. Antibodies known to be specific for CD11b (H5A4) or CD18 (PLM2) were used to distinguish between the two molecules [Hildreth et al., 1983, 1989]. When lysates of cells transfected with the full-length CD11b gene were immunoprecipitated with the H5A4 MAb, two species of CD11b could be identified (Fig. 2, lane 3), a 165 kD band and 155 kD band. These species were similar to those reported to occur in lymphoid cells [Ho and Springer, 1983; Sanchez-Madrid et al., 1983] and appeared to represent the intracellular high mannose precursor form of CD11b and the higher molecular weight, sialic acid containing, mature form of CD11b. The occurrence of the high molecular weight form was surprising and suggested that CD11b in the absence of CD18 might be transported to the cell surface. To verify that CD11b was transported to the plasma membrane, cell surface iodination studies were conducted. It was found (Fig. 2, lane 2) that only the high molecular weight form of CD11b could be visualized by cell surface iodination, and that the low molecular weight species could only be visualized by metabolic labeling (Fig. 2, lane 3). Thus, CD11b did not require heterodimer formation with CD18 for export to the surface of 293 cells.

When 293 cells were transfected with CD18 alone a single band of 87 kD could be detected by metabolic labeling (Fig. 2, lane 5), but not by cell surface iodination (Fig. 2, lane 4). This result suggests that CD18 was retained in an intracellular compartment and was not, by itself, exported to the cell surface. When 293 cells were transfected with both CD11b and CD18, two species of CD18 could be visualized, a 87 kD species and a 92 kD species (Fig. 2, lane 8). This result suggested that co-transfection with CD11b allowed CD18 to be exported to the plasma membrane. Cell surface iodination studies demonstrated that CD18 appeared on the cell surface only after cotransfection with CD11b. Since both the H5A4 MAb (Fig. 2, lane 6) and the PLM-2 MAb (Fig. 2, lane 7) co-precipitated both the CD11b and CD18 bands after cell surface labeling, it appeared that both molecules occur on the surface as a stable heterodimer. In addition these studies show that the only heterodimers that are visualized are those consisting of the high molecular weight forms of CD11b and CD18.

Expression of Truncated, Secreted Mac-1

Plasmids containing genes encoding truncated CD11b (rsCD11b) and truncated CD18 (rsCD18) were transfected into 293 cells and analyzed as described above. When cells were transfected with the rsCD11b gene alone, a 155 kD form of the truncated protein was found in the cell culture medium (Fig. 3, lane 1). Thus, like the membrane bound form, truncated CD11b is able to enter the protein export pathway and is released into the extracellular compartment. When the rsCD18 gene alone was

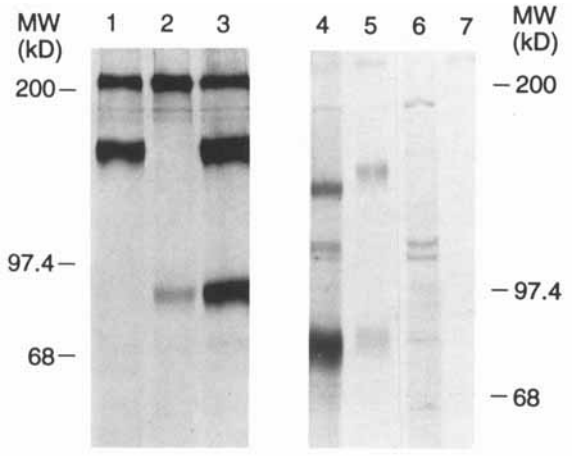

Fig. 3. Immunoprecipitation of truncated variants of CD11b, CD18, or Mac-1 heterodimers from transfected 293 cells. Cell were transfected with plasmids for the expression of rsCD11b alone (**lane 1**) or rsCD18 alone (**lane 2**) or were cotransfected with both the rsCD11b and rsCD18 expression plasmids (**lanes 3–5**). Three days after transfection, the cells were metabolically labeled with ^{35}S-methionine and growth conditioned cell culture medium (lanes 1,2,3,5,7) or cell lysates (lanes 4,6) were immunoprecipitated with a mixture of MAbs to CD11b (H5A4) and CD18 (PLM-2). Mock transfected cells served as controls for cell lysate (**lane 6**) or cell culture medium (**lane 7**) immunoprecipitations. The immunoprecipitated proteins were resolved by SDS-PAGE on 5 gels and visualized by autoradiography. The mobilities of molecular weight markers are indicated in the margins.

transfected into 293 cells, the majority of the protein was retained intracellularly as an 82 kD species (data not shown). However, upon long exposures (Fig. 3, lane 2), autoradiographs revealed that a trace amount of the truncated protein (84 kD) could be detected in the cell culture medium. When the rsCD18 and rsCD11b were transfected together, both proteins were exported into the cell culture medium (Fig. 3, lanes 3, 5). Like the membrane bound forms of CD11b and CD18, the low molecular weight species (145 kD, 82 kD) of each protein was only visualized intracellularly (Fig. 3, lane 4) whereas the high molecular weight forms occurred exclusively at the cell surface or in the cell culture medium.

Carbohydrate Analysis of rsCD11b and rsCD18

To account for the differences in molecular weight between the intracellular and extracellular proteins, the carbohydrate composition of the truncated molecules was examined. Figure 4A,B compares the molecular mobilities of truncated CD11b and CD18 before and after digestion with the Endoglycosidase H, which is specific for the high mannose form of N-linked carbohydrate, and N-glycanase, which is able to remove both the high mannose and complex (sialic acid containing) forms of N-linked carbohydrate [Tarentino et al., 1974, 1985]. It was found that intracellular CD11b and CD18 were both sensitive to Endo H digestion and yielded species of 115 kD and 70 kD, respectively (Fig. 4A, lane 2; 4B, lane 2). Because the mobilities of Endo H treated and N-glycanase treated samples were identical (Fig. 4A, lane 3; 4B, lane 3), it appeared that the glycosylation present on intracellular proteins was entirely the high mannose form of N-linked carbohydrate. Analysis of the extracellular, secreted forms of rsCD18 (84 kD) showed that it was completely resistant to Endo H digestion (Fig. 4B, lane 6), indicating that it had acquired the complex, sialic acid containing form of N-linked carbohydrate upon export to the extracellular compartment. Analysis of the secreted CD11b showed that it was only partially resistant to Endo H digestion yielding a 145 kD species and suggesting that it contained a mixture of simple and complex N-linked carbohydrate. Thus, the difference in molecular weights between the intracellular and secreted forms of CD11b and CD18 is attributable to a difference in glycosylation, where the intracellular form contains only the simple high mannose form of N-linked carbohydrate, and the secreted forms contain the mature, sialic acid containing complex form of N-linked carbohydrate.

Co-immunoprecipitation analysis of these samples showed that only a trace amount of CD11b could be co-precipitated with the PLM-2 (anti-CD18) MAb from lysates of cells co-transfected with the rsCD11b and rsCD18 plasmids (Fig. 4B, lane 1). Similarly, rsCD18 could not be co-precipitated from these cell lysates with a MAb to CD11b (H5A4) (Fig. 4A, lane 1). Thus, there was no steady state accumulation of rsCD11b/rsCD18 heterodimers within the transfected cells. In contrast, the PLM-2 MAb clearly co-immunoprecipitated rsCD11b along with rsCD18 when the growth conditioned cell culture media from the transfected cells were analyzed (Fig. 4B, lane 5). Similarly, the H5A4 MAb co-precipitated rsCD18 along with rsCD11b from the same cell culture media (Fig. 4A, lane 5). Thus, it appears that the majority of the rsCD11b and rsCD18 present in transfected cells occurred as free and uncomplexed species containing the high mannose form of N-linked carbohydrate. Because there was no intracellular

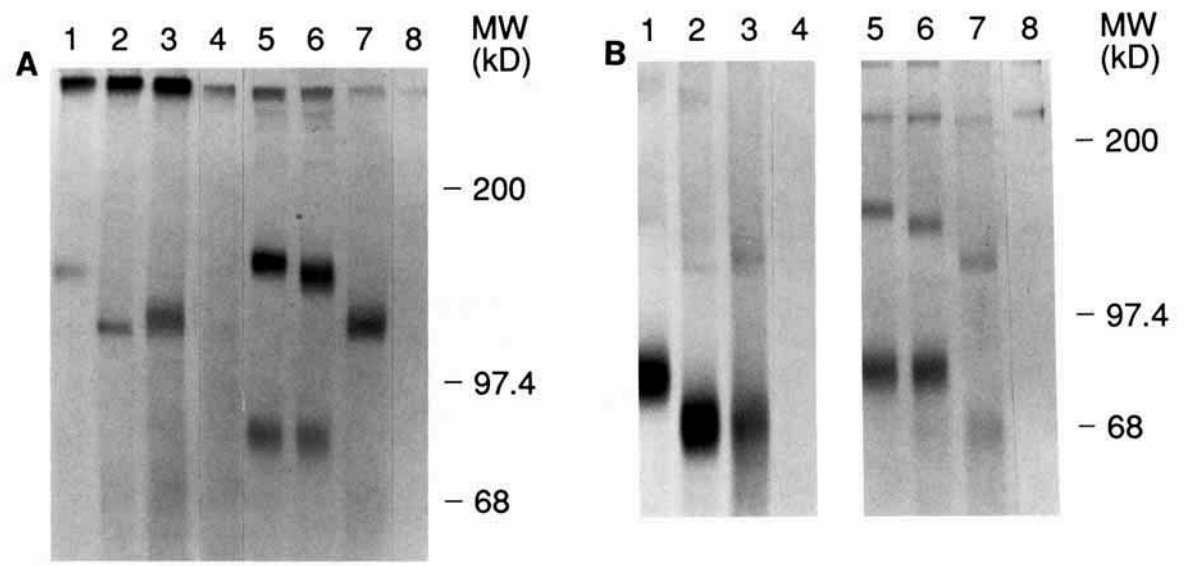

Fig. 4. Carbohydrate and co-immunoprecipitation analysis of intracellular and secreted rsMac-1 Cells (293) were co-transfected with a plasmid containing both rsCD11b and rsCD18 transcription units Three days after transfection, the cells were metabolically labeled with ^{35}S-methionine and the cell lysates (**lanes 1–4**) and cell culture medium (**lanes 5–8**) were immunoprecipitated with the H5A4 MAb specific for CD11b (**A**) or the PLM2 MAb specific for CD18 (**B**) The immunoprecipitated proteins were analyzed by SDS-PAGE on 7% gels after mock digestion (lanes 1,5), Endo H digestion (lanes 2,6), or N-glycanase digestion (lanes 3,7) Immunoprecipitations from mock transfected cells are shown in lanes 4 and 8 Proteins were visualized by autoradiography The mobilities of molecular mass markers are shown in the margins

accumulation of rsCD11b or rsCD18 containing complex carbohydrate, nor an intracellular accumulation rsCD11b/rsCD18 complexes, it appeared that once heterodimer formation and the acquisition of complex carbohydrate occurs, the CD11b/CD18 complex is rapidly and efficiently exported out of the cell into the culture medium.

Comparison of the intensities of the rsCD11b bands precipitated from cell culture media of 293 cells co-transfected with rsCD11b and rsCD18 (Fig. 4A, lane 5; 4B, lane 5) shows that the rsCD11b signal is greater with the H5A4 MAb than with the PLM-2 MAb. This result suggests that the extracellular culture medium contains a mixture of free rsCD11b and rsCD11b associated with rsCD18 in a heterodimeric complex.

Production of rsMAC-1 Purified From a Continuous Cell Line

In order to produce quantities of rsMac-1 sufficient for biological and structural studies, a continuous 293 cell line secreting rsMac-1 was constructed. The plasmid (pRKCD18T/CD11bT) used to express rsMac-1 contained both rsCD18 and rsCD11b transcription units in tandem (Fig. 1). Co-transfection of pRKCD18T/CD11bT and another plasmid (pSVDHFR) containing the selectable marker dihydrofolate reductase which allowed for the isolation of a permanent cell line that constitutively secreted rsMac-1. Cell culture medium from the cell line was harvested, and several CD18 specific MAbs were evaluated for their ability to purify the rsMac-1 by immunoaffinity chromatography (Fig. 5). During the course of these studies, one MAb, MHM.23, was identified that possessed the unique property of reacting only with mature, fully glycosylated rCD18 within the rMac-1 or rsMac-1 complex and did not bind to monomeric or intracellular rCD18 (data not shown). To be certain that this binding activity was not a peculiarity of the recombinant protein, the specificity of this antibody was evaluated with native Mac-1. Figure 5B compares the reactivity of three CD18 specific MAbs, MHM.23, PLM-2, and H-52, with native CD18 expressed in the U937 cell line. It was found that H-52 (Fig. 5B, lane 1) and PLM-2 (Fig. 5B, lane 2) reacted with both the high molecular weight, mature form CD18 as well as the lower molecular weight (high mannose) precursor. In contrast, MHM.23 (Fig. 5B, lane 3) reacted only with the mature form. All three antibodies co-immunoprecipitated CD11b as well as the other beta 2 integrin, p150,95 (CD11c). These studies suggested that the antibody used

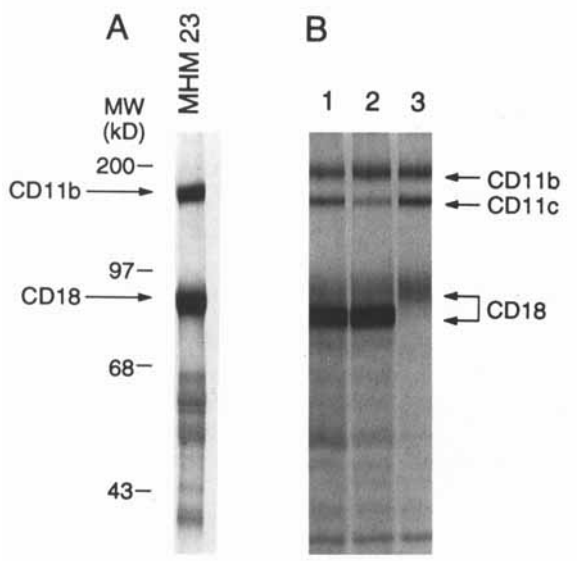

Fig. 5. Specificity of antibodies used for the purification of secreted Mac-1. **A:** Growth conditioned cell culture medium from the rsMac-1 cell line was harvested and fractionated on an affinity column consisting of MHM.23 coupled to control pore glass. The column was eluted at acid pH and the eluted protein was analyzed by SDS-PAGE and visualized by silver staining. The mobilities of molecular weight marked are indicated in the margin. **B:** The U937 promyelocyte cell line was treated with PMA (2 ng/ml) for 3 days and then metabolically labeled with ^{35}S-methionine. Cell lysates were then immunoprecipitated with the CD18 specific MAbs, PLM-2 (**lane 1**), H52 (**lane 2**), or MHM.23 (**lane 3**).

to purify rsMac-1 possessed the unique property of reacting only with the mature form of CD18 within the native or recombinant CD11b/CD18 complex. Thus, the MHM.23 MAb appeared well suited for the purification of rsMac-1 because it reacted with only the mature form of CD18 and did not bind free CD18 precursor that might be released into cell culture media as a consequence of inadvertent cell lysis. Purified rsMac-1 obtained by MHM.23 immunoaffinity chromatography of growth conditioned cell culture media and visualized by silver staining is shown in Figure 5A.

Kinetics of Activated Neutrophil Binding to HUVECs

The biological activity of rsMac-1 was examined using an assay where the binding of neutrophils (PMNs) labeled with the fluorescent dye BCECF to human umbilical vein endothelial cells (HUVECs) was measured. In initial studies, the binding properties of PMNs activated by two distinct stimuli—opsonized zymosan (OpZy) and the granulocyte chemotactic peptide, formyl-

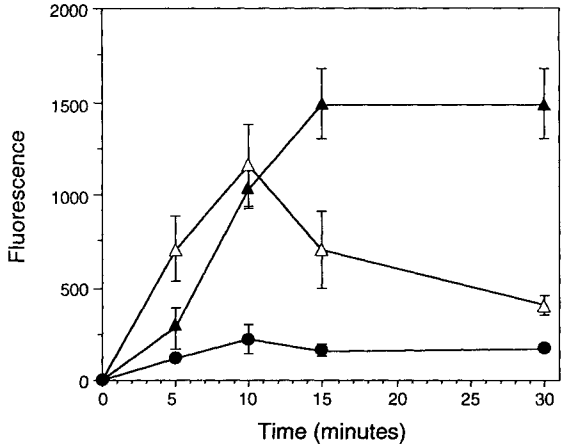

Fig. 6. Kinetics of PMN adhesion to HUVECs by different activators. PMNs (2×10^5 cells per 0.1 ml) were added to HUVEC monolayers. PMNs activated by either fMLP (10^{-7} M) (open triangles) or OpZy (500 μg/ml) (shaded triangles) were compared. Baseline binding of PMNs to HUVECs in the absence of inhibitors is indicated by shaded circles. Adherence was monitored at 5, 10, 15, and 30 min post-activation at 37°C.

methionyl-leucyl-phenylananyl peptide (fMLP)—were examined (Fig. 6). The binding of PMNs activated by fMLP was maximal at 10 min post-activation and then progressively declined to baseline. In contrast, the binding of PMNs activated by OpZy reached maximum binding at 15 min and maintained a plateau level for an additional 15 min. Based on the kinetics of the fMLP response, it was concluded that comparative binding studies would be performed at 10 min post-activation.

Binding of Activated PMNs to Unactivated HUVECs

To assess the role of Mac-1 on activated PMN binding to unactivated HUVECs, studies with MAbs (H52 and 904) known to block Mac-1 function were performed. It was found (Fig. 7) that the CD18 specific MAb, H52, was able to inhibit the binding of PMNs activated by both stimuli in a concentration dependent fashion, with a 50% inhibitory concentration (IC_{50}) in the 10^{-8} M range. In control experiments using an isotype matched MAb or MAbs specific for CD11a (data not shown), no inhibition of activated PMN binding to HUVECs was detected. When a $F(ab')_2$ fragments of the 904 MAb were examined, the CD11b antibody was found to be comparable to the CD18 MAb with respect to blocking OpZy activated PMN binding, but was significantly less effective (IC_{50} in the 10^{-6} to

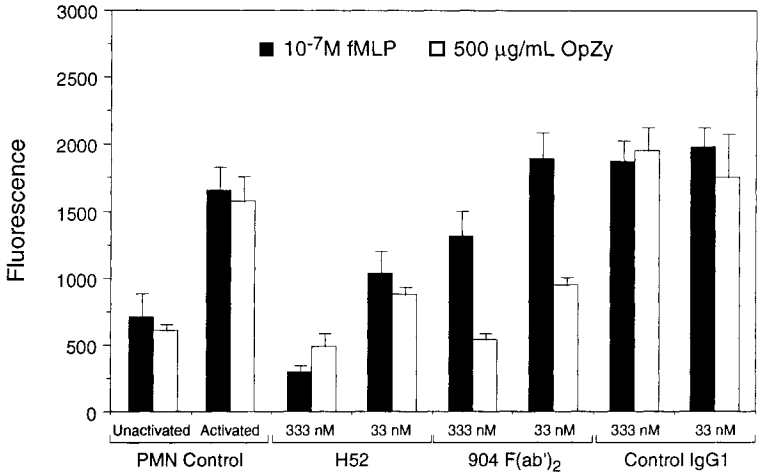

Fig. 7. Inhibition of activated PMN binding to HUVECs by MAbs to CD11b and CD18. PMNs (2×10^5 cells per 0.1 ml) were added to HUVEC monolayers. PMNs were activated with either fMLP (10^{-7} M) or OpZy (500 µg per ml) in the presence or absence of a MAb to CD18 (H52) or CD11b (904 $F(ab')_2$), or isotype matched control MAb (anti-human growth hormone IgG1). Cell adherence was determined following a 10 min incubation at 37°C.

10^{-7} M range) than the CD18 specific MAb in blocking the binding of fMLP activated PMNs. Thus, the CD11b MAb blocked all of the activation induced binding of OpZy activated PMNs at an antibody concentration of 333 nM, whereas this concentration of MAb blocked approximately 40% of the binding by fMLP activated cells.

To further explore the nature of these adhesive interactions, rsMac-1 was tested in the assays described above. It was found that rsMac-1 was effective in blocking the binding of PMNs activated by either OpZy (Fig. 8A) or fMLP (Fig. 8B) to unactivated HUVECs. Because rsMac-1 was able to reduce the binding of fMLP activated PMNs to baseline levels, it appeared to be significantly more potent than the 904 MAb in this assay. In contrast, rsMac-1 was somewhat less effective in blocking the binding of OpZy activated PMNs to HUVECs in that it reduced binding by approximately 60% as compared to the 904 preparation which reduced binding to baseline values. In control experiments the H52 MAb, but not the control MAb, blocked the binding of PMNs activated by both stimuli to HUVECs. Thus, these studies confirmed that the binding of OpZy and fMLP activated PMNs to HUVECs is entirely Mac-1 dependent and demonstrated that rsMac-1 exhibited inhibitory activity comparable to CD18 or CD11b specific MABs.

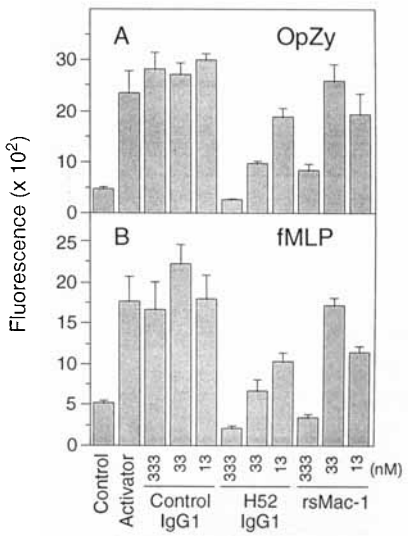

Fig. 8. Inhibition of activated PMN binding to HUVECs by rsMac-1. PMNs were activated and analyzed for binding to HUVECs by the same method described in Fig. 7. The ability of rsMac-1 to inhibit binding of PMNs induced by the activators OpZy **(A)**, or fMLP **(B)** was compared to that of the anti-CD18 MAb, H52, or control IgG1.

Binding of Unactivated Neutrophils to Activated and Unactivated HUVECs

In other studies (Fig. 9) the ability of soluble Mac-1 to inhibit the binding of unactivated PMNs to unactivated and TNF-α activated HUVECs was examined. TNF is known to be a

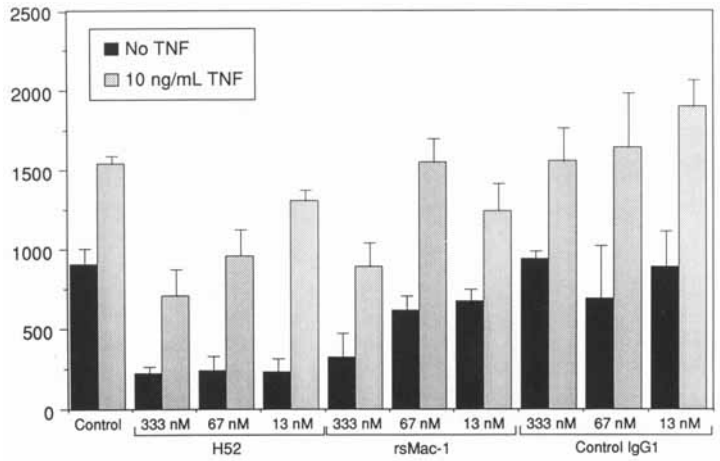

Fig. 9. Inhibition of unactivated PMN binding to TNF-α stimulated HUVECs by rsMac-1. HUVEC monolayers were stimulated with human TNF-α at 10 ng per ml for 24 h at 37°C. Non-stimulated PMNs (2×10^5 cells per 0.1 ml) were added to HUVECs and allowed to adhere for 10 min at 37°C. Non stimulated HUVECs were used to monitor the level of basal binding. The isotype matched control MAb was an anti-human growth hormone IgG1.

pro-inflammatory cytokine and is a potent inducer of ICAM, the putative ligand for Mac-1, on endothelial cells. In these studies the inhibitory activity of the CD18 specific MAb, H52, was compared to that of rsMac-1. It was found that H-52 and rsMac-1 were effective in inhibiting unactivated PMN binding to unactivated HUVECs. The 50% inhibitory concentration (IC_{50}) for H52 appeared to be less than 13 nM, whereas the IC_{50} for rsMac-1 blocking the binding of unactivated PMN binding to unactivated HUVECs occurred in the range between 67 and 333 nM. When binding to activated endothelial cells was examined, the estimated IC_{50} for the H52 monoclonal appeared to be in the range of 100 nM, whereas the estimated IC_{50} for soluble Mac-1 was in the 300–500 nM range. Thus rsMac-1 was an effective inhibitor of PMN binding to HUVECs, but less effective than the CD18 specific MAb, H52.

DISCUSSION

In this study we describe the biosynthesis of membrane bound and secreted forms of the beta 2 integrin, Mac-1, in the 293 human kidney adenocarcinoma cell line. Transfection studies showed that both full-length and truncated CD11b are exported to the cell surface in 293 cells and do not require CD18 to facilitate export. This result was unexpected since granulocytes from patients with a defect in the expression of CD18 (leukocyte adhesion deficiency [LAD]) reportedly fail to express cell surface CD11b [Arnaout et al., 1984; Springer et al., 1984; Anderson and Springer, 1987]. In addition transfection studies with other integrins (e.g., IIbIIIa) failed to observe export of integrin alpha chains to the cell surface in COS cells in the absence of the corresponding beta chain [O'Toole et al., 1989]. One explanation for these results may relate to the fact that Mac-1 normally accumulates in secretory granules in PMNs prior to being exported to the cell surface [Todd et al., 1984]. It is possible that CD11b might possess a sorting signal that serves to direct it to secretory granules when expressed in granulocytes, but by default is directed to the cell surface in cells that lack secretory granules. Another possibility is that CD11b expressed in 293 cells is chaperoned to the surface by forming a heterodimer with a beta chain of another integrin (e.g., beta 1 or beta 3). Although inappropriate complex formation with endogenous integrins was found to account for the unexpected export of GPIIa in 293 cells [Bodary et al., 1989], this mechanism is unlikely to account for the export of CD11b, since it has not been possible to demonstrate that another protein is co-immunoprecipitated membrane bound or secreted CD11b in 293 cells metabolically labeled with ^{35}S-cysteine or ^{35}S-methionine, or surface labeled with ^{125}I. Previously Larson et al. [1990] studied the expression of full-length CD11a, CD11b, and CD18 in COS cells and reported that the expression of CD11a was twentyfold greater in the presence of CD18 than with the

isolated alpha chain alone. These authors did not comment on the magnitude of isolated CD11b expression. Dana et al. [1991] suggested that truncated variants of CD11b and CD18 were secreted into the cell culture media of transiently transfected COS cells; however, data was not shown.

Immunoprecipitation analysis of the proteins synthesized in the rsMac-1 cell line suggested that there was a large excess of intracellular rsCD18 compared to rsCD11b. Indeed, pulse chase studies (data not shown) showed that radiolabeled amino acids were more slowly incorporated into rsCD18 in rsMac-1 heterodimers than rsCD11b. Large intracellular pools of CD18 have also been observed in leukocytes expressing native CD18 [Sanchez-Madrid et al., 1983; Ho and Springer, 1983]. Because the majority of intracellular CD18 was derivatized exclusively with the high mannose form of N-linked carbohydrate and did not possess the complex carbohydrate characteristic of Golgi apparatus (GA) processing, the pool of free monomeric CD18 appears to be located in the endoplasmic reticulum or a pre-GA compartment. The observation that only a trace amount of intracellular CD11b/CD18 heterodimers could be detected by co-immunoprecipitation from cell lysates with MAbs to CD11b or CD18 suggests that once heterodimers were formed, they are selectively exported to the GA, where they acquire complex carbohydrate, and then rapidly and efficiently exported to the extracellular compartment. The biosynthesis of rsMac-1 differs from that reported for native Mac-1 [Ho and Springer, 1983] in lymphoid cells in that heterodimers containing the high mannose form of N-linked carbohydrate are not readily detected.

In the present studies we describe the isolation of a continuous cell line that constitutively secreted a mixture of monomeric CD11b along with stable heterodimeric rsCD11b/rsCD18 complexes (rsMac-1). A monoclonal antibody (MHM.23) was identified that allowed for the selective purification of rsMac-1 heterodimers and an effective immunoaffinity purification process for rsMac-1 was developed. Purified rsMac-1 was able to inhibit the binding of fMLP and OpZy activated PMNs to unactivated HUVECs as well as the binding of unactivated PMNs to TNF-α activated HUVECs. In both cases, the IC_{50} of rsMac-1 was found to be in the 10^{-7} M range. A previous study by Dana et al. [1991] showed that rsMac-1 present in cell culture supernatants of transiently transfected COS cells was able to bind C3bi and to inhibit the binding of unactivated PMNs to HUVECs 4 h after activation with IL-1. The present studies confirm that biologically active rsMac-1 can be produced in transfected cells and show that it is able to inhibit activated PMNs binding to unactivated HUVECs as well as unactivated PMN binding to activated HUVECs. The adhesive interactions mediating the binding of unactivated PMNs to activated HUVECs that we have described (24 h after activation) differed from those studied by Dana et al. [1991] where binding was measured at 4 h post-activation. Luscinskas et al. [1989] previously showed that PMN binding at 4 h post-activation was due to both ELAM-1 (Lec-CAM) and CD18 dependent (Leu-CAM) mechanisms, whereas binding at 24 h post-activation, as was measured in the present studies, was entirely CD18 dependent. Thus the previous studies showed that rsMac-1 could inhibit interactions that were partially Leu-CAM dependent, whereas the present studies showed that rsMac-1 could inhibit completely Leu-CAM dependent adhesive interactions.

Since beta 2 integrins play such an important role in inflammatory responses, it has been proposed that inhibitors of beta 2 integrins might be useful in the treatment of acute inflammatory disorders such as reperfusion injury [Arfors et al., 1987; Simpson et al., 1988], hypovolemic shock [Mileski et al., 1990], and acute lung injury [Ismail et al., 1987]. Potential inhibitors of beta 2 integrin function include antibodies to integrin alpha chains (CD11a, CD11b, CD11c), antibodies to the integrin beta 2 chain (CD18), antibodies to the ligands for beta 2 integrins (ICAM-1, ICAM-2), and soluble receptors. Because the present study utilized purified protein, it was possible, for the first time, to directly compare the inhibitory activity of rsMac-1 to potential antibody inhibitors (e.g., MAbs to CD11b or CD18). In most cases, the rsMac-1 was less potent than CD18 or CD11b directed MAbs with the estimated IC_{50} for Mac-1 measured in the 10^{-7} M range, whereas those of the MAbs CD11b or CD18 were found to be in the 10^{-8} M range. However, rsMac-1 exhibited comparable, if not better, inhibitory activity than the 904 MAb in blocking the binding of fMLP activated PMNs to unactivated HUVECs. Thus, rsMac-1 might represent a significant alternative to MAbs as inhibitors of CD11b/CD18 mediated adhesion.

It has been suggested [Altieri and Edgington, 1988a; Altieri, 1991] that Mac-1 and LFA-1 [Dromsfield et al., 1992] can assume activated (high affinity) and inactive (low affinity) conformations. Moreover, it has been shown that phosphorylation of sequences in the cytoplasmic tail of the closely related integrin, CD11a, is required for activation [Hibbs et al., 1991]. The fact that rsMac-1 possesses inhibitory activity in the 10^{-7} M range demonstrates that the truncated secreted molecule was isolated in a biologically relevant conformation, and that the transmembrane domains and cytoplasmic tails are not required for binding. However, the present studies do not rule out the possibility that the rsMac-1 we have purified is actually in the low affinity state and that its binding activity could be increased by the appropriate activators. Studies by Altieri et al. [1988b, 1991] suggested that the binding of ligands such as ADP and ions such as Mn^{+2} can activate Mac-1 function. Thus, the IC_{50} values that we have measured may be minimal estimates of the rsMac-1 inhibitory activities.

In conclusion, we have described the construction of a cellular substrate and a purification strategy for the isolation of a functionally active variant of the CD11b/CD18 heterodimer. While the recombinant protein was active in several biological assays, we cannot be certain we have achieved the highest specific activity possible for this preparation. Further studies, employing different purification strategies and activating cofactors, will be required before the activity of rsMac-1 as an inhibitor of granulocyte function is fully understood. The availability of rsMac-1 will facilitate analysis of the role of Mac-1 in complex adhesive interactions involving multiple adhesive molecules, and should help to clarify the role of cofactors (e.g., divalent cations) and signal transduction mechanisms in the activity of beta 2 integrins.

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