Expression of a soluble and functional form of the human β2 integrin CD11b/CD18

(recombinant β2 integrins/complement receptors/leukocyte-endothelial adhesion/inflammatory reactions/reperfusion injury)

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ABSTRACT Polymorphonuclear cells and monocytes (phagocytes) are a critical component of host defense against infections. However, these cells also play a significant role in host tissue damage in many noninfectious diseases, such as ischemia-reperfusion injury syndromes and rejection of transplanted organs. The leukocyte adhesion molecule family CD11/CD18 (β2 integrins) is critical to the function of polymorphonuclear cells and monocytes in inflammation and injury. Inherited deficiency of CD11b/CD18 impairs phagocyte chemotaxis, adhesion and transmigration across endothelium, and clearance of invading microorganisms through phagocytosis and cell-mediated killing. Furthermore, murine monoclonal antibodies directed against the CD11b/CD18 (CR3) heterodimer have been shown to reduce, by 50%-80%, phagocyte-mediated ischemia-reperfusion injury in several organ systems, such as the myocardium, liver, and gastrointestinal tract and to inhibit development of insulin-dependent diabetes mellitus in nonobese diabetic (NOD) mice. Expression of CD11b/CD18 in a soluble and functional form might therefore be potentially useful as an anti-inflammatory agent. We have now expressed a recombinant soluble heterodimeric form of this human β2 integrin, normally expressed as two noncovalently associated membrane-bound subunits. The secreted receptor exhibited direct and specific binding to its ligand, iC3b, the major complement C3 opsonin, and inhibited binding of polymorphonuclear cells to recombinant interleukin 1-activated endothelium.

There is increasing awareness of the critical role of phagocytic cells, polymorphonuclear cells (PMN), and monocytes in tissue destruction in many clinical disorders including myocardial infarction, certain immune-complex glomerulonephritides, blistering skin diseases, allograft rejection, and ulcerative colitis (1). Interventions aimed at attenuating this harmful role may have great therapeutic benefit. However, given the myriad of proteinases, oxidants, and cationic proteins produced by phagocytes after activation with chemoattractants and cytokines and during phagocytosis (2), this goal has proven elusive.

Recently, another approach has been suggested by unraveling the molecular basis of an inherited disease (leukocyte adhesion molecule deficiency) manifested clinically by life-threatening recurrent bacterial infections in which affected individuals have a global deficiency in phagocyte-mediated acute inflammatory responses due to lack of expression of CD11/CD18 (β2 integrins) (3, 4). CD11/CD18 are adhesion receptors that promote interaction of leukocytes with each other (5, 6), with endothelial cells (during transmigration) (7-9), and with specific opsonins deposited on invading bacteria and rejected or hypoxic tissues (10, 11). The CD11/CD18 family consists of three heterodimeric surface membrane glycoproteins, each with a distinct α subunit (CD11a, b, or c) covalently associated with an identical β subunit (CD18) (12, 13). As in other integrins, association of the CD11 and CD18 subunits is required for normal surface-membrane expression and function of these receptors (14, 15). The CD11b/CD18 (CR3) heterodimer is a major β2 integrin on PMN and monocytes and mediates many of the proinflammatory functions in these cells (3, 16-18). Murine monoclonal antibodies (mAbs) directed against CD11b/CD18 reduce the degree of ischemia-reperfusion injury by 50%-80% in several animal models of phagocyte-dependent acute tissue injury (19-21) and prevent development of insulin-dependent diabetes mellitus in susceptible mouse strains (22) through prevention of phagocyte accumulation in damaged tissues and their interaction with complement iC3b. These antibodies, however, usually elicit an antoglobulin response in humans and are thus of limited therapeutic usefulness. We therefore considered that antagonism of phagocyte interactions with inflamed endothelium and with iC3b might also be achieved by using a soluble and functional form of human CD11b/CD18.

In this paper we report the expression of a recombinant soluble form of CD11b/CD18 in mammalian cells. The soluble receptor remains complexed as a heterodimer as revealed by immunoprecipitation using subunit-specific mAbs. Furthermore the soluble heterodimer binds specifically to iC3b in a divergent cation-dependent manner similar to that of the wild-type receptor and inhibits binding of PMN to recombinant interleukin 1 (rIL-1)-activated endothelium.

MATERIALS AND METHODS

Reagents. Activated thiol-Sepharose (ATS) was bought from Sigma. Human complement C3 and factors I and H were purified as described (23, 24). Purified human C3 was exposed to ATS in the presence of l-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Worthington) to convert it to C3b covalently linked to ATS (ATS-C3b). The concentration of C3b was 4.5 mg/ml of settled Sepharose. ATS-iC3b was generated by proteolytic cleavage of ATS-C3b with purified human complement factors H and I (24, 25). Conversion of C3b to iC3b was confirmed by SDS/PAGE analysis under reducing conditions. Human PMN were isolated from EDTA-anticoagulated blood by centrifugation over Ficoll/Hypaque as described (26). Purified cells were surface labeled with 125I (New England Nuclear) using Iodo-Gen (Pierce) as described (27). rIL-1 was provided by Biogen.

Abbreviations: PMN, polymorphonuclear cells; rIL-1, recombinant interleukin 1; CR3, complement receptor type 3; ATS, activated thiol-Sepharose; mAb, monoclonal antibody; HUVE, human umbilical vein endothelial cells; mCD11b/CD18, membrane-bound receptor CD11b/CD18; sCD11b/CD18, sCD11b, and sCD18, secreted CD11b/CD18, CD11b, and CD18, respectively.

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Generation of In-Frame Stop Codons in CD11b and CD18 cDNAs. We used site-directed mutagenesis to introduce in-frame translational stop codons at the predicted extracellular boundaries of the corresponding transmembrane domains of CD11b and CD18 (Fig. 1). Asn-700 in CD18 and Leu-1090 in CD11b were replaced by translational stop codons in the wild-type cDNAs. In-frame stop codons in full-length CD11b cDNA and CD18 cDNA (29,31) were generated by using the gapped-duplex mutagenesis technique (32), modified as described (29). Briefly, the PAT-X plasmid containing the partial CD18 cDNA clones J19 [provided by Alex Law, Oxford University (31)] was linearized with HindIII or digested with Nco I to generate a 1331-base-pair (bp) gap. These two plasmids were mixed with an excess of the synthetic and 5'-end-phosphorylated 18-mer (O-18 N/st; 5'-agcggccctagatcgccg) containing desired nucleotide substitutions (upcase letters). The mixture was denatured by boiling and renatured by stepwise cooling. Rehybridized DNA (containing the single-stranded region to which the mutant 18-mer is hybridized) was primer-extended to fill the gap and used to transform Escherichia coli strain BMH 71-18 mut I. (29) Plasmids containing the mutation were identified by differential hybridization with 32P-labeled wild-type or mutant 18-mers, and the DNA was used to retransform E. coli JM109. Positive colonies were identified after rehybridization, sequenced to verify the mutation, and then used to replace the corresponding fragment in wild-type full-length CD18 cDNA cloned in the pπH3M expression vector (29). A stop codon was similarly introduced in CD11b cDNA by using a Bluescript plasmid vector containing the full coding region of membrane-bound CD11b cDNA (30). A mixture of Kpn I-linearized and gapped (by removing a Sma I fragment, I048-bp long) CD11b cDNAs were mixed with an excess of the synthetic mutant 18-mer (O-18 L/st; 5'-caaccccTAgcgtctcat), processed as detailed above, and the desired cDNA (confirmed by sequencing) was subcloned into the πH3M expression vector.

Production of Mutant CD11b and CD18 in COS Cells. Each plasmid was transfected into COS cells using DEAE-dextran as described (29,33). Forty to 50% confluent COS M6 monkey kidney cells in 100-mm tissue-culture dishes were washed in serum-free medium and transfected using DEAE-dextran/chloroquine and supercoiled mutated CD11b cDNA and mutated CD18 cDNA constructs, each at 2 µg/ml. In some transfections, COS cells were transfected with either mutated CD11b or CD18 cDNA. Sham-transfected cells were used as controls. To detect secreted cell products, cells from each 100-mm dish, representing ~3 × 10⁶ cells, were pulsed with 0.1 mCi (1 Ci = 37 GBq) of [35S]methionine for 16 hr at 37°C in a humidified 5% CO₂ incubator. Supernatants were centrifuged to remove nonadherent cells and debris and then stored at 4°C or used for immunoprecipitations, ligand binding, or inhibition studies.

Binding of Radiolabeled Membrane-Bound and Truncated Receptors to ATS-iC3b. This binding was done as described (25) with minor modifications. Briefly, 1 × 10⁸ 125I-surface-labeled PMN (a source of radiolabeled-membrane-bound receptor, mCD11b/CD18) were solubilized in 1 ml of phosphate-buffered saline/0.5% Nonidet P-40/2 mM phenylmethylsulfonyl fluoride. The detergent-soluble fraction was then diluted 3-fold in distilled water/0.5% Nonidet P-40/2 mM MgCl₂/0.33 mM CaCl₂. One milliliter of the diluted lysate was then mixed with either 200 µl of settled ATS-C3b or ATS-iC3b. After 90 min at room temperature, each sample was transferred to a 10-ml Econo column (Bio-Rad) and washed with 10 ml of 5 mM Tris/24 mM NaCl/1 mM KCl, pH 7.4/100 mM NaCl (washing buffer) containing 0.5% Nonidet P-40. The column was then eluted in 1-ml fractions with 0.4 M NaCl in the same buffer. Fractions containing peak radioactivity were pooled, dialyzed extensively against water, lyophilized, and then analyzed on SDS/PAGE under reducing conditions (34). Transfected cell supernatants were processed similarly. The recombinant CD11b/CD18 was radiolabeled by metabolically labeling CD11b/CD18 cDNA-transfected COS cells (and COS cells transfected with mutated CD11b, or mutated CD18 cDNA or sham-transfected cells) with 35S)methionine. Supernatants from labeled transfected COS cells were collected (each pooled from two semi-confluent 100-mm petri dishes each containing ~3 × 10⁶ cells) and used as sham control, a source of secreted CD11b/CD18 (sCD11b/CD18) secreted CD11b (sCD11b), or secreted CD18 (sCD18). Each pool (15 ml) was concentrated to 1 ml using collodion bags (10,000 M, cut-off; Schleicher & Schuell). One-hundred microliters was used for immunoprecipitations to detect the radiolabeled sCD11b/CD18 heterodimer, sCD11b, or sCD18 monomers. The rest was diluted 3-fold in distilled water/2 mM MgCl₂/0.33 mM CaCl₂ or 10 mM EDTA, and 1 ml was incubated with ATS-C3b or ATS–iC3b. Samples were then processed as described above. ATS elution was accomplished by using washing buffer containing 0.1% Nonidet P-40 and 0.4 M NaCl.

Immunoprecipitations and Quantitation of Recombinant Secreted Receptor. Subunit-specific mAbs 44a (anti-CD11b) (10,35) or TS18 (anti-CD18) (36) were used to immunoprecipitate CD11b/CD18 from PMN or COS cell supernatant. Typically, 100 µl of [35S]labeled PMN detergent lysate or 100 µl of metabolically labeled COS cell supernatant (following a 10-fold concentration) were used in each case. The 44a and TS18 mAbs were provided by R. F. Todd III (University of Michigan, Ann Arbor) and S. Burakoff (Dana–Farber Cancer Institute, Boston), respectively. Immunoprecipitations were done as described (37). To determine the fraction of the secreted receptor expressed as a heterodimer, intensity of CD11b and CD18 bands immunoprecipitated from equal volumes of supernatants by the anti-CD11b and anti-CD18 mAbs was compared by densitometry with an LKB Ultrascan XL densitometer (Pharmacia LKB).

The concentration of secreted receptor in supernatants from two separate transfections was quantified by using an RIA. Five-tenths milliliter of CD11b/CD18 receptor-containing super-
PMN-Adhesion to rIL-1-Treated Endothelium. Adherence of purified human PMN to confluent monolayers of human umbilical vein endothelial cells (HUVE) pretreated with rIL-1 (10 units/ml for 4 hr at 37°C) was done as described (8) with the following modifications: PMN were labeled with carboxyfluorescein (Molecular Probes) by incubating 4 × 10^6 cells with 30 μg of carboxyfluorescein in 1 ml of Tris-buffered saline for 10 min on ice, followed by three washes. HUVE were preincubated with undiluted supernatants from transfected COS cells, in triplicate wells for 10 min at 37°C, before labeled PMN (5 × 10^5 cells per well) in an equal volume were added, and incubation was continued for an additional 10 min. Some human PMN were preincubated with NS1 (a negative control ascites), 44a mAb, or TS18 mAbs (at 1:100 dilution) for 5 min at room temperature before use. At the end of the adhesion assay, HUVE were washed twice, and HUVE-associated fluorescein was harvested in distilled water/0.1% SDS/0.1 M NaOH and measured (at excitatory wavelength, 490 nm; emitted wavelength, 500 nm) using a fluorometer (SLM 8000; SLM Aminco, Urbana, IL). Typically, 25–32% of added PMN adhered to endothelium in buffer alone or the NS1 control mAb under these conditions (ref. 8 and data not shown). PMN binding with NS1 or supernatant from sham-transfected COS cells was normalized to 100 arbitrary units, and relative binding in the presence of mAb 44a, mAb TS18, or sCD11b/CD18 was then calculated. The data are expressed as the mean of four separate experiments representing three separate transfections and the variance as the SEM. Significance was determined by the Student’s t test.

RESULTS

Expression of a Soluble CD11b/CD18 Heterodimer in COS Cells. To determine whether COS cells can express a secreted protein heterodimer, we cotransfected the mutated human CD11b and CD18 cDNA constructs into COS cells. After metabolic labeling, harvested supernatants were used for immunoprecipitation with anti-CD11b or anti-CD18 mAbs. Fig. 2 shows that both subunits of the receptor were immunoprecipitated from mutated CD11b/CD18-transfected but not from sham-transfected COS cell supernatants. The detergent-soluble fraction of the pulse-labeled cells contained little or no radiolabeled recombinant CD11b/CD18 (data not shown), indicating that the bulk of the synthesized receptor was secreted. The sCD11b/CD18 heterodimer had an apparent molecular mass of 149 kDa and 84 kDa for its CD11b and CD18 subunits, respectively (Fig. 2), compared with 155 kDa and 94 kDa for the membrane-associated forms (10, 35), in agreement with an expected loss of 45 and 69 amino acids from CD11b and CD18, respectively. MAbs directed against either the sCD11b or sCD18 subunits immunoprecipitated both subunits (Fig. 2). Quantitative analysis of the immunoprecipitated bands by using densitometry indicated that ~66% of the secreted material is present in an aβ complex. In two separate transfections, the total amount of sCD11b detected in 1 ml of culture supernatant was 20 ng ± 1.

sCD11b/CD18 Binds to iC3b. Both 44a and TS18 mAbs, which reacted with sCD11b/CD18, are known to be directed to functional epitopes in membrane-bound receptor (mCD11b/CD18) (13, 16, 36), suggesting that the recombinant secreted heterodimer may be functional. We therefore measured the ability of sCD11b/CD18 to bind to its well-characterized ligand iC3b, the major opsonic fragment of complement C3, using mCD11b/CD18 as a positive control. The 125I-labeled mCD11b/CD18 bound specifically to iC3b (Fig. 3A, lanes b and d) but not to C3b (Fig. 3A, lanes a and c), confirming reports (39, 40). Under the same conditions, metabolically labeled supernatants containing the sCD11b/CD18 heterodimer bound to ATS-iC3b (Fig. 3B, lane b). Binding was specific in that no binding was observed to ATS-C3b (Fig. 3B, lane c). No comparable binding activity to iC3b was detected in supernatants from the sham-transfected COS cells (Fig. 3B, lane a). Binding of mCD11b/CD18 and sCD11b/CD18 to iC3b was inhibited in the presence of EDTA (data not shown). Binding of sCD11b/CD18 to iC3b was also critically dependent on the presence of the recombinant receptor as a heterodimer because neither sCD11b nor sCD18 subunits, expressed separately, bound to iC3b-Sepharose (Fig. 4).

tsCD11b/CD18 Inhibits PMN Binding to Inflamed Endothelium. The ability of sCD11b/CD18 to inhibit binding of human PMN to inflamed endothelium was also examined and compared with levels of inhibition found using anti-CD11b or anti-CD18 mAbs (8, 9). 44a and TS18 mAbs inhibited adhesion of PMN to rIL-1-treated endothelium by 32 ± 9.3% and 72 ± 6.4% (mean ± SEM, n = 4), respectively (Fig. 5), in agreement with our previous data (8, 9). Supernatants containing sCD11b/CD18 (~7 ng of sCD11b added to each well containing 2.7 ng of surface-expressed receptor, or ~3-fold molar excess of secreted to membrane-bound receptor) were also effective in blocking PMN adhesion to rIL-1-induced endothelium (58 ± 7.2% inhibition, P < 0.001 compared to supernatants from sham-transfected cells) (Fig. 5).
DISCUSSION

This report demonstrates the synthesis and expression of a functional and soluble form of a recombinant integrin heterodimer. The evidence that the recombinant β2 integrin CD11b/CD18 can be expressed in a water-soluble form is supported by the observations that a truncated form of the receptor of the expected molecular size is present in supernatants from the transfected cells. That the bulk of the expressed receptor is in a heterodimeric form is shown by the ability of mAbs directed against either the CD11b or CD18 subunits to immunoprecipitate both subunits (Fig. 2). These findings also suggest that neither the cytoplasmic end nor the transmembrane regions of the CD11b or CD18 subunits are required for heterodimer formation. We cannot exclude the possibility, however, that these regions may improve the stability of this complex.

Our ability to express a recombinant sCD11b/CD18 heterodimer contrasts with the significant difficulties being encountered in expressing heterodimeric forms of other receptors (41) and, perhaps, reflects special features in the requirements for formation of an αβ complex in integrins. The present findings agree with recent molecular cloning studies (29, 42, 43) and EM studies of integrins (44) that suggest that αβ association requires direct binding of the N-terminal halves of the two subunits, forming a mushroom-like head with the remainder of each subunit forming a free tail embedded in the plasma membrane.

The secreted receptor reacted with mAbs directed against functional epitopes or domains (13, 16, 36) suggesting that the overall conformation of the native form of the receptor is maintained. This suggestion was directly confirmed by examination of the ligand-binding properties of the recombinant receptor. sCD11b/CD18 bound to its ligand, iC3b, under similar conditions to those previously used to show direct and specific binding of mCD11b/CD18 (Fig. 3). Binding of sCD11b/CD18 to iC3b was divalent cation-dependent and specific in that no binding was detected to C3b. Binding also required expression of the secreted receptor in its heterodimeric form because no binding activity was displayed by its secreted subunits when expressed separately (Fig. 4). The

**Fig. 3.** Binding of membrane (A) and secreted (B) CD11b/CD18 to iC3b. (A) Autoradiograph of a SDS/6% PAGE of PMN-derived 125I-surface-labeled glycoproteins eluted from ATS-C3b (lane a) and ATS-iC3b (lane b). Eluants from ATS-C3b (lane a) contained complement receptor type 1 (CR1) (250 kDa) and the C3-binding regulatory protein gp45/70 (45–70 kDa) (39, 40). Eluants from ATS-iC3b (lane b) contained two additional proteins at 155 kDa and 94 kDa, representing mCD11b/CD18 heterodimer. CD11b/CD18 was immunoprecipitated with 44a mAb from material eluted from ATS-iC3b (lane d) but not from ATS-C3b (lane c). Molecular mass standards (arrowheads) were myosin, phosphorylase b, and bovine serum albumin. (B) Autoradiograph of an SDS/8% PAGE showing specific binding of metabolically labeled sCD11b/CD18 heterodimer to iC3b. sCD11b/CD18 was eluted from ATS-iC3b (lane b) but not from ATS-C3b (lane c). No specific radiolabeled material was present in eluant of ATS-iC3b exposed to sham-transfected COS cells (lane a). Molecular mass standards (arrowheads) are as in Fig. 2. Gel exposure to x-ray film (with an intensifying screen) was at −80°C for 5 days. Similar results were seen with supernatants from two other transfections.

**Fig. 4.** Autoradiograph of a SDS/10% PAGE evaluating binding of COS cell supernatants containing metabolically labeled sCD11b, sCD18, or sCD11b/CD18 heterodimer to iC3b. Binding activity was observed only in supernatants from COS cells expressing sCD11b/CD18 but not in those expressing either sCD11b or sCD18 alone. Culture supernatants contained equivalent amounts of sCD11b, sCD18, or sCD11b/CD18, as determined by immunoprecipitation studies (data not shown). Molecular mass standards (arrows) are the same as for Fig. 2.

amination of the ligand-binding properties of the recombinant receptor. sCD11b/CD18 bound to its ligand, iC3b, under similar conditions to those previously used to show direct and specific binding of mCD11b/CD18 (Fig. 3). Binding of sCD11b/CD18 to iC3b was divalent cation-dependent and specific in that no binding was detected to C3b. Binding also required expression of the secreted receptor in its heterodimeric form because no binding activity was displayed by its secreted subunits when expressed separately (Fig. 4). The
secreted heterodimer also significantly inhibited binding of PMN to rIL-1-activated endothelium.

β2 integrins have been suggested to require the cytoplasmic portion for enhanced adhesion to various ligands. Stimulus-induced phosphorylation of CD18 has been directly demonstrated (45) and occurs with kinetics similar to those of stimulus-induced adhesive interactions (46). The ability of sCD11b/CD18 to block β2-integrin-mediated cell adhesion indicates that the ligand-binding properties themselves are intact at least with regard to interaction with IC3b and rIL-1-activated endothelium. Similar findings have also been observed in other adhesive receptor families such as the cadherins (47). The observed posttranslational modifications in mCD11/CD18 receptors may enhance their affinity to ligands through interactions with cytoskeleton and/or expression of conformational epitopes or binding sites that may also become expressed in the recombinant receptor. Comparison of the binding affinities of secreted vs. membrane-bound integrins can now directly address this question.

The purified sCD11b/CD18 described in this report should be useful in identifying other putative ligands for this receptor that appear to mediate binding of phagocyte to endothelium (8, 9) and to a variety of microorganisms (48). It should also help in better delineation of the areas involved in heterodimer formation. Generation of larger quantities of this receptor from stably transfected cell lines should also permit a detailed evaluation of its functional activity in a large number of adhesive interactions mediated by CD11b/CD18 (e.g., phagocytosis, chemotaxis, binding to clotting factors, opsonized-zymosan-induced oxygen free radicals and proteolytic-enzyme release) (3). The anti-adhesive and complement IC3b-binding activities of sCD11b/CD18 demonstrated in this report make it (or a derivative) an attractive anti-inflammatory candidate, which could effectively block phagocyte emigration into inflamed organs as well as complement-dependent tissue injury. These effects may be additive when used in combination with the secreted form of the monomeric receptor CR1 (49). This approach should also apply to other integrins important in inflammatory and thrombo-occlusive disorders (50–52).

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