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Molecular cloning and adhesive properties of murine platelet/endothelial cell adhesion molecule 1

(immunoglobulin gene superfamily/CD31/leukocytes/membrane protein)

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ABSTRACT **We describe the isolation and characterization of a functional murine platelet/endothelial cell adhesion molecule (PECAM) 1 cDNA clone from a mouse lung library. At the nucleotide level, the coding sequence of murine PECAM-1 is 73% identical to human PECAM-1, and at the amino acid level, the sequence is 79% homologous to its human counterpart. Southern hybridization reveals that one copy of the gene exists in the mouse genome; Northern hybridization reveals a single mRNA species in mouse lung tissue. COS-7 and mouse L cells transfected with murine PECAM-1 expressed a 130-kDa glycoprotein on their surfaces that reacted with anti-murine PECAM-1 monoclonal antibody and comigrated on SDS/PAGE with human PECAM-1. Stable L-cell transfectants aggregate with each other in a PECAM-dependent, homophilic manner.**

Cell adhesion molecules (CAMs) belonging to the immunoglobulin gene superfamily perform essential roles in a wide range of immune responses. These CAMs possess the immunoglobulin fold and sequence pattern (1). They are believed to participate in a variety of homophilic and heterophilic cellular interactions (2), including those that occur during development [neural CAM (N-CAM)] (3, 4) and inflammation and wound healing [intercellular adhesion molecule 1 (ICAM-1) and vascular CAM (VCAM-1)] (5, 6).

Human platelet/endothelial CAM (PECAM) 1 is a 130- to 135-kDa membrane glycoprotein that is expressed on platelets, endothelial cells, monocytes, neutrophils, and certain T-cell subsets (7–10). Peptide sequence analysis has revealed that PECAM-1 is a member of the immunoglobulin supergene family. Within its extracellular domain, there are six immunoglobulin-like conserved homologous units of the C2 subclass (8). The structure of PECAM-1 is similar to other CAMs such as ICAM-1, VCAM-1, and N-CAM.

An adhesive function for human PECAM-1 has been demonstrated in cell–cell adhesion by a series of *in vitro* experiments. First, PECAM-1 is concentrated at the contact regions between endothelial cells (7) and transfected COS cells (11). Second, anti-PECAM antibodies inhibit the endothelial monolayer formation (11, 12). Third, mouse L cells stably transfected with PECAM-1 aggregate in a PECAM-dependent manner that can be blocked by anti-PECAM antibodies (11). Human PECAM-1 appears to mediate heterophilic (13) as well as homophilic adhesion (11).

In vitro experiments have suggested roles for PECAM in the inflammatory response. Ligation or crosslinking of PECAM on the surface of naive T cells activates β_1 integrins (14); crosslinking PECAM on the surface of monocytes and neutrophils activates β_2 integrin activity (W.A.M. and M. E. Berman, unpublished results). Recently, a critical function for PECAM has been demonstrated in the transendothelial migration of monocytes and neutrophils (29).

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Although the function of PECAM-1 *in vitro* has begun to be understood, its *in vivo* role is yet to be established. To study the function of PECAM *in vivo*, we sought to identify the murine counterpart of human PECAM. In this paper we report the isolation and sequencing of a full-length cDNA clone encoding murine PECAM-1.† Cell lines transfected with this clone express functional murine PECAM-1.

MATERIALS AND METHODS

Cells and Antibodies. COS-7 and mouse L cells were purchased from the American Type Culture Collection. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). L cells were cultured in RPMI medium with 10% FBS. Hamster monoclonal antibody (mAb) 2H8 recognizing murine PECAM-1 was provided by Steven Bogen (24). Murine mAb hec7 recognizes human PECAM-1 (7, 8).

Library Screening, Clone Isolation, and Characterization. Hybridization of human PECAM-1 probe to the murine lung cDNA library (Clontech) and clone characterization were as described in Sambrook *et al.* (15). Full-length human PECAM cDNA was labeled with ^{32}P by the random-primer labeling system (16) and hybridized in 6× standard saline citrate (SSC)/5× Denhardt's solution/0.1% SDS/50% formamide/denatured salmon sperm DNA (100 μg/ml) at 42°C to the murine lung cDNA library. Filters were washed twice with 2× SSC/0.1% SDS at 42°C for 30 min, then with 0.5× SSC/0.1% SDS at 65°C for 30 min. The filters were exposed to x-ray film overnight. Clones containing hybridizing sequences were purified, and the inserts were subcloned into the M13mp18 and M13mp19 bacteriophage and the Bluescript plasmid vectors (Stratagene) for further characterization (15). Subclones were characterized by restriction enzyme mapping, and the cDNA inserts were sequenced by the dideoxynucleotide chain termination method (17) using a Sequenase kit (United States Biochemical). All sequences were obtained on both strands. Alignments were performed by DNASIS and PROSIS programs (Hitachi Software Engineering) based on both protein and DNA sequence homologies.

Southern and Northern Blot Analysis. Genomic DNA was isolated from BALB/c mouse liver cells (15). Approximately 10 μg of DNA was digested with various restriction enzymes and run on a 0.8% agarose gel for 10 hr; the gel was blotted onto nitrocellulose (15). The murine PECAM-1 cDNA was labeled with [^{32}P]dCTP and hybridized to the filter.

Total RNA was isolated from BALB/c mouse lung (15). Approximately 15 μg of this RNA was run on a 1.0% agarose gel containing formaldehyde, blotted onto nitrocellulose, and probed with the murine PECAM-1 cDNA. The conditions for

Abbreviations: CAM, cell adhesion molecule; PECAM, platelet/endothelial CAM; mAb, monoclonal antibody; FBS, fetal bovine serum; CFSE, 5- and 6-carboxyfluorescein diacetate, succinimidyl ester.

†The cDNA sequence reported in this paper has been deposited in the GenBank data base (accession number L06039).

hybridization and washing for both Southern and Northern blots were as described for the library screen above.

Transient Transfection of COS-7 Cells with Murine PECAM. A 2.54-kb cDNA fragment containing the entire coding sequence for murine PECAM-1 was removed from the λgt11 vector and subcloned into the *Hin*dIII and *Not* I restriction sites of the pCDM8 vector (Invitrogen) to produce plasmid pmPECAM-1. COS-7 cells were resuspended in cold DMEM to about 2×10^7 cells per ml, and 0.5 ml was transferred to a 0.4-cm electrode Gene Pulser cuvette (Bio-Rad). The cells were transfected with 20 μg of pmPECAM-1 or PECAM/CDM8 [human PECAM-1 cDNA clone (13)] in a Gene Pulser electroporation device at 250 mV and 960 μF (18, 19). After electroporation, the COS-7 cells were plated on 100-mm Petri dishes in DMEM with 10% FBS and incubated in 5% CO_2 at 37°C for an additional 36 hr.

Stable Transfection. Murine PECAM-1 cDNA was subcloned into the pRC/CMV vector (Invitrogen) to make pcmPECAM-1. The L cells prepared as described above for COS cells were transfected with 20 μg of pcmPECAM-1 (linearized by restriction enzyme *Nru* I) in a Gene Pulser electroporation device at 240 mV and 960 μF (13). L cells were plated on six 100-mm Petri dishes in DMEM with 10% FBS. After 2 days, the medium was replaced with DMEM containing 10% FBS and G418 (Geneticin; GIBCO) at 0.5 mg/ml. After ≈2 weeks, G418-resistant clones were isolated and expanded. The clones expressing murine PECAM-1 were identified by indirect immunofluorescence staining using mAb 2H8. The most positive staining clones were subcloned by limiting dilution and then further characterized by fluorescence-activated cell sorting analysis (see below) using the murine PECAM-1-specific mAb 2H8.

Flow Cytometry. Flow cytometry was performed as described (13) using fluorescein isothiocyanate-labeled goat anti-hamster antibody or rabbit anti-mouse antibody.

Metabolic Labeling, Immune Precipitation, and Immunofluorescence. COS-7 cells transfected with murine or human PECAM-1 cDNA in pCDM8 were split and replated onto 30-mm Petri dishes. Metabolic labeling, immune precipitation, SDS/PAGE, and fluorography were performed as described (7). Immune complexes of murine PECAM-1 and hamster mAb 2H8 were retrieved by protein A-Sepharose using an intermediate layer of rabbit anti-hamster antibody (The Jackson Laboratory).

Aggregation of L-Cell Transfectants. These assays were performed as described for L cells expressing human PECAM (11, 13). Briefly, stable murine PECAM-1-transfected L cells (MPL5) and control cells (D6) were resuspended nonenzymatically in Hanks' balanced salt solution (HBSS) containing 10 mM EDTA at 37°C and pH 7.4 and washed twice in cold HBSS. In antibody-blocking experiments, the cells were incubated at this point for 30 min on ice with mAb supernatants. In the "mixed aggregation" experiments, cells were incubated at this point for 20 min on ice in the dark with or without 5- and 6-carboxyfluorescein diacetate, succinimidyl ester (CFSE). At the end of these incubations, the cells were resuspended to a concentration of 10^6/ml in warm HBSS (37°C) with or without 1 mM $MgCl_2$ and 2 mM $CaCl_2$.

One-milliliter aliquots of cells were added to a 24-well tissue culture tray precoated with human serum albumin to prevent nonspecific sticking (20, 21). The tray was rotated (90 rpm) at 37°C. Aggregation was stopped by adding glutaraldehyde to a final concentration of 2%. Aggregation was examined by random 10-μl aliquots from each sample on a hemacytometer grid under phase-contrast optics. The number of single cells or aggregates (of at least three cells) was counted in nine squares.

To determine whether aggregation involved homophilic or heterophilic adhesion, 0.5 ml of each of two cell populations (one fluorescently labeled with CFSE, the other unlabeled) were combined in the tissue culture well. The aggregation assay was carried out as described above. The cells were examined under UV light with fluorescein filters using a Nikon Microphot with a UFX-II camera system.

FIG. 1. Nucleotide sequence and predicted protein sequence of the cDNA encoding murine PECAM-1. Underlined sequences indicate putative leader and transmembrane sequences, respectively. Seven closed triangles indicate consensus recognition sequences for potential N-linked glycosylation sites. Cysteine residues marked by boldface type are thought to participate in disulfide bond formation within individual immunoglobulin homology units.

RESULTS

Isolation and Characterization of Murine PECAM-1 cDNA Clones. A mouse lung cDNA library (Clontech) was screened with a full-length human PECAM-1 probe. Fourteen positive clones from 10^6 plaques were subcloned into plasmids and sequenced. The P228 clone is 2534 bp long and has an open reading frame of 2181 nt, a 5′ untranslated sequence of 98 nt, and a 3′ untranslated region of 255 nt (Fig. 1). The P228 clone encodes a protein of 727 aa with a highly hydrophobic N-terminal amino acid sequence, which probably functions as a leader sequence, and a hydrophobic region typical of transmembrane domains (Fig. 1, residues 591–609). We have assigned the bond between aa 17 and 18 (from the initiator methionine) as the probable site of cleavage of the signal peptide from mature murine PECAM-1. This site conforms well with the (−3, −1) rule (22). The molecular mass of the resulting mature 710-aa deduced polypeptide chain is ≈80 kDa. There are seven predicted asparagine-linked glycosylation sites distributed over the molecule (Fig. 1). The full molecular size shown in immunoprecipitation is about 130 kDa; thus, carbohydrate residues must account for 40% of the molecular size of murine PECAM-1, as they do for the human form (8). The predicted structure of murine PECAM-1 appears to be a typical type 1 transmembrane protein. There are 573 aa in the N-terminal extracellular domain, 19 aa in the membrane-spanning domains, and 118 aa in the C-terminal cytoplasmic domains. The cytoplasmic domain contains a tyrosine residue (Fig. 1, aa 702) that could potentially serve as a phosphorylation site for tyrosine kinase. However, in human platelet PECAM, phosphorylation has only been detected on cytoplasmic serine residues (23).

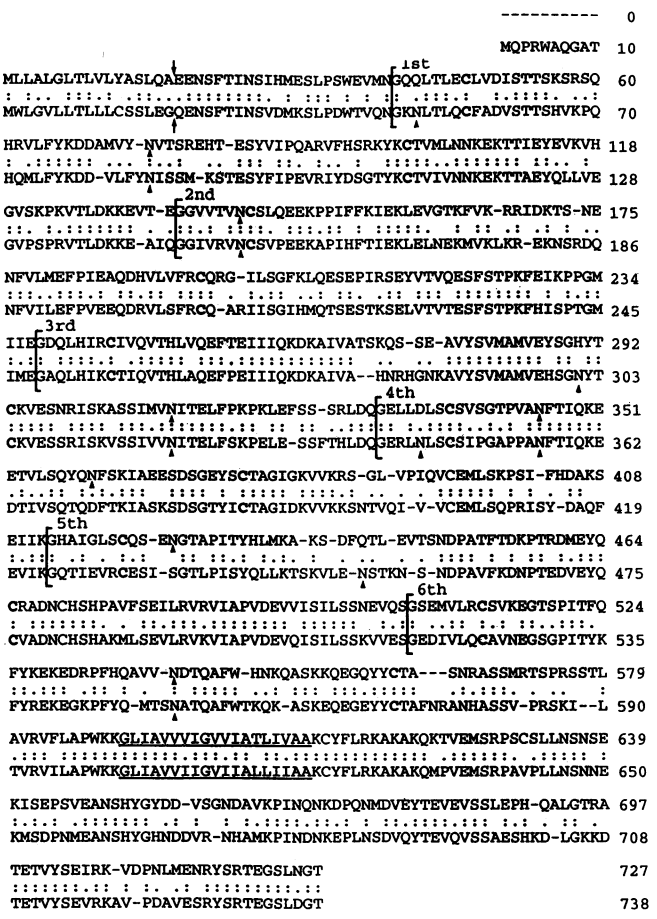

FIG. 2. Amino acid comparison of murine (upper sequence) and human (lower sequence) PECAM-1. Identical amino acids are indicated by two dots; conserved substitutions are indicated by one dot. Dashes indicate insertions to maximize homology. The conserved cysteine residues are printed in boldface type. The six C2-type immunoglobulin domains are indicated by vertical brackets. The putative N-linked glycosylation sites are marked by closed triangles. The probable cleavage site of the signal peptide is marked by an arrow.

Searches of GeneBank data base (Release 74.0) and the Genpept data base (Release 74.0) did not reveal any significant similarities to other proteins. Comparison with the p228 insertion sequence and the human PECAM-1 revealed significant homology at both the nucleotide and protein levels (Fig. 2). The overall nucleotide identity of the coding region with the human PECAM-1 is 73%. The overall amino acid identity with the human PECAM-1 is 63% (79% homology considering conservative substitutions). The immunoglobulin domain alignment of murine PECAM-1 is quite similar to that of human PECAM-1; all cysteine residues are conserved. This provides strong evidence that the cloned insertion sequence is a murine homolog of human PECAM-1.

Genomic Southern blot analysis revealed that the murine PECAM gene is present as a single copy. Northern blot analysis of transcripts in BALB/c lung tissue demonstrated that only a single transcript about 4.5 kb hybridized with a full-length mouse PECAM probe (data not shown).

Expression of Murine PECAM-1 in Transfected Cells. COS-7 cells were transfected with the murine PECAM-1 cDNA subcloned into the mammalian transient expression vector pCDM8. Approximately 25% of the cells expressed murine PECAM-1 on their surface by immunofluorescence (data not shown). Murine PECAM-1 expression was also demonstrated by immunoprecipitation from [^{35}S]methionine-labeled extracts from transfected COS-7 cells using an anti-murine PECAM-1 mAb (Fig. 3). Protein bands at 110 and 130 kDa were detected in transfected COS-7 cells (lane M2) that migrated identically to the material immunoprecipitated from positive control extracts of COS-7 that had been transfected with a human PECAM-1 cDNA clone (lane H2). The lower band probably represents an incompletely or alternatively glycosylated precursor protein (7). No material was immunoprecipitated by negative control mAb (lanes H1 and M1). mAb to murine PECAM did not recognize human PECAM and vice versa (Fig. 3, lanes H3 and M3).

Murine PECAM cDNA cloned into mammalian expression plasmid pRC/CMV (Invitrogen) was transfected into murine L cells, which do not express PECAM on the cell surface.

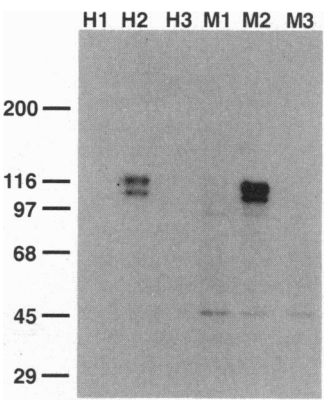

FIG. 3. Expression of murine PECAM by transfected COS cells. COS-7 cells transfected with human (H) or murine (M) PECAM-1 were metabolically labeled overnight and lysed in nonionic detergent. Immunoprecipitation followed by SDS/PAGE and fluorography was performed as described in *Materials and Methods* using the following antibodies: lane H1, hec1 (a nonbinding mouse IgG2a that is an isotype match for hec7); lane H2, hec7 (anti-human PECAM); lane H3, 2H8 (anti-mouse PECAM); lane M1, hamster anti-mouse CD3; lane M2, 2H8 (anti-mouse PECAM); lane M3, hec7 (anti-human PECAM). Molecular size markers (in kDa) are indicated.

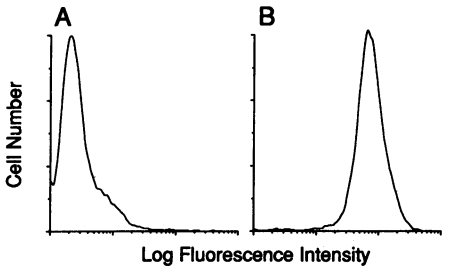

FIG. 4. Surface expression of murine PECAM by stable transfectants. MPL5, a clone of L cells stably transfected with murine PECAM, was analyzed for surface expression of murine antigens by fluorescence-activated cell sorting after immunofluorescence labeling. (*A*) Line MPL5 does not bind mAb 2E6, which recognizes murine CD18. (*B*) Line MPL5 stains intensely with 2H8, indicating definite surface expression of murine PECAM.

Sixty stably transfected clones were produced, eight of which expressed high levels of PECAM.

Surface PECAM staining of a representative positive clone, MPL5, is illustrated in Fig. 4. A single population of cells stained brightly with the mAb against murine PECAM (Fig. 4*B*) but was unstained by control mAb (Fig. 4*A*). MPL5 cells did not stain with irrelevant mAb (data not shown).

Murine PECAM-1 Mediates the Aggregation of Transfected L Cells. L cells expressing murine PECAM (MPL5) rapidly formed aggregates in suspension, whereas cells derived from the same L-cell parent expressing only the neomycin-resistance gene (D6) did not (Fig. 5*A*). Distinct aggregation was observed within 5 min (data not shown), and aggregation increased during the testing period (45 min). In the absence of divalent cations, >50% of transfected cells were in aggregates by 45 min. The presence of calcium and magnesium raised the aggregation efficiency to 80%. As a further demonstration of specificity, anti-murine PECAM mAb 2H8 blocked aggregation of MPL5 cells (Fig. 5*B*).

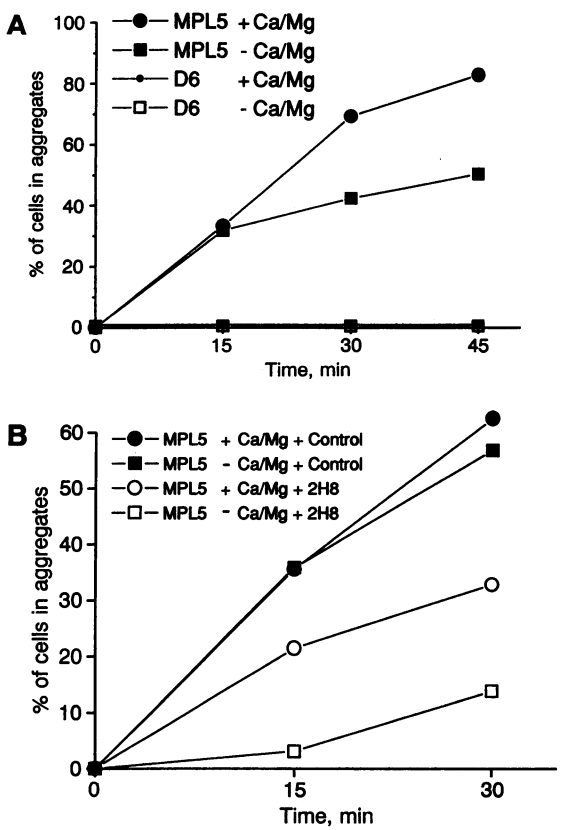

FIG. 5. Murine PECAM mediates L-cell aggregation. (*A*) L cells stably transfected with murine PECAM (MPL5) or control stable transfectants (D6) were nonenzymatically resuspended in the presence or absence of divalent cations and subjected to the standard aggregation assay. Aggregation was observed only in cells expressing mouse PECAM. (*B*) Nonenzymatically resuspended MPL5 cells were incubated for 30 min on ice with 2H8 or negative control mAb supernate (hec7), washed free of unbound antibody, and subjected to the aggregation assay with or without divalent cations. Standard deviations of measurements were <10%.

Murine PECAM Mediates Homophilic Adhesion. To identify whether adhesion of murine PECAM was homophilic or heterophilic, a mixed aggregation experiment was performed similar to that defined for other CAMs (21). Typical results are presented in Fig. 6. When unlabeled control cells and fluorescently labeled murine PECAM transfectants are mixed, all of the cells in the aggregates fluoresce (NP*); hence all express PECAM. When labeled control cells are mixed with unlabeled PECAM transfectants, all of the cells in the aggregates are unlabeled PECAM transfectants (PN*). As a control, an equal mixture of labeled and unlabeled PECAM transfectants yielded aggregates with both labeled and unlabeled cells, demonstrating that the CFSE labeling procedure did not influence aggregation (PP*).

The results quantitated in Fig. 7 are typical of three separate experiments. These data demonstrate that cells bearing murine PECAM form aggregates only with other PECAM-bearing cells.

DISCUSSION

Structure of Murine PECAM-1. The sequence identity between human and murine PECAM-1 is 73% at the DNA level and 63% at the protein level (79% considering conservative amino acid substitutions). Murine PECAM-1 is also organized with six extracellular immunoglobulin-like domains linked tandemly. The human and murine protein sequences are easily aligned to place these domains in similar

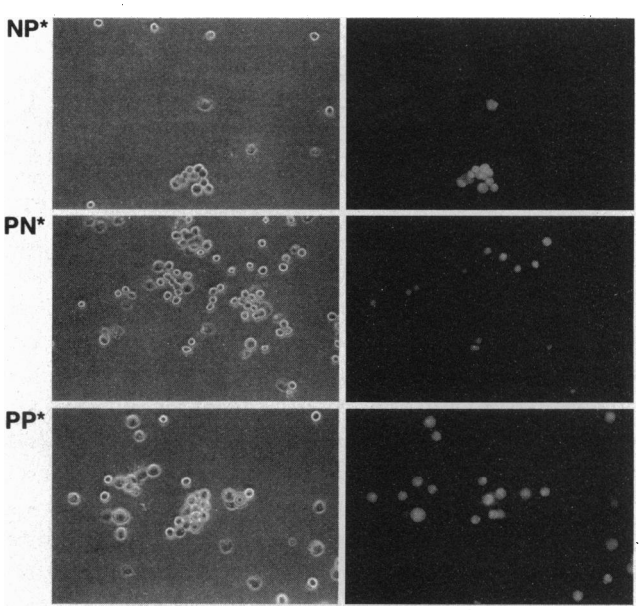

FIG. 6. Murine PECAM-1 mediates homophilic aggregation. Equal numbers of murine PECAM-positive L cells (P) were mixed with control L-cell transfectants (N) in the presence of divalent cations for 30 min at 37°C. Aggregates were examined by phase-contrast (*Left*) and fluorescence (*Right*) microscopy. The top row shows unlabeled murine PECAM-negative cells (N) mixed with an equal number of CFSE-labeled murine PECAM-positive cells (P*). The middle row indicates murine PECAM-positive cells (P) mixed with equal numbers of CFSE-labeled murine PECAM-negative cells (N*). The bottom row (PP*) indicates the equal mixture of unlabeled and labeled murine PECAM-positive L cells.

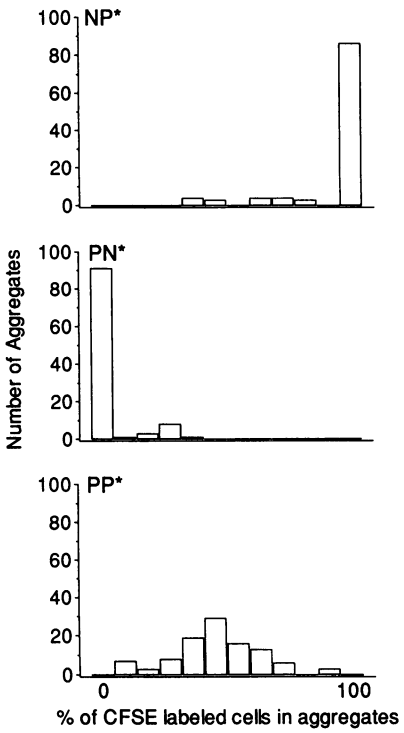

FIG. 7. Quantitation of homophilic aggregation by murine PECAM. One hundred eight aggregates (of at least three cells) were randomly chosen from each of the samples in a mixing experiment similar to that shown in Fig. 6. The percentage of labeled cells in each aggregate is plotted in increments of 10%. Aggregates with 0% and 100% labeled cells are plotted in the indicated columns at the ends of the histogram. The abbreviations are those used in Fig. 6.

positions; the six pairs of cysteine residues that form the disulfide bridges are entirely conserved (Fig. 2). The tertiary structure of murine PECAM-1 and its potential function(s) are likely to be very similar to that of human PECAM-1.

During the preparation of this manuscript, Bogen *et al.* (24) identified murine PECAM-1 using mAb 2H8 and reported its N-terminal sequence. This same publication reported the cloning of murine PECAM and the cDNA sequence corresponding to the N-terminal decapeptide (24). Our cDNA sequence in this region matches the one reported, and our predicted N-terminal protein sequence is compatible with the actual N-terminal peptide sequence of murine PECAM-1. At this writing, the full sequence of their clone has not been published and is not available in the EMBL/GenBank data base, so a further sequence comparison is not possible.

Homophilic Adhesion of Murine PECAM. COS and L cells transfected with a eukaryotic expression vector containing murine PECAM-1 cDNA both resulted in the specific expression of a 130-kDa surface glycoprotein (Figs. 3 and 4) that was bound by anti-murine PECAM mAb 2H8 and that comigrated with human PECAM by SDS/PAGE. Moreover, murine PECAM-1 functioned as a cell–cell adhesion molecule: L-cell transfectants expressing murine PECAM adhered to each other in a PECAM-dependent manner in a standard Takeichi-type aggregation assay (20), whereas control transfectants did not (Fig. 5*A*). Furthermore, this aggregation (particularly in the absence of divalent cations) could be inhibited by murine PECAM-1-specific mAb 2H8 (Fig. 5*B*). Binding of murine PECAM-1 in the aggregation assay is apparently homophilic (Figs. 6 and 7) and can occur in the absence of calcium and magnesium (Fig. 5*A*). Similar aggregation is found in a number of well-documented immunoglobulin superfamily proteins (4, 25–28).

In contrast, *human* PECAM-1-transfected L cells aggregate in a divalent cation-dependent (11, 13) and heterophilic (13) manner in the same assay. Expression of human PECAM in murine cells may affect the posttranslational modification and/or adhesion preference of human PECAM. Alternatively, the difference in the number (nine in human, seven in mouse) and location (see Figs. 1 and 2) of glycosylation sites may be responsible for this difference. Whatever the explanation, heterophilic aggregation of human PECAM precludes the use of this assay to study potential binding of human PECAM to murine PECAM.

The observed differences in adhesion mechanism underscore the importance of obtaining the murine clone. The murine PECAM-1 cDNA clone will be an invaluable tool in the elucidation of the function of this CAM *in vivo*. Mammalian expression systems can serve as the source of membrane-bound or soluble antigen for the generation of high-affinity blocking mAb. Antisense probes and recombinant soluble molecules can now be generated to specifically study the function of PECAM in well-established murine models of acute and chronic inflammation *in situ* and *in vivo*.

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