Platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) is a 130-kDa integral membrane glycoprotein expressed on endothelial cells, platelets, and leukocytes. Experiments analyzing the aggregation of mouse L-cells stably transfected with full-length PECAM-1 cDNA have demonstrated that PECAM-1 is capable of cell-surface-dependent heterophilic aggregation. In this report the ligand interactions involved in the aggregation process were studied. This aggregation was inhibited by heparin and chondroitin sulfate, but not by other glycosaminoglycans. Enzymatic removal of cell surface glycosaminoglycans confirmed a PECAM-1-glycosaminoglycan interaction and suggested that this interaction involved glycosaminoglycans on adjacent cells. PECAM-1 contains a glycosaminoglycan consensus binding sequence in the second immunoglobulin-like domain of the molecule’s extracellular domain. A comparable region in the related adhesion protein N-CAM has been shown to mediate the adhesive properties of N-CAM. Cells expressing mutant PECAM-1 protein missing the second domain failed to aggregate. Synthetic peptides mimicking the consensus glycosaminoglycan binding sequence, L-K-R-E-K-N, inhibited aggregation. These results demonstrate that PECAM-1-mediated aggregation is dependent on the binding of PECAM-1 to specific glycosaminoglycans on adjacent cells via a glycosaminoglycan consensus binding sequence in the second immunoglobulin-like homology domain.

**PECAM-1**

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\*\*\*\* The abbreviations used are: PECAM-1, platelet/endothelial cell adhesion molecule-1; CAM, cell adhesion molecule; GAG, glycosaminoglycan; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; mAb, monoclonal antibody; bp, base pair(s); SOE, splicing by overlap extension; HBSS, Hanks’ balanced salt solution; PAGE, polyacrylamide gel electrophoresis.

The sequence homology of PECAM-1 to other members of the Ig superfamily that function as cell adhesion molecules (CAMs) suggested that PECAM-1 might also function in a similar capacity (Newman et al., 1990). This concept is supported by the observations that PECAM-1 is concentrated at intercellular borders of adjacent cultured endothelial cells (Muller et al., 1989; Albelda et al., 1990) and that antibodies against the bovine homolog (endo-CAM) impair the ability of endothelial cells to form confluent monolayers (Albelda et al., 1990). Direct evidence that PECAM-1 functions as a cell adhesion molecule was recently obtained in experiments showing that: 1) 3T3 and COS cells transfected with the full-length PECAM-1 cDNA formed cell junctions that were highly enriched in PECAM-1, in a manner reminiscent of that seen at endothelial cell-cell borders. Significantly, no localization of PECAM-1 was seen at the borders of transfected and nontransfected cells; 2) PECAM-1-transfected 3T3 cells co-cultured with human umbilical vein endothelial cells localized PECAM-1 to points of contact between transfected cells and endothelial cells; and 3) mouse L-cells stably transfected with PECAM-1 cDNA demonstrated calcium-dependent aggregation that was inhibitable by polyclonal anti-PECAM-1 antibodies (Albelda et al., 1991). Although it has been demonstrated that PECAM-1 functions as a cell adhesion protein, the molecular nature of PECAM-1-mediated interactions is still not well understood. In order to further characterize the nature of PECAM-1-mediated adhesion, we have recently examined the aggregation of PECAM-1-transfected mouse L-cells (Albelda et al., 1991) using an approach similar to that employed in the study of cadherins (Takeichi, 1991), CD44 (St. John et al., 1990), and members of the immunoglobulin superfamily (Edelman and Crossin, 1991; Fibin et al., 1990). In transfected mouse L-cells, PECAM-1-dependent L-cell aggregation was found to be temperature-sensitive and supported by calcium or magnesium (Muller et al., 1992). In addition, using a "mixed" aggregation assay, where a mixture of transfected and nontransfected cells were allowed to aggregate together, PECAM-1-transfected cells were found to bind both to transfected, as
well as nontransfected L-cells (Muller et al., 1992), suggesting that heterophilic interactions account for at least some PECAM-1-dependent aggregation. 

These observations raised questions concerning the nature of the ligand(s) for PECAM-1. One possibility is that glycosaminoglycans (GAGs) may be involved. This is suggested by the fact that PECAM-1, like the related Ig superfamily adhesion molecule N-CAM, contains a consensus GAG binding sequence (Cardin and Weintraub, 1989; Cole and Akeson 1989; Reyes et al., 1990) in its second Ig-like homology domain (Newman et al., 1990; Albelda et al., 1991). Furthermore, GAGs have been implicated in adhesive events mediated by other Ig superfamily molecules such as N-CAM and CD4 (Reyes et al., 1990; Probstmeier et al., 1988; Lederman et al., 1989). The present study addresses this question and presents evidence that PECAM-1 is capable of mediating heterophilic aggregation through interactions with specific glycosaminoglycans on adjacent cells via the putative GAG binding sequence in the second Ig-like homology domain.

**Experimental Procedures**

**Glycosaminoglycans and Glycosaminoglycan-degrading Enzymes**

The following glycosaminoglycans and glycosaminoglycan-degrading enzymes were purchased from Sigma: heparin (sodium salt, from porcine intestinal mucosa), heparan sulfate (sodium salt, from bovine urinary bladder), keratan sulfate (sodium salt, from bovine cornea), dermatan sulfate (sodium salt, from porcine skin), dextran sulfate (average molecular weight, 500,000), hyaluronic acid (sodium salt, from bovine trachea), heparinase I, chondroitinase ABC, and hyaluronidase (from Streptomyces hyalurolyticus).

**Synthetic Peptides Corresponding to the Glycosaminoglycan Binding Domain of PECAM-1**

Synthetic peptides used in this study were synthesized on a MILLGEN 0650 Automated Peptide Synthesizer using standard Fmoc (N-9-(fluorenlyl)methoxycarbonyl)-polyamide solid phase chemistry. Three peptides were synthesized: L-K-R-E-K-N corresponding to residues 150-155 of PECAM-1, the reverse sequence, N-K-E-R-K-L, and a scrambled peptide, R-E-K-N-L-K. All three peptides were of identical size and charge and were purified by preparative reverse phase high performance liquid chromatography before use. Amino acid compositions were verified by dansyl chloride analysis of HCl hydrolysates of each synthetic peptide. All three peptides were water-soluble and were stored in small aliquots in PBS at -20 °C until use.

**Cells**

COS-7, 3T3 cells, and mouse L-cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). COS-7 cells and 3T3 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. L-cells were cultured in RPMI medium with 10% fetal bovine serum. The procedures to transfect PECAM-1 cDNA into these mammalian cells have been described previously (Albelda et al., 1991). Fluorescence-activated Cell Sorting (FACS) Analysis L-cells or 3T3 cells transfected with wild-type or mutated PECAM-1 cDNAs were enzymatically removed from the T25 flasks with trypsin/EDTA, washed in PBS, and treated with various anti-PECAM-1 mAbs for 1 h at 4 °C. The primary antibody was then removed, the cells washed twice with ice-cold PBS, and a 1:200 dilution of fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody (Cappel, West Chester, PA) added for 30 min at 4 °C. After washing in cold PBS, flow cytometry was performed using an Ortho Cytofluorograph 50H cell sorter equipped with a 2150 data handling system (Ortho Instruments, Westwood, MA).

**Construction of Mutant PECAM-1 (PECAM-1L or PECAM-12) Lacking the First or Second Ig-like Domain**

Polymerase chain reaction (PCR) based site-directed mutagenesis was used to generate mutant cDNAs lacking the coding sequence for the first (N-terminal) extracellular Ig-like domain (PECAM-1L) or the second (N-terminal) extracellular Ig-like domain (PECAM-12). Specifically, the full-length PECAM-1 cDNA in the expression vector pESP-SVTEXP ("TEX/PECAM") (Albelda et al., 1991) was cut with two unique restriction endonucleases, Apol and BstEI (Fig. 1). Apol cuts early in the signal sequence at bp 159, whereas BstEI cuts at bp 819, a site between the second and third Ig-like domains. PCR-generated replacement sequences were then engineered for each construct and then ligated into the digested vector. The approach for each construct is outlined below and in Fig. 1.

**PECAM-1Δ**—This construct was generated using the PCR technique of gene splicing by overlap extension (SOE) (Horton et al., 1990) using the following strategy. PCR was used to generate the sequence from the Apol site to bp 246, located immediately 5' to the sequence for domain 1 (5' fragment). The forward primer (Primer 1: AGGTGGGCCCAAAGG) used to make this insert was complementary to the region of the signal sequence containing the Apol site. The reverse primer (Primer 2: GGCCACCGGTTGAGTTGAAAGG) was complementary to the sequence immediately 5' to domain 1 and contained added base pairs that overlapped the region immediately following domain 1. A second fragment (3' fragment) was produced containing the sequence following domain 1, beginning at bp 546 and extending to the BstEI site. The forward primer for this construct (Primer 3: ACAATCAACAGGATGCCCAGTCCC) was complementary to the sequence immediately following domain 1 and contained base pairs that overlapped the sequence immediately 5' to domain 1. The reverse primer (Primer 4: CACGGTGACAGTGTCCACT) included the BstEI site and was then joined together by SOE and amplified by second round PCR. The resulting mutated cDNA lacked the coding sequence for the first Ig-like domain of PECAM-1. After purification, the DNA produced by PCR SOE was cut with Apol and BstEI and then ligated into the previously digested TEX/PECAM vector.

**PECAM-1Δ**—In order to restore the signal sequence, reclamp domain 1 and place the DNA back in the proper reading frame, PCR was used to generate a small insert that included the missing portion of the signal sequence as well as the domain for signal sequence and contained Apol and BstEI at the 5' and 3' ends, respectively. The forward primer (AGGTGGGCCAAAAGG) used to make this insert was complementary to the region of the signal sequence containing the Apol site. The reverse primer (CAGGCGTAGCAGTGACAGTGTCCACT) was complementary to a region between domains 1 and 2 in its 3' end and contained added base pairs at its 5' end that reconstituted the BstEI cloning site. The resulting mutated DNA lacked the coding sequence for the second Ig-like domain of PECAM-1. After purification, the DNA produced by PCR was cut with Apol and BstEI and then ligated into the similarly digested TEX/PECAM vector.

**Cell Labeling**

In order to test the effect of GAG-degrading enzymes on the integrity of PECAM-1, transfected cells were surface-labeled with 3H using previously described procedures (Albelda et al., 1989). Surface-labeled cells were then exposed to heparinase (5 units/ml) as described below. After heparinase treatment, membrane protein extracts were prepared by adding three volumes of TNC (0.01 M Tris acetate, pH 8.0, 0.5% Nonidet P-40, 0.5 mM CaCl2) containing 2 mM phenylmethylsulfonyl fluoride to the pellet, mixed on ice for 15 min, and then centrifuging for 30 min at 12,000 x g. The resulting supernatant was used immediately or stored at -20 °C until use. The remaining pellet was reconstituted with 100 mM Tris acetate, pH 8.0, 0.5% Nonidet P-40, 0.5 mM CaCl2, and 2 mM phenylmethylsulfonyl fluoride, mixed on ice for 15 min, and then centrifuged for 30 min at 12,000 x g. The resulting pellet was reconstituted with 100 mM Tris acetate, pH 8.0, 0.5% Nonidet P-40, 0.5 mM CaCl2, and 2 mM phenylmethylsulfonyl fluoride. Following incubation, free biotin was removed by washing with cell wash buffer. Membrane protein extracts were then prepared as described above.
Preparation of PECAM Δ1

Preparation of PECAM Δ2

FIG. 1. Construction of mutant PECAM-1 cDNA. The full-length PECAM-1 cDNA in the expression vector pESP-SVTEXP (TEX/PECAM) (Albelda et al., 1991) is depicted schematically in the figure. Open and hatched boxes represent, respectively, the immunoglobulin-like homology units and the interconnecting regions. The solid boxes represent the signal sequence and the transmembrane domain. TEX/PECAM was cut with two unique restriction endonucleases, ApaI and BstEII. ApaI cuts early in the signal sequence at bp 159, whereas BstEII cuts at bp 819, a site between the second and third Ig-like domains. Simultaneously, PCR-generated replacement sequences were then engineered for each construct, digested with ApaI and BstEII, and then ligated into the digested vector. The sequences for the five primers (+) used are: 1) AGGTGGCCCAAGG; 2) GGGCACCTCGTGTGTTGGAAG; 3) ACAATCAACGGGAGTCCC; 4) TCACTGAGCTGCCCCAAGTTT; 5) TCACTGAGCTGCCCCAAGTTT.

Immunoprecipitation

Nonionic detergent extracts were prepared as described in the methods. Cells were harvested by trypsinization, washed once with cold PBS, and resuspended at a concentration of 1 × 10⁶ cells/ml in HBSS. Samples were disrupted by boiling for 3 min. Immunoprecipitation was performed as described in the Methods. The samples were then dissolved with sample loading buffer (62.5 mM Tris base, 2% SDS, 10% glycerol, pH 6.8), denatured by boiling for 3 min, and electrophoresed on 8% SDS-PAGE gels, and processed for enhanced chemiluminescence study (Schimmenti et al., 1992) or autoradiography using Kodak XR-5 x-ray film.

Aggregation of L-cell Transfectants

The aggregation assay used, a variation of the system developed by Takeichi (1977) for the study of cells expressing cadherin molecules, has been described in detail previously (Albelda et al., 1991, Muller et al., 1992). Stable L-cell transfectants, which had been plated (8–10 × 10⁶ cells/75-cm² flask), were removed from the flasks by trypsinization and EDTA for 3 min. Immunoprecipitation of [35S]-surface-labeled PECAM-1-transfected cells, as well as FACS analysis using anti-PECAM-1 antibodies, demonstrated that PECAM-1 was not altered by this treatment (data not shown). After neutralization of the trypsin with media containing serum, the cells were washed twice with 10 mM EDTA in PBS, pH 7.2, and twice with HBSS without divalent cations. Cells were then resuspended to a concentration of approximately 1 × 10⁶ cells/ml in HBSS with 1 mM Ca²⁺. Glycosaminoglycans, enzymes, peptides, and antibodies were added as indicated to give the desired concentration (see results). After the cells had been dispersed to a single cell suspension, 1-ml aliquots were transferred to wells in a 24-well non-tissue culture plates (Costar Corp., Cambridge, MA) that had been incubated previously with 2% BSA in HBSS for at least 1 h and washed thoroughly with HBSS immediately before use. This treatment prevented nonspecific binding to the bottom of the well (Takeichi, 1977). The 24-well plates containing the suspended L-cells were rotated on a gyratory platform (100 rpm) at 37 °C for 45 min. Aggregation was quantified by examining representative aliquots from each sample on a hemocytometer grid using phase contrast optics. The number of cells in aggregates of greater than three cells, as well as the total number cells, were counted from four 1-mm² areas. Data were expressed either as the percent of total cells present in aggregates (%) aggregation = A/A × 100, where A = sample aggregation − mean background aggregation, and A = mean control aggregation − mean background aggregation.

Mixed Aggregation Assay

To determine whether the PECAM-1-dependent L-cell aggregation was mediated by homophilic or heterophilic mechanisms, "mixed aggregation" assays were performed (Muller et al., 1992). In these experiments L-cell aggregation was performed as described above, except that two populations of cells, control and transfected, were used. One set of cells was labeled prior to aggregation by incubating for 30 min at 37 °C, in 10 ml of 33 μM 5-(and 6-) carboxyfluorescein diacetate succinimide ester (CSFE; Molecular Probes, Eugene OR) in HBSS (Bronner-Fraser, 1985). Each set of cells, one labeled, and the other unlabeled, were resuspended at 1 × 10⁶ cells/ml. One-half-ml aliquots from each were combined in the
wells of a 24-well non-tissue culture plate and allowed to aggregate as described above. After the aggregation was complete, the cells were viewed with fluorescence microscopy. The number of fluorescent cells in each aggregate of a given size was counted. Quantitative analysis of the aggregating cell populations was performed as described by Sieber and Roseman (1981).

Treatment of L-cells with GAG-degrading Enzymes

The effects of the GAG-degrading enzymes, heparinase, chondroitinase ABC, and hyaluronidase on aggregation were determined by pretreatment of PECAM-1-transfected or control-transfected L-cells with each enzyme. Cells were enzymatically removed from T75 flasks followed by digestion with the GAG-degrading enzymes at concentrations shown by others to be effective in removing cell surface GAGs. Specifically, cells were treated with heparinase (5 units/ml; Massia and Hubbell, 1992), chondroitinase ABC (50 milliunit/ml; Yamada et al., 1980; Faassen et al., 1992), or hyaluronidase (100 milliunit/ml; Yamada et al., 1980; Munaim et al., 1991) for 30 min at 37°C. The cells were then pelleted gently and the enzyme removed. Washed cells were then used in the aggregation assay as described above.

RESULTS

Specific Glycosaminoglycans Inhibit PECAM-1-mediated Aggregation—Because of the presence of a GAG binding consensus sequence in PECAM-1 and the similarities of PECAM-1 to N-CAM (which is known to have GAG binding properties), the role of GAGs in PECAM-1-mediated L-cell aggregation was examined by determining the ability of PECAM-1-transfected L-cells to form aggregates in the presence of a variety of GAG’s.

In the presence of 1 mM Ca²⁺, approximately 25–35% of PECAM-1-transfected L-cells became incorporated into aggregates of four cells or greater (Fig. 2A). This aggregation was reduced to base-line levels of 5–10% in the absence of Ca²⁺. This background level of aggregation was similar to that seen in L-cells transfected with neomycin-resistance plasmid alone (Abel et al., 1991, Muller et al., 1992).

Heparin, at concentrations of 50–500 µg/ml, markedly inhibited calcium-dependent aggregation in a concentration-dependent fashion (Fig. 2A). Heparin sulfate and chondroitin 6-sulfate also reduced aggregation at a concentration of 50 µg/ml (Fig. 2B). The dose-response relationship of these GAGs was very similar to that seen with heparin (data not shown). In contrast, other GAGs and sulfated polysaccharides, including dermatan sulfate, dextran sulfate, keratan sulfate, and hyaluronate had no inhibitory effect on aggregation, even at concentrations up to 500 µg/ml (Fig. 2B), thus ruling out nonspecific inhibition related to the simple charge characteristics of GAGs. Under our experimental conditions, heparin (up to 500 µg/ml) did not lower measured ionized calcium and increasing the calcium concentration to 3 mM did not reverse the inhibitory effect of heparin (data not shown). Together, these data indicate that the inhibition of aggregation by heparin is not due to the chelation of calcium.

PECAM-1-mediated L-cell Aggregation Is Inhibited by Removal of Specific GAGs from the Cell Surface—In order to confirm that the glycosaminoglycans, heparin, heparan sulfate, or chondroitin 6-sulfate participated in PECAM-1-mediated aggregation, PECAM-1-transfected or control L-cells were digested with GAG-degrading enzymes and then tested for their ability to form aggregates. As shown in Fig. 3A, the addition of heparinase (5 units/ml) reduced the ability of PECAM-1-transfectants to aggregate by approximately 80%. A smaller (42% inhibition), but significant effect, was seen following digestion with chondroitinase ABC, but not hyaluronidase (Fig. 3A), confirming the specificity of the PECAM-1-GAG interaction. The possibility that this inhibition might be due to proteases contaminating the enzyme preparation was addressed in two ways. First, transfected cells were surface labeled with ¹²⁵I and subsequently digested with heparinase. Control cells were incubated with buffer alone. Immunoprecipitation of extracts of these cells using anti-PECAM-1 mAb showed that digestion with heparinase had no effect on the behavior of the PECAM-1 protein on SDS-PAGE (Fig. 3B). Second, aggregation experiments were performed in which the cells were pretreated with a high concentration of heparin (500 µg/ml), heparinase (5 units/ml), or both (to neutralize the GAG-digesting effects of the enzyme). After washing, the cells were allowed to aggregate as described above. Under these conditions, heparinase but not heparin/heparinase treatment inhibited aggregation (data not shown). Together these data indicate that the reduction in aggregation was mediated by GAGs and was not caused by proteolysis of PECAM-1 or other cell surface proteins during the digestion procedure.

Mixed Aggregation Experiments Support a Role for GAG Binding—To study the binding mechanisms involved in PECAM-1-dependent L-cell aggregation, a “mixed” aggregation assay was used (Muller et al., 1992). In these experiments, either the transfected or control L-cells were fluorescently labeled prior to the addition of equal numbers of unlabeled cells. After incubation, the composition of each four-, five-, and six-cell aggregate was determined microscopically (Fig. 4).

By analyzing the frequency distribution of fluorescent and non-fluorescent cells within the four-, five- and six-cell aggregates, it is possible to theoretically predict whether PECAM-1-mediated interactions are homophilic or heterophilic in nature (Sieber and Roseman, 1981: Fig. 5).

Fig. 6A summarizes the actual data obtained from a mixed aggregation assay in which the distribution of fluorescent and non-fluorescent cells in the five cell aggregates was analyzed as described above. A normal distribution of cellular aggregates was obtained, consistent with the previously observed heterophilic mechanism of interaction (Muller et al., 1992). Similar results were obtained counting four- or six-cell aggregates (data not shown). It should be noted, as demonstrated in Fig. 4, that in many of the aggregates, the control-transfected L-cells were on the periphery of the aggregates, indicating that they were not trapped nonspecifically in aggregates of PECAM-1-transfectants.

In order to confirm our observations regarding the role of GAGs in PECAM-1-mediated L-cell aggregation, and to address the issue of whether the PECAM-1-GAG interaction was occurring on the same cell (i.e. a molecule of PECAM-1 interacting with an adjacent GAG molecule on the same cell) or on different cells (i.e. a molecule of PECAM-1 binding to a GAG molecule on an adjacent L-cell), further mixed aggregation studies were conducted in which either PECAM-1-transfected or control-transfected L-cells were digested with heparinase prior to mixing. If PECAM-1 was interacting with a heparin-like proteoglycan molecule on the surface of an adjacent L-cell, then heparinase pretreatment of control-transfected cells should prevent them from interacting with the transfected cells and allow only the PECAM-1-transfectants to form aggregates with one another. This would alter the frequency distribution of fluorescently labeled versus nonlabeled cells from a heterophilic random-type distribution to a homophilic distribution (see Fig. 5). Or, the other hand, treatment of the PECAM-1 transfectants with heparinase should not prevent them from aggregating with the control-transfected control L-cells, as the PECAM-1 on the surface of the transfectants would still be available to bind to heparan proteoglycans on the surface of the control-transfected control.
PECAM-1-mediated Aggregation Is GAG-dependent

Fig. 2. The effect of glycosaminoglycans on PECAM-1-mediated aggregation. The aggregation assay using PECAM-1-transfected L-cells was performed as described under “Experimental Procedures,” with GAGs added to the aggregating cell mixture. A, heparan dose response. PECAM-1-transfected L-cells were allowed to aggregate in the presence of 1 mM calcium and various concentrations of heparin. These data presented are representative of four experiments done in triplicate. Background corresponds to conditions in which no calcium or glycosaminoglycans were present. Heparin inhibited aggregation in a dose-dependent manner. Similar results were obtained for heparan sulfate and chondroitin 6-sulfate. B, comparison of the effects of different glycosaminoglycans. The GAG concentrations used in these experiments were 50 μg/ml. Similar results were seen at concentrations up to 500 μg/ml. Background aggregation was 5-10%; control aggregation was 25-35%. The means and standard deviations were computed on the basis of at least two separate experiments done in triplicate.

Functional Significance of a GAG Recognition Sequence on PECAM-1—The amino acid sequences -X-B-B-X-B-X- and -X-B-B-X-X-B-X- (where B and X, respectively, correspond to basic and nonbasic residues) represent consensus sequences for glycosaminoglycan recognition (Cardin and Weintraub, 1989). As noted above, PECAM-1 contains such a sequence, L-K-R-E-K-N, at residues 150-155 of the second Ig-like homology unit of its extracellular domain. To establish the functional significance of this region, two mutant DNA constructs were produced: one lacking the second Ig-like domain (PECAMAB) and a control construct lacking the first Ig-like domain (PECAMAl). After sequencing to verify the correct structure, each construct was transfected into L-cells and clones showing high expression were selected. Fig. 7 shows the FACS profiles of representative L-cell clones expressing wild-type (Fig. 7A), PECAMAl (Fig. 7B), and PECAMAB (Fig. 7C) constructs. All three clones expressed similar amounts of protein on their surfaces. This level of PECAM-1 expression is similar to that seen on cultured endothelial cells (data not shown). Fig. 7D demonstrates an immunoprecipitation of each of the constructs with anti-PECAM-1 antibody. The transfected wild-type PECAM-1 has a molecular size of 130 kDa, identical to native PECAM-1 found in endothelial cells (Albelda et al., 1991). Both mutants, as expected, are smaller and have similar molecular sizes of approximately 100 kDa.

Having verified that the mutant molecules expressed in L-cells were of the appropriate size and that they were expressed at equivalent levels as wild-type PECAM-1, the ability of the
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above control levels. Because it could be argued that the mutation induced in the extracellular domain of PECAM-1 resulted in a nonspecific loss of function, we also produced a "control" mutant missing the first Ig-like domain (PECAMΔ1). In contrast to PECAMΔ2, cells transfected with the PECAMΔ1 construct were able to aggregate almost normally (Fig. 8). These experiments thus show that the second Ig-like domain is required for PECAM-1-mediated L-cell aggregation.

To further confirm the functional significance of the GAG recognition sequence, synthetic peptides containing the amino acids present in this sequence were prepared and their effects on aggregation examined (Fig. 9). The 6-amino acid peptide (L-K-R-E-K-N, PECAM-1 150–155) that exactly mimicked the sequence in PECAM-1 reduced aggregation by 70%, whereas a scrambled peptide (R-E-K-N-L-K) and an irrelevant peptide had no effect (Fig. 9A). The peptide inhibition of aggregation occurred in a concentration-dependent manner (Fig. 9B). Interestingly, a peptide with the reverse sequence (N-K-E-R-K-L) also strongly blocked aggregation (Fig. 9A).

DISCUSSION

The widespread and strong expression of PECAM-1 on endothelial cells, platelets, and leukocytes suggests that it plays an important role in vascular biology. Our previous data indicate that it may function as a cell adhesion molecule. In order to better understand PECAM-1-mediated adhesion, the aggregation of mouse L-cell fibroblasts transfected with PECAM-1 was analyzed. Although an artificial system, the L-cell aggregation assay allows one to study a given cell adhesion molecule in isolation from other cell adhesion molecules on the cell surface. It has been used successfully by many investigators to define the adhesive characteristics of a number of cell adhesion molecules, including cadherins (Takeichi, 1991), immunoglobulin superfamily cell adhesion molecules (Edelman and Crossin, 1991; Filbin et al., 1990), and CD44 (St. John et al., 1990). These findings have, in many instances, subsequently been shown to have in vivo significance. For example St. John et al. (1990) used a mixed aggregation assay, similar to the one described here, to show that CD44 participated in heterophilic binding and speculated that a component of the extracellular matrix may be involved. This speculation was confirmed with the demonstration of CD44 as one of the principal cell surface receptors for hyaluronate (Aruffo et al., 1990).

The data presented here indicate that PECAM-1 mediates heterophilic L-cell aggregation that is dependent on restricted classes of glycosaminoglycans (heparin, heparan sulfate, and to a lesser extent, chondroitin sulfate) expressed on adjacent cells. The significance of the finding that both heparin and chondroitin sulfate are involved in PECAM-1-mediated aggregation is unclear, although it raises the possibility that the proteoglycan involved contains both of these GAGs. The binding of heparin and chondroitin sulfate to inhibit aggregation indicates that the inhibition seen is not related simply to the charge characteristics of GAGs. These findings are consistent with studies that have shown that adhesive phenomena involving other Ig superfamily CAMs can be blocked by GAGs. The binding of retinal probe cells to retinal cell monolayers, an assay for N-CAM mediated adhesion, is blocked by heparin, but not by chondroitin sulfate (Cole et al., 1986). A soluble form of N-CAM (N-CAM 110) binds to immobilized heparin in a manner that is inhibited by heparin and chondroitin sulfate (Probstmeier et al., 1989). Dextran sulfate, heparin, and fucoidan, but not chondroitin sulfate, inhibit the binding of recombi-

**Fig. 3. Effect of glycosaminoglycan-degrading enzymes.** A, PECAM-1-transfected cells were allowed to aggregate in the presence of glycosaminoglycan-degrading enzymes. Enzyme concentrations used correspond to concentrations at which the enzyme has been reported to remove cell surface GAGs. Heparinase (5 units/ml) and chondroitinase ABC (50 milliunits/ml) were inhibitory, while hyaluronidase (100 milliunits/ml) had no effect. The means and standard deviations were calculated on the basis of at least two experiments done in triplicate. Background aggregation was 5–10%; control aggregation was 25–35%. B, to determine the effect of heparinase on PECAM-1, cells were 125I-surface-labeled, treated with either buffer alone (lane A) or heparinase in buffer (lane B) before protein extraction, immunoprecipitated with monoclonal anti-PECAM-1 antibody, and subjected to SDS-PAGE under reducing conditions followed by autoradiography. Heparinase treatment did not alter the mobility (130 kDa) of the protein. Molecular masses in kilodaltons are shown on the right.

**Fig. 4. Mixed aggregates.** Examples of L-cell aggregates taken from a mixed aggregation experiment in which equal numbers of PECAM-1-transfected and control (neo-transfected) L-cells were mixed together. In this experiment, the PECAM-1-transfectants were fluorescently labeled and thus appear white in the figure. One representative microscopic field is shown along with two larger aggregates from adjacent fields (arrows). Bar = 100 μm.

PECAMΔ1- and PECAMΔ2-transfected L-cells to aggregate was compared with L-cells transfected with wild-type PECAM-1. As shown in Fig. 8, the mutant construct lacking the GAG-binding region (PECAMΔ2) was unable to aggregate in the presence of glycosaminoglycan-degrading enzymes.
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Fig. 5. Predicted frequency distribution of mixed aggregates. A, a homophilic mechanism would result in a nonrandom frequency distribution (markedly shifted to the right) as the PECAM-1-transfectants preferentially adhere with each other. B, a heterophilic mechanism would result in aggregates consisting of nearly equal mixtures of control-transfected and transfected cells and thus approximate a normal distribution. This distribution would be shifted slightly toward the right, since initial cell-cell binding always begins with a PECAM-1-transfected cell.

nant human immunodeficiency virus gp120 to recombinant CD4 (Lederman et al., 1989). Dextran sulfate can also inhibit CD2-mediated Eresetting of human T cells and the binding of monoclonal anti-CD2 antibodies to CD2 (Parish et al., 1988).

Studies with GAG-digesting enzymes were particularly helpful. Specifically, inhibition of aggregation by enzymatic removal of GAGs provided independent evidence of the role of GAGs in PECAM-1-mediated cell adhesion. Additionally, the mixed aggregation experiments, in which either cell type was pretreated with heparinase, suggested strongly that this interaction may involve a proteoglycan on adjacent cells acting as a primary counter-ligand rather than a facilitator in which binding to the GAG promotes homophilic (PECAM-1-PECAM-1) adhesion. The latter mechanism has been invoked to explain the heparan sulfate proteoglycan dependence of N-CAM and L1-mediated neural cell adhesion (Cole et al., 1986; Kadmon et al., 1989).

The involvement of GAGs in PECAM-1-mediated heterophilic adhesion is consistent with the presence of a consensus GAG binding sequence in its second Ig-like homology domain. A similar region in N-CAM promotes a significant portion of the cell adhesion properties of N-CAM (Reyes et al., 1986). The importance of this GAG recognition site was demonstrated by the fact that deletion of the second Ig-like loop of PECAM-1 resulted in a protein that was unable to support L-cell aggregation. A similar deletion involving the first Ig-like loop had no such effect, making nonspecific alterations in protein structure induced by the deletions unlikely. A secondary and independent approach showed that synthetic peptides containing the consensus GAG binding sequence of PECAM-1 (L-K-R-E-K-N) inhibited PECAM-1-dependent L-cell aggregation, providing additional strong evidence that this region of the molecule is critical to its ability to mediate heterophilic adhesion (Fig. 6). A "scrambled" peptide containing the same amino acids was ineffective in blocking aggregation. The concentration of peptide found to block aggregation (400 µg/ml, ~0.5 mM) is similar to concentrations reported for other blocking peptides, including the RGD peptide (Hayman et al., 1985; Yamada and Kennedy, 1987), a laminin domain III peptide (Graf et al., 1987), and a GMP-140 lectin domain peptide which blocks adherence of neutrophils to immobilized GMP-140 (Geng et al., 1991). Interestingly, a peptide containing the reversed sequence (N-K-E-R-K-L) also inhibited aggregation to a degree comparable with the L-K-R-E-K-N peptide, a finding we attribute to the somewhat palindromic nature of the charge sequence of the blocking peptide. This is not unique, as a similar inhibition of cell adhesion by the "reversed" sequence of the bioactive peptide, RGDS, has also been observed (Yamada and Kennedy, 1987). Murine PECAM-1 also contains a GAG recognition sequence in the second Ig-like domain, further strengthening the idea that proteoglycans may play an important general role in PECAM-1-mediated intercellular adhesion (Baldwin et al., 1992).

The demonstration that PECAM-1-mediated L-cell aggregation involves binding to specific GAGs raises a number of interesting, but as yet unanswered, questions. The first is the identity of the proteoglycan(s) involved. The second relates to the physiologic implications of GAG binding to PECAM-1. GAG-dependent heterophilic mechanisms may underlie normal physiologic interactions between endothelial cells and platelets (Nader, 1991) and/or between endothelial cells, leukocytes, and the extracellular matrix (Gill et al., 1986). They may also have pathological consequences. It has been estimated that up to 30% of patients receiving therapeutic doses of heparin develop some degree of thrombocytopenia (Kelton, 1986), presumably due to the development of anti-platelet antibodies. Although it has been demonstrated that heparin binds to platelets in a reversible and saturable manner (Horne and Chao, 1989), the identity of this heparin-binding protein is still unknown. It is possible that the PECAM-1 found on platelets interacts with heparin and forms an antigen complex capable of inducing an immune response.

PECAM-1-mediated adhesion is complex. PECAM-1 appears to be capable of promoting both homophilic and heterophilic adhesion, depending on the conditions of the interaction. Such complex patterns have been recently suggested for a number of immunoglobulin superfamily cell adhesion molecules, including Ng-CAM (Grumet and Edelman, 1988), Nr-CAM (Mauro et al., 1992), and N-CAM (Murray and Jensen, 1992; Rao et al., 1992). When suspended cells are involved, as shown here, or as might occur when white cells or platelets interact with endothelial cells, the adhesive interaction may
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**Fig. 6.** Mixed aggregation studies. Mixed aggregation assays were performed in which equal numbers of control-transfected and transfected cells were mixed together after fluorescent labeling of one of the cell lines. After incubation, the number of labeled cells within each five-cell aggregate was counted. The data presented are representative of at least three experiments. A, with untreated cells, the distribution of aggregates reveals a normal distribution, with the majority of the aggregates being made up of mixtures of control-transfected and transfected cells. This pattern indicates heterophilic interactions. B, pretreatment of control-transfected cells with heparinase (5 units/ml) resulted in a change in the frequency distribution, with most aggregates being made up of non-enzyme-treated PECAM-1-transfected cells. C, heparinase pretreatment of the transfected cells, however, did not alter the frequency distribution; most aggregates still contained relatively equal mixtures of enzyme-treated PECAM-1-transfected and control-transfected cells. Similar results were obtained after analysis of four- and six-cell aggregates.

**Fig. 7.** Expression of mutant PECAM-1 cDNA. Cell surface expression of PECAM-1, PECAMΔ1, and PECAMΔ2 by transfected cell lines was assessed by fluorescence-activated cell sorting using mAb 4G6 (A, B, C). For each cell line >80% of the cells were positive. To confirm that the proper-sized protein was produced, the transfected cells were biotinylated, immunoprecipitated with anti-PECAM-1 antibodies, and analyzed by SDS/PAGE (D). Neomycin-transfected control L-cells expressed no PECAM-1 protein (lane 1). Extracts of cells expressing either PECAMΔ1 (lane 3) or PECAMΔ2 (lane 4) were precipitated by the anti-PECAM-1 monoclonal antibody at a molecular mass of approximately 100 kDa, slightly less than that of the wild-type PECAM-1 (130 kDa; lane 2).

**Fig. 8.** Effect of the deletion of domain 2 on aggregation. Aggregation of PECAM-1-transfected cells was compared with that of transfectants lacking domain 1 (PECAMΔ1) or domain 2 (PECAMΔ2) in the presence or absence of calcium. Cells expressing PECAMΔ2 did not aggregate, whereas cells expressing PECAMΔ1 retained aggregating capability.
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FIG. 9. Effect of synthetic peptides. Aggregation of PECAM-1-transfected cells was conducted in the presence of synthetic peptides mimicking the GAG recognition sequence. A, the six amino acid peptide L-K-R-E-K-N (PECAM-1 150–155), which exactly mimics the sequence of PECAM-1, and the reverse sequence peptide, N-K-E-R-K-L (400 pg/ml), markedly inhibited aggregation, whereas no effect was seen with an scrambled peptide or an irrelevant peptide (constructed to mimic the terminal 26 amino acids of the β1 integrin subunit). Background aggregation was 5–10%; control aggregation was 25–35%. The means and standard deviations were calculated on the basis of at least two experiments done in triplicate. B, dose response of PECAM-1 150–155. PECAM-1-transfected L-cells were allowed to aggregate in the presence of 1 mM calcium and various concentrations of PECAM-1 150–155. These data presented are representative of two experiments done in triplicate. Background corresponds to conditions in which no calcium or synthetic peptide was present. PECAM-1 150–155 inhibited aggregation in a dose-dependent manner. Similar results were obtained for the reverse peptide.

In summary, the data presented here further expand our knowledge of the adhesive mechanisms of PECAM-1, confirming its ability to function as a heterophilic adhesion molecule interacting with proteoglycans. Specifically, this adhesive function of PECAM-1 appears to involve a consensus GAG binding sequence in the second Ig-like homology domain.

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been presented for PECAM-1 (Albelda et al., 1991). In this case, PECAM-1 was concentrated only at borders between COS cells or fibroblasts transfected with PECAM-1 cDNA and never when transfected cells were juxtaposed with non-transfected cells. Although these data are consistent with homophilic interaction, the experiments presented here suggest the possibility that this effect could be due to GAGs in the extracellular matrix serving to bridge the interaction between PECAM-1 on adjacent cells rather than the direct binding of PECAM-1 molecules with one another.

Despite our increasing understanding of PECAM-1-mediated adhesion in vitro, the actual physiologic roles of this molecule and its mechanism of action in vivo remain unknown. The strong constitutive expression of PECAM-1 on endothelium and many types of leukocytes (Muller et al., 1989; Albelda et al., 1990) suggests that either PECAM-1-mediated cell adhesion is a relatively weak interaction or an activational event is required for function in vivo, in a manner similar to that seen with activation of integrins and selectins on white cells in response to inflammatory stimuli (Osborn, 1990). It is also possible that engagement of PECAM-1 with its ligand is not a primary adhesive event, but rather an activational signal for other adhesion molecules. Antibody-induced cross-linking of surface PECAM-1 has been shown to activate both β1 and β2 integrins on the surface of T-cells (Tanaka et al., 1992) and β2 integrins on neutrophils and monocytes (Berman and Muller, 1992).
