Deletions in the Cytoplasmic Domain of Platelet-Endothelial Cell Adhesion Molecule-1 (PECAM-1, CD31) Result in Changes in Ligand Binding Properties

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Abstract. Platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) is a member of the immunoglobulin superfamily present on platelets, endothelial cells, and leukocytes that may function as a vascular cell adhesion molecule. The purpose of this study was to examine the role of the cytoplasmic domain in PECAM-1 function. To accomplish this, wild-type and mutated forms of PECAM-1 cDNA were transfected into murine fibroblasts and the functional characteristics of the cells analyzed. Wild-type PECAM-1 localized to the cell–cell borders of adjacently transfected cells and mediated heterophilic, calcium-dependent L-cell aggregation that was inhibitable by a polyclonal and two monoclonal anti-PECAM-1 antibodies. A mutant protein lacking the entire cytoplasmic domain did not support aggregation or move to cell–cell borders. In contrast, both forms of PECAM-1 with partially truncated cytoplasmic domains (missing either the COOH-terminal third or two thirds of the cytoplasmic domain) localized to cell–cell borders in 3T3 cells in a manner analogous to the distribution seen in cultured endothelial cells. L-cells expressing these mutants demonstrated *homo*philic, calcium-*in*dependent aggregation that was blocked by the polyclonal anti-PECAM-1 antibody, but not by the two bioactive monoclonal antibodies. Although changes in the cytoplasmic domain of other receptors have been shown to alter ligand-binding affinity, to our knowledge, PECAM-1 is the first example of a cell adhesion molecule where changes in the cytoplasmic domain result in a switch in the basic mechanism of adhesion leading to different ligand-binding specificity. Variations in the cytoplasmic domain could thus be a potential mechanism for regulating PECAM-1 activity in vivo.

P ECAM-1 (CD31) is a newly described 130-kD glycoprotein expressed on endothelial cells, platelets, and most leukocytes (Muller, 1992*a*; Newman and Albelda, 1992; DeLisser et al., 1993*b*). Although the full range of physiological functions of PECAM-1 have not yet been defined, a number of lines of evidence indicate that it functions in the process of cell–cell adhesion. On cultured endothelial cells, PECAM-1 localizes to cell–cell borders. Antibodies directed against PECAM-1 inhibit initial endothelial junction formation (Albelda et al., 1990). PECAM-1 cDNA transfected into COS cells or 3T3 fibroblasts localizes exclusively at cell–cell borders of adjacently transfected cells (Albelda et al., 1991). PECAM-1 cDNA transfected into mouse L-cells confers the ability to aggregate in a calcium-dependent, heterophilic manner (Albelda et al., 1991; Muller et al., 1992*b*; DeLisser et al., 1993*a*). This categorization of PECAM-1 as a cell-adhesion molecule is supported by experiments showing that it is involved in biological processes

such as cell migration (Schimmenti et al., 1992), angiogenesis (Merwin et al., 1993), leukocyte-endothelial adhesion (Tanaka et al., 1992), and leukocyte transendothelial migration (Muller et al., 1993).

Cloning studies have revealed that PECAM-1 is a member of the immunoglobulin superfamily (Ig-SF) (Newman et al., 1990; Simmons et al., 1990; Stockinger et al., 1990; Zehnder et al., 1992) with homologies to other Ig-SF cell adhesion molecules such as VCAM-1, ICAM-1, CEA, and NCAM. As such, it contains six Ig-like homology units in its extracellular domain, a typical hydrophobic transmembrane region, and a cytoplasmic domain containing 118 amino acids (Newman et al., 1990). A heparin-binding sequence on Ig-like domain 2 appears to be important in mediating heterophilic glycosaminoglycan-dependent cell–cell adhesion (DeLisser et al., 1993*a*).

The function and behavior of many types of cell adhesion molecules including integrins (Marcantonio et al., 1990; Chan et al., 1992; O'Toole et al., 1991), selectins (Kansas et al., 1993), CD44 (Lesley et al., 1992), as well as other members of the Ig-SF (Carpén et al., 1992; Shin et al., 1991;

Powell et al., 1991) are influenced by their cytoplasmic domains. The cytoplasmic region of PECAM-1 is also likely to play an important role in the function of the molecule. The ability of PECAM-1 to move to cell–cell borders suggests a requirement for cytoskeletal interaction (Albelda et al., 1991). In addition, Newman et al. (1992) have found PECAM-1 associated with the cytoskeleton of activated platelets. To date, however, there is little understanding of how the structure of the cytoplasmic domain of PECAM-1 contributes to the functional properties of the molecule. Therefore, to examine the role of the cytoplasmic domain in PECAM-1-mediated intercellular adhesion, wild-type, and mutated forms of PECAM-1 cDNA were transfected into murine fibroblasts and the functional characteristics of the cells analyzed. Our results indicate that the cytoplasmic domain is required for PECAM-1 to localize to cell–cell borders and to support aggregation. Surprisingly, however, we also found that partial deletions of the cytoplasmic domain markedly altered the functional characteristics of binding, converting PECAM-1-mediated aggregation from a *het*-erophilic to *homo*philic process. These findings demonstrate that alterations in the cytoplasmic domain result in changes in the extracellular region that affect the functional characteristics of PECAM-1 binding and suggest a potential mechanism for regulating PECAM-1 activity in diverse biological processes.

Materials and Methods

Construction of PECAM-1 Mutant cDNAs

PECAM-1 mutants (Fig. 1) were constructed by manipulation of wild-type PECAM-1 cDNA that had been subcloned into the pESP-SVTEXP expression vector (designated "TEX/PECAM") as previously described (Albelda et al., 1991).

Cytoplasmic Deletion Mutant-1 (CDM1) and Cytoplasmic Deletion Mutant-2 (CDM2). To prepare constructs missing approximately the terminal third of the cytoplasmic domain (CDM1) and the terminal two thirds of the cytoplasmic domain (CDM2), we took advantage of a unique Bgl II site present at bp 2247 and a unique Tth111 I site at bp 2143 (Fig. 1 A). Wild-type TEX/PECAM was reacted with the appropriate restriction enzyme. The linearized plasmids were then treated with mung bean nuclease to make the ends blunt and ligated to a synthetic multiple reading frame termination linker (SMURFT-Pharmacia LKB Biotechnology, Piscataway, NJ). Bacteria were transformed with the ligation mixture and plasmid DNA isolated. Since this linker also contains an Xba I site, we were able to confirm the presence of the linker by the presence of this site. Constructs with the appropriate restriction digest pattern were then sequenced to confirm that the appropriate stop codon had been inserted.

Cytoplasmic Deletion Mutant-3 (CDM3). To prepare a construct missing the entire cytoplasmic domain, a modification of the PCR was used, taking advantage of the fact that additional nucleotide sequences can be added to the 5' end of PCR primers without affecting the ability of the reaction to amplify these sequences.

The full length TEX/PECAM was cut with the restriction enzymes Nhe I and Asu II (Fig. 1 A). Each of these enzymes has a unique restriction site. The Nhe I site is at bp 1825 and the Asu II site is in the 3' linker sequence. The larger 6.0-kb fragment, containing the vector plus the cDNA coding for most of the extracellular domain was purified using low-melt agarose. A 5' PCR primer identical to the coding strand of PECAM-1 from nucleotides 1820 to 1834 was synthesized. This region, near the transmembrane domain, contains the unique Nhe I restriction site. A 3' PCR primer was constructed to contain 17 bp complementary to the terminal portion of the transmembrane domain (bp 2003-1986). The 5' portion of this primer was designed to contain an "in frame" stop codon, a unique Aoc I restriction site as well as an Asu II restriction site. These primers were used to generate a 200-bp fragment that was purified, reacted with the restriction enzymes Nhe I and Asu II, and repurified. The digested PCR fragment was then ligated to the previously digested vector and the resultant DNA mixture

used for transformation. Plasmid DNA was isolated from the resultant colonies and the appropriate DNA, as identified by the presence of a new Aoc I site, was sequenced.

Transfection of Mutant cDNAs

Purified cDNA was cotransfected with a neomycin resistance gene into COS cells and 3T3 cells using calcium phosphate as described previously (Albelda et al., 1991). 3T3 cell lines expressing high levels of the PECAM-1 cDNAs were selected using G418 (Geneticin, GIBCO BRL, Gaithersburg, MD) followed by subcloning.

Unlike our success with 3T3 cells, we were unable to reliably obtain stable lines expressing high levels of PECAM-1 in mouse L-cells using the pESP-SVTEXP expression vector. We therefore subcloned the constructs into the pcDNAI/Neo vector (Invitrogen, San Diego, CA) followed by transfection into L-cells and selection and subcloning in G418 as previously described (Albelda et al., 1991).

Antibodies

The following anti-PECAM-1 monoclonal antibodies were used: mAbs PECAM-1.1, PECAM-1.2, and PECAM-1.3, (provided by Dr. Peter Newman, Blood Center of Southeastern Wisconsin, Milwaukee, WI) generated by immunizing mice with PECAM-1 purified from human platelets (Newman et al., 1992; Albelda et al., 1991); mAb hec7 (provided by Dr. William Muller, Rockefeller University, New York, NY) (Muller et al., 1989); mAb HDO3 (provided by Dr. Stephen Shaw, National Cancer Institute, Bethesda, MD), and mAb 4G6 generated in mice after immunization with PECAM-1-transfected 3T3 cells. Each of these antibodies immunoprecipitated the characteristic 130-kD protein from human umbilical vein endothelial cells and selectively recognized 3T3 cells transfected with PECAM-1.

Immunofluorescent Staining

Transfected 3T3 cell lines expressing wild-type and mutant forms of PECAM-1 in >90% of cells were plated at 4×10^4 cells/cm^2 on glass coverslips coated with 1% gelatin in PBS (Difco Labs., Detroit, MI). After the cells had grown to confluence, cells were fixed with 3% paraformaldehyde for 20 min, and then permeabilized with ice-cold 0.5% NP-40 for 1 min. After extensive washing, anti-PECAM-1 mAb 4G6 was added for 1 h. After rinsing, the coverslips were stained with a 1:200 dilution of FITC-labeled anti-mouse antibody (Cappell, West Chester, PA) for 1 h. Cells were viewed on a Zeiss phase-epifluorescent microscope using a 63× planapochromat oil-immersion lens numerical aperture 1.4 and photographed using TMAX film at 3200 ASA.

Fluorescence-activated Cell Sorting Analysis

L-cells transfected with wild-type or mutant PECAM-1 cDNAs were enzymatically removed for T25 flasks with trypsin/EDTA, washed in medium containing 10% FBS, 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 FITC-labeled goat anti-mouse secondary antibody (Cappell) added for 30 min at 4°C. After washing in cold PBS, flow cytometry was performed using a Cytofluorograph 50H cell sorter equipped with a 2150 data handling system (Ortho Instruments, Westwood, MA).

Cell Labeling

To metabolically label cellular proteins, confluent T25 flasks were incubated in medium containing 100 μCi [^{35}S]methionine for 18 h as previously described (Albelda et al., 1990).

For ^{32}P-labeling, confluent monolayers of L-cell transfectants in six well plates were incubated at 37°C in phosphate-free RPMI with 5% dialyzed FCS for 18 h. After the monolayers were washed twice with PBS, the cells were then incubated for 4 h with phosphate-free RPMI with 10% dialyzed serum and 250 μCi/ml sodium ortho[^{32}P] phosphate (Amersham Corp., Arlington Heights, IL). After incubation, the monolayers were washed twice with cold PBS and the membrane proteins extracted by adding 300 μl of TNC (0.01 M Tris acetate, pH 8.0, 0.5 % NP-40 and 0.05 mM CaCl$_2$) containing 2 mM PMSF and 10 mM sodium orthovanadate to the wells on ice for 20 min. The resulting extracts were then used immediately or stored at -20°C until use.

Immunoprecipitation

Nonionic detergent extracts were preadsorbed for 30 min at 4°C with protein-A-conjugated Sepharose beads (Pharmacia Fine Chemicals). After removal of the beads, the appropriate antibody was added to the precleared supernatants, together with fresh protein A–Sepharose beads for 1 h at 4°C. After the immunoprecipitation, the beads were washed five times with 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5% deoxycholate, and 0.1% SDS. The sample was then dissolved with electrophoresis loading buffer (62.5 mM Tris base, 2% SDS, 10% glycerol, pH 6.8), electrophoresed on 6% polyacrylamide gels, and processed for autoradiography using Kodak XR-5 X-ray film at −70°C.

Aggregation of L-Cell Transfectants

The aggregation assay used in these studies has been described in detail previously (Albelda et al., 1991; Muller et al., 1992b; DeLisser et al., 1993a). Briefly, stable L-cell transfectants, which had been plated (8-10 × 10⁶ cells/75-cm² flask) and grown overnight, were removed from the flasks by treating the cells with low dose trypsin/EDTA for 3 min. Fluorescence-activated cell sorting (FACS)[1] analysis using various anti-PECAM-1 antibodies, demonstrated that PECAM-1 was not altered by this treatment (data not shown). The cells were washed twice with 10 mM EDTA in PBS, pH 7.2, and twice with HBSS without divalent cations. Cells were finally resuspended to a concentration of ∼10⁶/ml in HBSS with or without 1 mM Ca⁺². Heparin (sodium salt, from porcine intestinal mucosa; Sigma Chem. Co., St. Louis, MO) and antibodies were added as indicated to give the desired concentration. 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 plastic tray (Costar Corp., Cambridge, MA) that had been previously incubated 2% BSA in HBSS for at least 1 h and washed thoroughly with HBSS immediately before use to prevent nonspecific binding to the tissue culture dish. The non-tissue culture trays 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 hemacytometer grid using phase contrast optics. The number of single cells (cells in aggregates ≤3) remaining versus those present in aggregates of greater than three cells were counted from four 1-mm squares. At least 600 cells were counted from each sample. Data were expressed as the percent of total cells present in aggregates.

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., 1992b; DeLisser et al., 1993a). In these experiments, L-cell aggregation was performed by mixing nontransfected and transfected cells, with one of the two cell types fluorescently labeled before mixing. After the cell line designated for labeling had been washed once with EDTA, it was resuspended in HBSS to a total volume of one ml. One ml of rhodamine-conjugated dye solution at a final concentration of 1 μM (Sigma Immunochemicals, St. Louis, MO), in buffer provided by the manufacturer, was added, followed by incubation at room temperature for 5–10 min. Labeling was terminated by adding an equal volume FBS and by washing the cells with HBSS. The second EDTA wash and the two HBSS washes were then completed as previously described. Each set of cells, one labeled, and the other unlabeled, were resuspended at 10⁶ cells/ml. Aliquots of 0.5 ml were combined in the wells of a 24-well non-tissue culture plate and allowed to aggregate as described above. After the aggregation was completed, the cells were viewed under epifluorescence. 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).

Results

Construction and Characterization of Mutated Forms of PECAM-1 Lacking Regions of the Cytoplasmic Domain

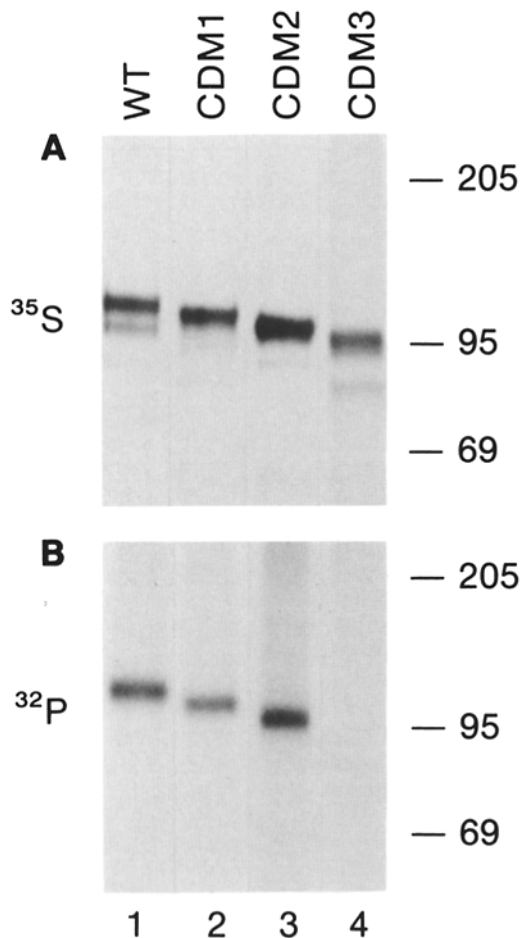To study the function of the cytoplasmic domain, three trun-

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1. *Abbreviation used in this paper*: FACS, fluorescence-activated cell sorting.
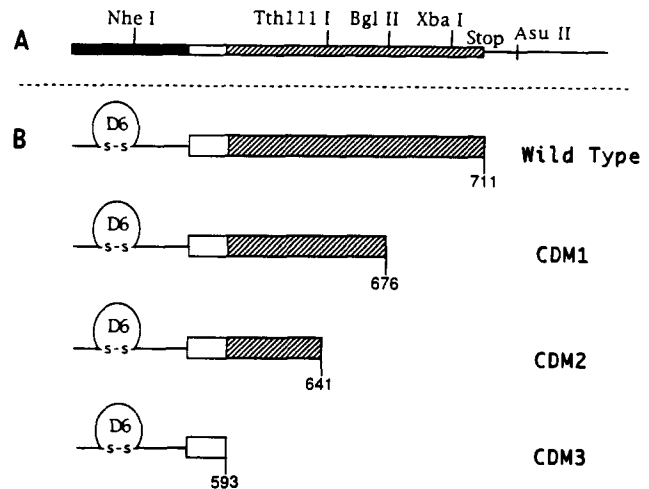
Figure 1. Construction of PECAM-1 cytoplasmic domain deletion mutants. (A) A schematic representation of the cDNA sequence for the 6th Ig-like unit and the transmembrane and cytoplasmic domains of PECAM-1 in the expression vector pESP-SVTEXP. The solid bar represents the 6th Ig-like domain. The open bar represents the transmembrane region. The hatched bar represents the cytoplasmic domain. Stop codons were inserted at two convenient, unique restriction enzyme sites, Bgl II and Tth111 I to create, respectively, the mutants CDM1 and CDM2. CDM3 was constructed by digesting the wild-type PECAM-1 cDNA with Nhe I and Asu II restriction enzymes (to remove the sequence coding the cytoplasmic domain), followed by the ligation of a PCR-generated fragment into the digested vector to reconstitute the sequence for the transmembrane domain. (B) Wild-type and the three cytoplasmic domain deletion mutants. The terminal third of the cytoplasmic domain is deleted from CDM1; the terminal two thirds of the cytoplasmic domain is missing from CDM2; and the entire cytoplasmic domain is absent from CDM3.

cated forms of PECAM-1 were created by inserting stop codons at specific locations in the coding sequence of the cytoplasmic domain of wild-type PECAM-1 as outlined in the methods (see Fig. 1). Cytoplasmic deletion mutant-1 (CDM1) was truncated at amino acid 676 and contained about 2/3 of the cytoplasmic domain. Cytoplasmic deletion mutant-2 (CDM2) was truncated at amino acid 641 and contained about 1/3 of the cytoplasmic domain. Cytoplasmic deletion mutant-3 (CDM3) was truncated at amino acid 593, immediately after the transmembrane region (Fig. 1 B). Each mutant construct was confirmed by sequencing. These constructs, along with wild-type PECAM-1 cDNA were subsequently transfected into mouse 3T3 cells and L-cells for analysis. Fig. 2 A illustrates the relative sizes of each of the PECAM-1 constructs. Transfected L-cells were metabolically labeled with [³⁵S]methionine and immunoprecipitated with an anti-PECAM-1 monoclonal antibody that did not react with non-transfected cells (data not shown). Each of the truncation mutants was of the appropriately smaller size than the wild-type molecule.

Previous studies have demonstrated that the cytoplasmic domain of PECAM-1 is constitutively phosphorylated on serine and threonine residues (Newman et al., 1992; Zehnder et al., 1992). To determine if these constructs maintained the ability to be phosphorylated, L-cells containing each of the PECAM-1 constructs were incubated in radiola-
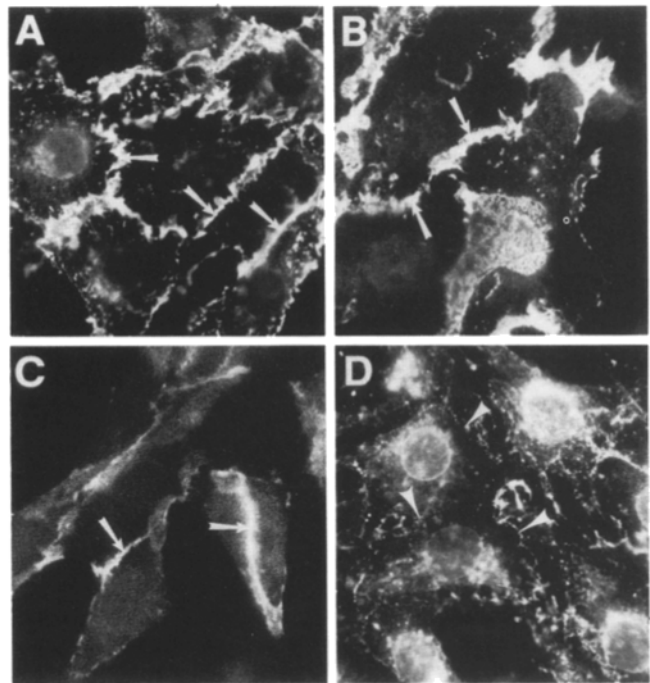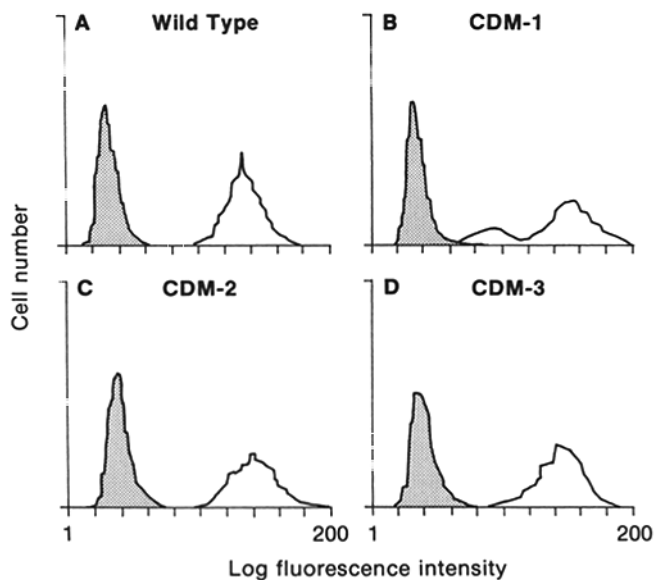
Figure 3. Localization of wild-type and mutant PECAM-1 on 3T3 cells. 3T3 clones expressing wild-type and each cytoplasmic deletion construct were plated onto glass coverslips, allowed to grow to near confluence, fixed and stained with an anti-PECAM-1 mAb followed by a secondary FITC-labeled anti-mouse antibody. Wild-type PECAM-1 (*A*), as well as CDM1 (*B*) and CDM2 (*C*), concentrated at intercellular borders (*arrows*). CDM3 (*D*) demonstrated little tendency to localize at cell–cell contacts (*arrowheads*).

Figure 2. Immunoprecipitation of wild-type (WT) and mutant PECAM-1 expressed in L-cell transfectants. (*A*) Transfected L-cells were metabolically labeled with [³⁵S]methionine and immunoprecipitated with anti-PECAM-1 monoclonal antibody. The size of the mutant constructs varied from 95 to 120 kD. (*B*) L-cell transfectants were labeled with ortho [³²P]phosphate, extracted with non-ionic detergent in the presence of sodium vanadate and immunoprecipitated with anti-PECAM-1 monoclonal antibody. Wild-type, CDM1, and CDM2 demonstrated similar levels of constitutive levels of phosphorylation. As expected, CDM3 was not phosphorylated. Numbers on the right represent molecular weight markers in kilodaltons.

beled sodium orthophosphate for 4 h, extracted in non-ionic detergent in the presence of sodium vanadate, and immunoprecipitated with anti-PECAM-1 monoclonal antibody. Duplicate wells were labeled with [³⁵S]methionine and processed identically (Fig. 2 *A*). As shown in Fig. 2 *B*, wild-type PECAM-1 in L-cells was constitutively phosphorylated (lane *1*). As expected, the CDM3 mutation lacking the cytoplasmic domain was not labeled (lane *4*), even though these cells were synthesizing the truncated protein (Fig. 2 *A*, lane *4*). Both CDM1 and CDM2, however, were clearly labeled by [³²P] at levels similar to wild type.

The Cytoplasmic Domain Is Required for PECAM-1 Localization to Cell–Cell Borders

After transfection into Cos-7 and 3T3 cells, wild-type PECAM-1 localizes to cell–cell borders of adjacent trans-

fected cells in a manner analogous to the distribution seen in cultured endothelial cells (Albelda et al., 1991). The requirement of the cytoplasmic domain for PECAM-1 intercellular localization was determined by immunofluorescence analysis of 3T3 clones expressing each truncated form of PECAM-1 (Fig. 3). Fig. 3 *A* shows wild-type PECAM-1 localized to cell–cell borders, as expected (Albelda et al., 1991). In contrast, the construct missing the entire cytoplasmic domain (CDM3), showed only a minimal tendency to localize at cell–cell contact sites (Fig. 3 *D*). Although somewhat difficult to quantitate, cells transfected with either CDM1 (Fig. 3 *B*) or CDM2 (Fig. 3 *C*) retained their ability to move to cell–cell borders.

Deletions of the Cytoplasmic Domain Alter the Binding Characteristics of PECAM-1

When transfected into murine L-cell fibroblasts, wild-type PECAM-1 mediates calcium- and glycosaminoglycan-dependent, heterophilic aggregation (Albelda et al., 1991; Muller et al., 1992*b*; DeLisser et al., 1993*a*). Therefore, each cytoplasmic deletion mutant was subcloned into the pcDNAI-Neo vector and transfected into L-cells. After selection in G418, clones were isolated, and levels of PECAM-1 expression on the cell surface were determined by FACS analysis (Fig. 4). Each clone expressed PECAM-1 on greater than 90% of the cells at similar fluorescence intensities. Multiple independent clones of each mutant expressing high levels of protein were chosen for further study.
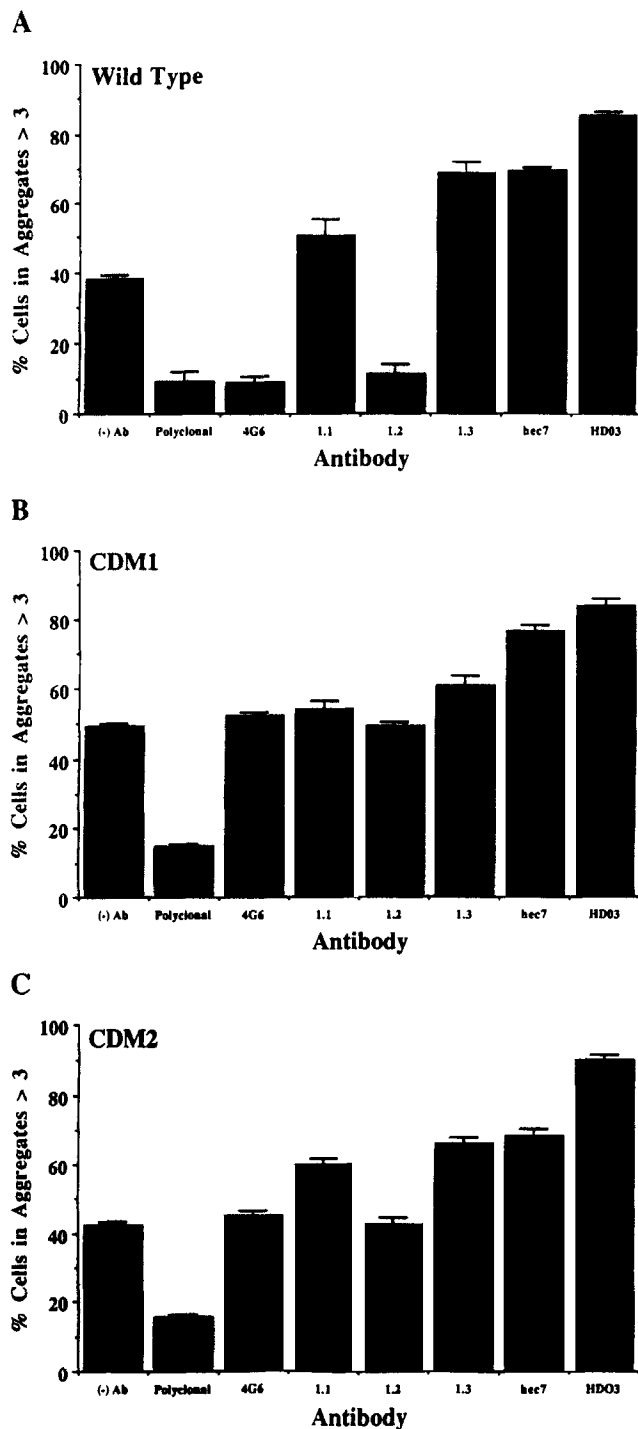
Figure 4. Expression of wild-type and mutant PECAM-1 L-cell transfectants. Cell surface expression of L-cells transfected with wild-type PECAM-1, CDM1, CDM2, and CDM3 constructs were assessed by fluorescence-activated cell sorting using an anti-PECAM-1 polyclonal antibody (A, B, C, and D). For each L-cell transfectant, >90% of the cells were positive.

Each construct was tested for its ability to support L-cell aggregation in the presence and absence of calcium (Fig. 5). In Fig. 5 A, as expected, wild-type PECAM-1 demonstrated calcium-dependent aggregation. Sham transfectants demonstrated minimal aggregation in the presence or absence of calcium (see Albelda et al., 1991). In contrast, the CDM3 construct that was lacking the entire cytoplasmic domain, was unable to support L-cell aggregation in either the presence or absence of calcium. Although removal of the entire cytoplasmic domain completely eliminated the ability of PECAM-1 to support aggregation, deletions of specific portions of the cytoplasmic domain led to surprising alterations in the adhesive properties of the PECAM-1 molecule (Fig. 5). Cells transfected with constructs lacking either the terminal one third of the cytoplasmic domain (CDM1) or the terminal two thirds of the cytoplasmic domain (CDM2) aggregated equally well in both the presence and absence of calcium (Fig. 5 A). In both cases, even though the cells expressed comparable levels of PECAM-1 on their surface (Fig. 4), the cells expressing truncated forms of the molecule consistently displayed higher levels of aggregation than cells transfected with wild-type PECAM-1.

The effects of temperature and heparin on mutant and wild-type aggregation in the presence of calcium were also studied. As shown in Fig. 5 B, cells transfected with either CDM1 or CDM2, like wild-type transfectants were unable to aggregate at low temperatures suggesting that this process requires metabolic energy (see also Muller et al., 1992b). Heparin (50 μg/ml) blocked the aggregation mediated by the wild-type PECAM-1, as previously demonstrated (DeLisser et al., 1993a). In contrast, it did not significantly affect the aggregation mediated by CDM1 or CDM2 (Fig. 5 C), even at concentrations up to 500 μg/ml (data not shown).
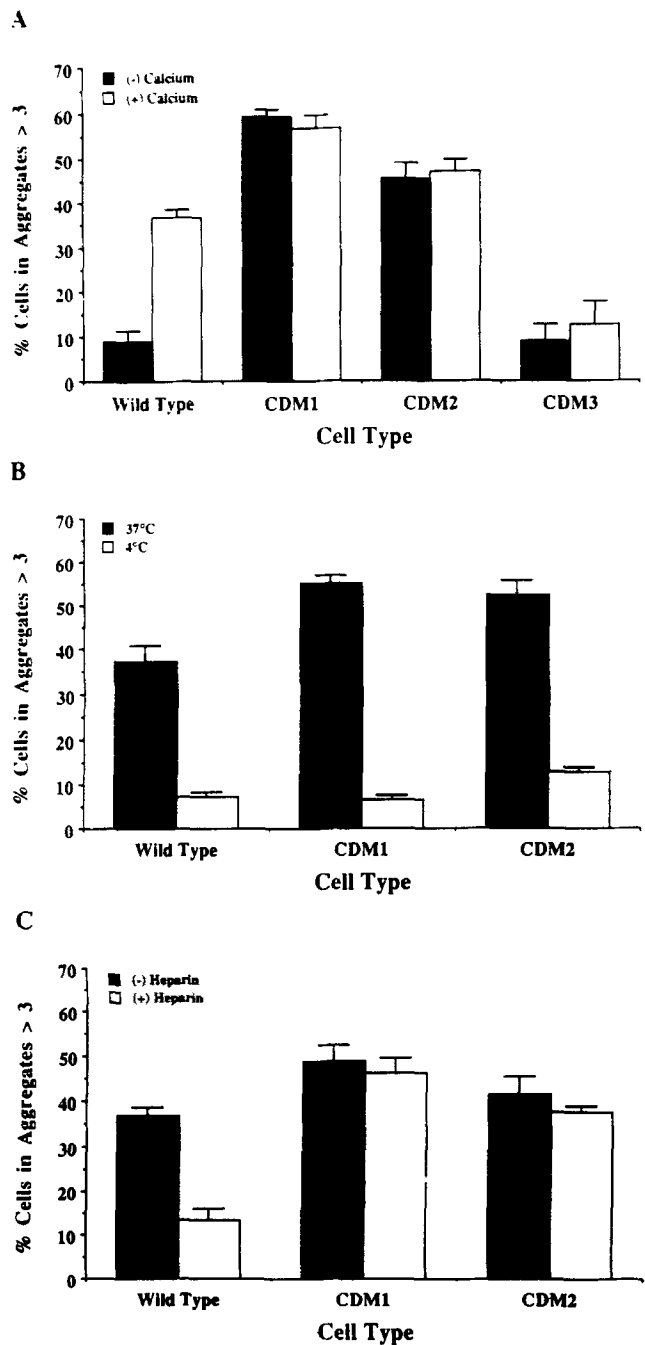
Figure 5. Aggregation of wild-type and mutant PECAM-1 expressing L-cell transfectants. The aggregation assay using L-cell transfectants was performed as described in Materials and Methods. (A) Calcium dependence. Wild-type and mutant transfected L-cell were allowed to aggregate in the absence or presence of 1 mM calcium. Wild-type transfectants demonstrated calcium-dependent aggregation while CDM1 and CDM2 displayed calcium-independent aggregation. CDM3 did not aggregate. Subsequent experiments were performed to study the effect of temperature and heparin on the aggregation of wild-type, CDM1, and CDM2 in the presence of calcium. (B) Effect of lowering temperature. Lowering the temperature to 4°C blocked the aggregation of all three constructs. (C) Effect of heparin. Heparin (50 μg/ml) inhibited the aggregation of wild-type, but not CDM1- or CDM2-expressing cells. Similar results were seen at concentrations up to 500 μg/ml. For CDM1 and CDM2, similar results were obtained for experiments performed in the absence of calcium. The data presented are representative of at least three experiments done in duplicate and of at least two independent clones.

A

B

C

Figure 6. Effect of Anti-PECAM-1 antibodies on the aggregation of wild-type and mutant PECAM-1 L-cell transfectants. One polyclonal and six monoclonal (4G6, PECAM-1.1 [1.1], PECAM-1.2 [1.2], PECAM-1.3 [1.3], hec7, HD03) were studied for their effect on the aggregation process in the presence of calcium (*A*, *B*, and *C*). The polyclonal antibody was able to block the aggregation of all three constructs. Two monoclonal antibodies (mAb 4G6 and mAb PECAM-1.2) inhibited wild-type dependent aggregation (*A*) but had no effect on the aggregation mediated by CDM1 (*B*) or CDM2 (*C*). The data presented are representative of at least two experiments done in duplicate. For CDM1 and CDM2, similar results were obtained for experiments performed in the absence of calcium.

Two Monoclonal Antibodies Block Wild Type– But Not CDM1- or CDM2-dependent Aggregation

Since aggregation mediated by wild-type PECAM-1 can be inhibited by a polyclonal antibody made against platelet PECAM-1 (Fig. 6 *A*; see also Albelda et al., 1991), the ability of this antibody to block CDM1- and CDM2-mediated aggregation was also tested. As shown in Fig. 6, *B* and *C*, the polyclonal antibody was able to inhibit the aggregation induced by the mutant PECAM-1 forms, confirming that the interaction was specific and PECAM-1 dependent.

To further explore the nature of the binding mediated by the different forms of PECAM-1, we tested a panel of monoclonal antibodies for their ability to inhibit aggregation. Six mAbs were screened. All recognized wild-type PECAM-1 and each of the mutant forms on FACS analysis and by immunoprecipitation (data not shown). When tested with L-cells transfected with wild-type PECAM-1, two of the six mAbs (PECAM-1.2 and 4G6) were able to inhibit aggregation (Fig. 6 *A*). In contrast, when these same antibodies were used in aggregation assays using cells transfected with CDM1 and CDM2, no inhibition was observed (Fig. 6, *B* and *C*). The failure to block aggregation was not due to a loss in the ability of the antibodies to bind to the mutant proteins since all demonstrated roughly equivalent binding on FACS analysis (Table I). Similar results were obtained with experiments performed in the absence of calcium. Three of the antibodies (hec7, PECAM-1.3 and HD03) appeared to induce aggregation. However, this effect was probably due to antibody cross-linking of PECAM-1, as Fab fragments of these antibodies failed to enhance aggregation (data not shown). These results indicate that the binding observed in the cells transfected with the altered PECAM-1 constructs use different epitopes from those used in the wild-type PECAM-1.

CDM1 and CDM2 Mediate Homophilic Aggregation

The experiments described above suggested that the aggregation mediated by the mutant constructs was fundamentally different than that of the wild-type PECAM-1 molecule. To determine if the ligand-binding specificity (i.e., heterophilic vs homophilic) was different, a mixed aggregation assay was used (Muller et al., 1992*b*; DeLisser et al., 1993*a*). In these

Table I. FACS Analysis of Wild-Type and Mutant PECAM-1 L-cell Transfectants Using Anti-PECAM-1 Polyclonal and 4G6 and PECAM-1.2 Monoclonal Antibodies

| Cell type	Mean fluorescence intensity			
	Irrelevant control mAb	Polyclonal antibody	mAb 4G6	mAb 1.2
Wild-type	35	136	97	97
CDM1	37	139	96	112
CDM2	40	138	103	118
CDM3	38	141	92	88

The ability of an irrelevant control mAb, a polyclonal anti-PECAM-1 antibody, and two blocking monoclonal antibodies (4G6 and PECAM-1.2) to bind to L-cells expressing wild-type PECAM-1 and mutant constructs was assessed by fluorescence-activated cell sorting analysis. The mean (log) fluorescence intensity for each antibody was determined for each cell type. The polyclonal antibody, mAb 4G6 and mAb PECAM-1.2 bind equally well to wild-type and the mutant constructs. Similar results were noted for the other four non-blocking monoclonal antibodies.

studies, equal mixtures of non-transfected L-cells and L-cells transfected with PECAM-1 were allowed to aggregate in the presence of calcium, after fluorescent labeling of one of the cell lines. After 45 min, the composition of aggregates of specific sizes were determined and a frequency distribution tabulated. As reported in detail previously (DeLisser et al., 1993a), and shown in Fig. 7 A, the presence of transfected and non-transfected cells in the majority of the aggregates is characteristic of heterophilic aggregation. That is, PECAM-1 on one cell is interacting with a non-PECAM-1 ligand. In marked contrast, the pattern of aggregation for both the CDM1 (Fig. 7 B) and CDM2 (Fig. 7 C) transfected cells was characterized by aggregates composed almost entirely of transfected cells, resulting in distribution curves shifted markedly to the right. This pattern is characteristic of homophilic aggregation (PECAM-1 on one cell interacting with PECAM-1 on an adjacent cell) and has been observed previously for cadherins (Jaffe et al., 1990; Muller et al., 1992b). Similar results were obtained for 4- and 6-cell aggregates and when experiments were conducted in the absence of calcium.

Discussion

The purpose of this study was to evaluate the role of the cytoplasmic domain in PECAM-1 function. To accomplish this, full length and truncated versions of PECAM-1 cDNA were transfected into murine fibroblasts and their ability to promote cell–cell adhesion was analyzed. Wild-type PECAM-1 accumulated at cell–cell borders in transfected 3T3 cells and supported L-cell aggregation in a heterophilic, calcium-dependent manner that was inhibitable by heparin. Aggregation was blocked by a polyclonal anti-PECAM-1 antibody and two specific monoclonal antibodies. A PECAM-1 mutant with a complete deletion of the cytoplasmic domain showed minimal localization to adjacent cell–cell borders and did not promote L-cell aggregation. In marked contrast, two mutants with partial deletions of their cytoplasmic domains displayed markedly altered adhesion properties. Both truncated proteins retained the ability to move to cell–cell borders; however, the CDM1 mutant (lacking the carboxyl-terminal one third of the cytoplasmic domain) and the CDM2 mutant (lacking the carboxyl-terminal two thirds of the cytoplasmic domain) supported homophilic, calcium-, and heparin-independent aggregation. Although aggregation was still blocked by the polyclonal anti-PECAM-1 antibody, the monoclonal antibodies that inhibited heterophilic binding had no effect on homophilic aggregation mediated by either truncated form of PECAM-1, despite their ability to bind to the mutated PECAM-1 molecules as demonstrated by FACS.

Truncation of the cytoplasmic domain has been shown to affect the function of a number of cell adhesion molecules, including integrins, selectins, CD44, and immunoglobulin superfamily members. In most cases, deletions of the cytoplasmic domain have changed the ability of receptors to interact with elements of the cytoskeletal complex (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Nagafuchi and Takeichi, 1988; Lesley et al., 1992). Although O'Toole et al. (1991) have presented data to show that deletion of the cytoplasmic domain of the integrin α_{IIb} subunit could lead to an increase in the affinity of the $\alpha_{IIb}\beta_3$
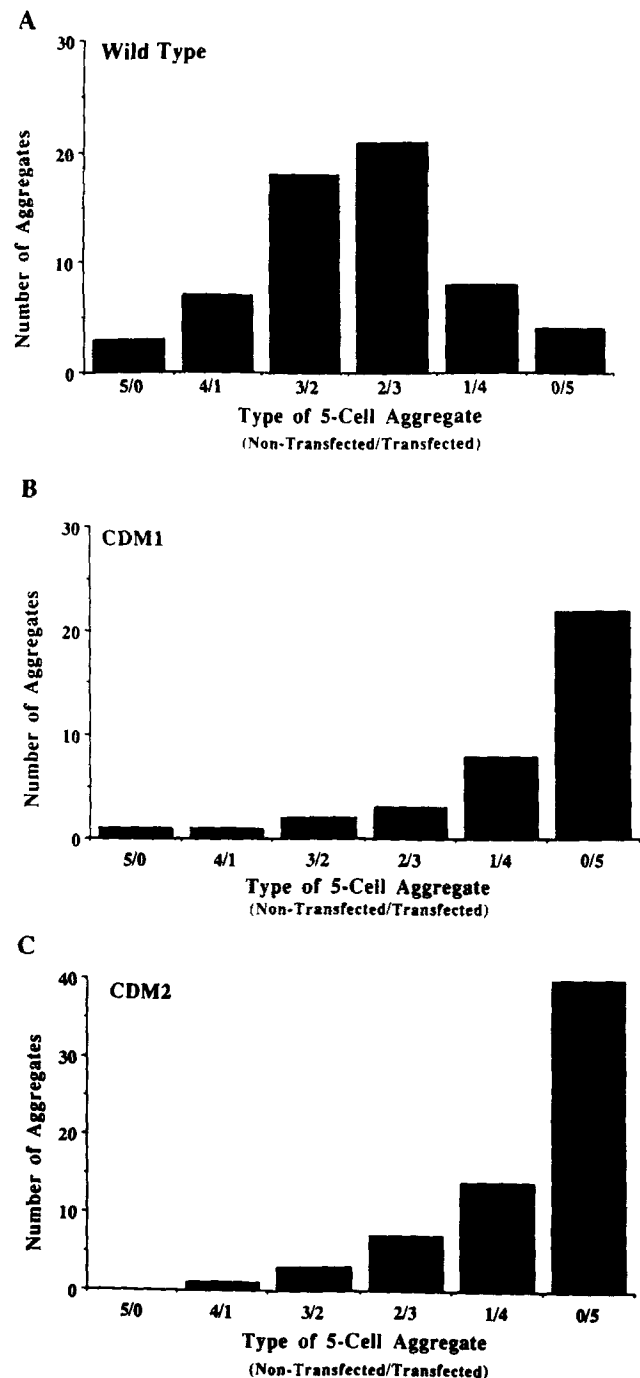
Figure 7. Mixed aggregation studies. Mixed aggregation assays were performed in which equal numbers of non-transfected and transfected L-cells were mixed together after fluorescent labeling of one of the cell lines. After incubation, the number of labeled cells within each 5 cell aggregate was counted. The data is representative of at least three experiments. (A) When wild-type PECAM-1 L-cell transfectants were mixed with non-transfected L-cells the majority of aggregates were made up of mixtures of transfected and non-transfected cells. A "normal" distribution is noted, indicative of a heterophilic interaction (see DeLisser et al., 1993a). Similar mixed aggregation experiments with CDM1 (B) and CDM2 (C) yielded aggregates that were composed primarily of transfected cells. The frequency distribution is shifted toward the right, reflecting a homophilic interaction.

receptor for its ligand, our data demonstrate a complete change in the nature of the adhesion process accompanying partial deletion of the cytoplasmic domain of PECAM-1. To our knowledge, this is the first example in which alterations in the cytoplasmic domain result in changes in ligand specificity as well as cation dependence.

How might these alterations in the cytoplasmic domain of PECAM-1 lead to changes in the functional properties of the molecule? Although we currently have no clear answer to this question, data involving other adhesion molecules suggest a number of possibilities. Deletions in the cytoplasmic domain could cause changes in the overall folding of the molecule resulting in a change in the conformation of the extracellular portion of the molecule. The altered conformation would present different ligand-binding domains and therefore have altered divalent cation requirements. It is also possible that the changes in the extracellular region are related to an altered pattern of phosphorylation of the cytoplasmic domain in the mutants. The function of a number of adhesion molecules, such as the $\beta2$ integrins, are known to be affected by the state of cytoplasmic phosphorylation. For example, phosphorylation of LFA-1 by both protein kinase C-dependent and independent pathways regulate the binding affinity of this molecule for ICAM-1 (Hibbs et al., 1991; Hedman and Lundgren, 1992). There is circumstantial evidence to suggest that the phosphorylation state of PECAM-1 may be important in function. Increases in baseline levels of phosphorylation have been observed after activation of endothelial cells, platelets, and lymphocytes, and appear to involve exclusively phosphorylation of serine residues in the cytoplasmic domain (Newman et al., 1992; Zehnder et al., 1992). Although there do not appear to be any gross differences in the level of phosphorylation between the wild-type PECAM-1 versus the CDM1 or CDM2 mutants (Fig. 2 B), our results do not exclude the possibility that one or more of the serine residues in the proximal third of the cytoplasmic domain (S611, S620, S626, S634), common to both CDM1 and CDM2, may be differentially phosphorylated.

Another mechanism by which alterations in the cytoplasmic domain could affect PECAM-1 function might involve changes in the interaction of the truncated molecules with other cellular proteins. Cytoplasmic domain-cytoskeletal interactions have been shown to be important for a number of cell adhesion molecules (Nagafuchi and Takeichi, 1988; Otey et al., 1990; Burridge et al., 1988; Lacy and Underhill, 1987; Kansas et al., 1993) and have recently been demonstrated for platelet PECAM-1 (Newman et al., 1992). It is possible that the altered ability to interact with cytoskeletal structures could change the nature of PECAM-1 binding. Alternatively, the truncated mutants may have also lost the ability to associate with some, as yet unidentified, PECAM-1-associated proteins, analogous to molecules such as integrin modulating factor-1 (Hermanowski-Vosatka et al., 1992) or integrin-associated protein (Brown et al., 1990).

Whatever the mechanism, it appears that truncation of the PECAM-1 cytoplasmic domain leads to alterations in the conformation of the extracellular portion of the molecule as evidenced not only by altered dependence upon divalent cations and glycosaminoglycans, but also by the fact that monoclonal antibodies that block cell–cell aggregation mediated by intact PECAM-1 no longer block aggregation, despite being able to bind to the mutant PECAM-1 molecules (Table

I). Interestingly, the epitopes of these mAbs that block heterophilic aggregation, 4G6 and PECAM-1.2, map to domain 6 (Yan, H. C., H. M. DeLisser, and S. M. Albelda, manuscript in preparation) and are thus distinct from the heparin-binding sequence in domain 2. It is currently unclear how this region in loop 6 regulates heterophilic binding.

Although the deletions created in the cytoplasmic domain described in this study were artificially engineered, there is intriguing evidence to suggest that naturally occurring alterations in the cytoplasmic domain of PECAM-1 (i.e., alternative splicing) exist. Preliminary work (Kirschbaum, N. E., and P. J. Newman. 1993. Thromb. Haemostasis. 69:1010a) indicates that the gene for PECAM-1 is complex, containing 16 exons and extending over more than 75 kilobases of DNA. The transmembrane and cytoplasmic domain are encoded by a series of short exons (exons 9-16). Interestingly, these authors reported the isolation of an alternatively spliced cDNA clone missing 63 basepairs from the coding sequence for the cytoplasmic tail. The missing region corresponded exactly to exon 13 of the gene, a region of the molecule that is disrupted in our mutants (Newman, P. J., personal communication). Since alternative splicing may represent a means of efficiently changing the functional characteristics of a given protein (Smith et al., 1989), it is tempting to speculate that changes in the cytoplasmic domain of PECAM-1 generated by alternative splicing, might play a role in modulating the ligand interactions of PECAM-1 during vascular development or pathological conditions involving angiogenesis or leukocyte-endothelial interactions.

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