Alternative Splicing of a Specific Cytoplasmic Exon Alters the Binding Characteristics of Murine Platelet/Endothelial Cell Adhesion Molecule-1 (PECAM-1)*

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Horng-Chin Yan†‡, H. Scott Baldwin§, Jing Sun, Clayton A. Buck§, Steven M. Albeda§** and Horace M. DeLisser†‡

From the Department of Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104-4283, §The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104, the Cardiology Division, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, 19104-4283, and the Pulmonary Division, Department of Medicine, Tri-Service General Hospital, Taipei, Taiwan

Platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) is a membrane glycoprotein expressed on endothelial cells, platelets, and leukocytes. Analysis of PECAM-1 expression in the developing mouse embryo has revealed the presence of multiple isoforms of murine PECAM-1 (muPECAM-1) that appeared to result from the alternative splicing of exons encoding cytoplasmic domain sequences (exons 10-16) (Baldwin, H. S., Shen, H. M., Yan, H., DeLisser, H. M., Chung, A., Mickanin, C., Trask, T., Kirschbaum, N. E., Newman, P. J., Aebela, S., and Buck, C. A. (1994) Development 120, 2539-2553). To investigate the functional consequences of alternatively spliced muPECAM-1 cytoplasmic domains, L-cells were transfected with cDNA for each variant and their ability to promote cell aggregation was compared. In this assay, full-length muPECAM-1 and all three isoforms containing exon 14 behaved like human PECAM-1 in that they mediated calcium- and heparin-dependent heterophilic aggregation. In contrast, three muPECAM-1 variants, all missing exon 14, mediated calcium- and heparin-independent homophilic aggregation. Exon 14 thus appears to modulate the ligand and adhesive interactions of the extracellular domain of PECAM-1. These findings suggest that alternative splicing may represent a mode of regulating the adhesive function of PECAM-1 in vivo and provides direct evidence that alternative splicing involving the cytoplasmic domain affects the ligand specificity and binding properties of a cell adhesion receptor.

PECAM-12 (CD31) has the distinctive feature of being found on platelets, leukocytes, and endothelial cells (reviewed in DeLisser et al. (1994b)). Recent observations have implicated PECAM-1 in a number of important processes. PECAM-1 facilitates the diapedesis of leukocytes both in vitro and in vivo (Muller et al., 1993; Vaporiyan et al., 1993; Bogen et al., 1994), acts as a trigger for up-regulating integrins on leukocytes (Tanaka et al., 1992; Piali et al., 1993; Berman and Muller, 1995; Leavely et al., 1994), and thus appears to play a role in cell-cell interactions during an inflammatory response. It is also one of the first cell surface molecules to be expressed by endothelial and endocardial cells during embryonic development, suggesting that it may be involved in the establishment of the early cardiovascular system (Baldwin et al., 1994).

PECAM-1 is organized into an extracellular amino-terminal domain containing 6 immunoglobulin (Ig)-like repeats, a short hydrophobic transmembrane domain, and long cytoplasmic tail (Newman et al., 1990). The gene for human PECAM-1 (huPECAM-1) has recently been characterized (Kirschbaum et al., 1994) and is composed of 16 exons separated by introns ranging in size from 86 to more than 12,000 base pairs in length. Each of the six extracellular Ig homology domains is encoded by a single exon (exons 3-8). The transmembrane region is encoded by one exon (exon 9), while the cytoplasmic tail is encoded by a series of six short exons (exons 10-16). This multi-exon structure of the cytoplasmic domain is quite unusual for Ig superfamily members. A number of cytoplasmic domain variants of PECAM-1, presumably arising from alternative splicing, have been identified in huPECAM-1 (Goldberger et al., 1994; Kirschbaum et al., 1994) and in murine PECAM-1 (muPECAM-1) (Baldwin et al., 1994). Analysis of PECAM-1 expression in the developing mouse embryo documented the presence of at least six isoforms of muPECAM-1 that appeared to result from the alternative splicing of the exons encoding the cytoplasmic domain (Baldwin et al., 1994).

Although the role of these multiple isoforms of PECAM-1 is currently unknown, studies examining the binding characteristics of huPECAM-1 suggest that alterations in the cytoplasmic domain have important functional implications. Previous experiments showed that full-length huPECAM-1 promoted heterophilic aggregation in transfected L-cells in a divalent cation-dependent, heparin-sensitive manner (Albeda et al., 1991; Muller et al., 1992; DeLisser et al., 1993). In contrast, mutants of huPECAM-1 with partially truncated cytoplasmic domains mediated aggregation that was quite different in that it was homophilic, divalent cation-independent, and heparin-insensitive (DeLisser et al., 1994a). These findings raise the possibility that naturally occurring alternatively spliced forms of PECAM-1 might also function differently (Baldwin et al., 1994).

The purpose of this study was to analyze the adhesive properties of each of the muPECAM-1 isoforms detected in early mouse embryos (Baldwin et al., 1994) and, if possible, identify
specific regions of the cytoplasmic domain that determined the binding characteristics of the molecule. To accomplish this, L-cells were transfected with the cDNA for each variant and the functional properties of each isoform analyzed using the L-cell aggregation assay. These experiments documented that consistent changes in the aggregation properties of the cells were correlated with specific isoforms of muPECAM-1. Full-length muPECAM-1 and all isoforms containing peptide sequences encoded in cytoplasmic exon 14 mediated heterophilic, calcium-dependent, heparin-sensitive aggregation. In contrast, all isoforms missing this peptide sequence in their cytoplasmic domains demonstrated homophilic aggregation that was calcium-independent and heparin-insensitive. These findings provided direct evidence for the hypothesis that naturally occurring alternatively spliced PECAM-1 isoforms have different ligand specificity and suggest that alternative splicing may be a method of regulating the adhesive function of PECAM-1 in vivo. In addition, these results pinpoint exon 14 as a key region of the cytoplasmic domain that determines the ligand and adhesive interactions of PECAM-1.

**EXPERIMENTAL PROCEDURES**

Naming of the Cytoplasmic Domain Variants of muPECAM-1—A total of six isoforms of muPECAM-1 were isolated and studied; a full-length form and five alternatively spliced cytoplasmic domain variants. The full-length construct was designated muPECAM-1, while each of the five other isoforms was identified based on the exon that was deleted. For example, muPECAM-1-12,15 designates the isoform missing exons 12 and 15.

"Shot-gun" Cloning and Isolation of Alternately Spliced muPECAM-1 Isoforms—Dated ICR mice were purchased from Harlan Sprague-Dawley (Indianapolis IN). Using previously described methods (Chomczynski et al., 1987), total RNA was isolated from the pooled tissue culture media of mouse embryos. Poly(A)+ RNA, extracted by an oligo(dT) spin column (Micro Fast Tract, Invitrogen) was subsequently used as a template for reverse transcription (RT) in a reaction mixture of random and oligo(dT) primers and avian myeloblastosis virus reverse transcriptase as outlined in the manufacturer's instructions (cDNA Cycle Kit, Invitrogen). To polymerase chain reaction amplify the cytoplasmic domains of all possible muPECAM-1 isoforms from the reverse transcribed cDNA, the following primers were used: a sense primer (5'-CTCTAGTAC 9-1852CCAAGGCCAAACAGA1866-3') flanking the BstEII restriction site within the extracellular domain and an antisense primer (5'-CGATGCT9-1572AAGTTTAA1642-3') representing a region of muPECAM-1 homologous to exon 10; and antisense primer, 5'-AAGGGACCTCTGCC9-1840TCC4-3' representing a region in exon 16 of the cytoplasmic domain of the published sequence of the PECAM-1 gene. Candidate clones were then sequenced to confirm their identity using two different primer pairs from two different orientation: sense primers, 5'-TCTAGTAC 9-1852CCAAGGCCAAACAGA1866-3' and 5'-GGAAGGAAGAGAGGCC9-1620ATC4-3'; and antisense primers, 5'-AAGGGACCTCTGCC9-1840TCC4-3' and 5'-CGATGCT9-1572A9TCA1842-3'.

**Tissue Culture and Transfection of L-cells—**L-cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured in RPMI medium with 10% fetal bovine serum. The procedures to transfect PECAM-1 cDNA into these cells have been previously described (Albeda et al., 1991; DelLiser et al., 1994a).

**Immunoprecipitation—**For deglycosylation experiments, L-cells transfected were cultured in normal media with deoxyxymannojirimycin (40 μg/ml; Calbiochem Corp.; Diamond et al., 1991) or swainsine (0.1 mM; Sigma; Nguyen et al., 1992) for 48 h. The cells were then detached from the cell culture plates with trypsin-EDTA and replated with fresh media with inhibitor for an additional 24 h. To remove sialic acid residues, L-cell transfected were detached from cell culture plates with Enzyme Free Cell Dissociation Solution (Specialty Media, Lavallette, NJ), washed three times with phosphate-buffered saline with 1 mM MgCl2 and 0.3 mM CaCl2, and resuspended in the same buffer containing 0.2 unit/ml Vibrio cholera neuraminidase (Calbiochem Corp.) for 75 min at 37°C with gentle rocking (Diamond et al., 1991). Following washing, the cells were then used in aggregation experiments or biotinylated detergent extracts were prepared and immunoprecipitated as described above.

**Glycosylation Inhibition—**For deglycosylation experiments, L-cells transfected were incubated with 10 μg/ml trypsin-EDTA from Vibrio cholera neuraminidase for 1 h at 37°C. Following immunoprecipitation, the beads were washed and the primary supernatant was digested with BstEII and NotI and ligated into a pcDNAI/Neo vector (Invitrogen) containing muPECAM-1-12,15 that was also cut with the same enzymes. The resulting ligation mixture was then used to transform competent cells. Antibiotic resistant clones were initially screened by size following digestion of miniprep plasmid DNA with a unique restriction endonuclease. Promising clones were then screened by polymerase chain reaction with the following primer pair: sense primer, 5'-TCTAGTAC 9-1852CCAAGGCCAAACAGA1866-3'; and antisense primer, 5'-AAGGGACCTCTGCC9-1840TCC4-3'.

**Fluorescent Cell Sorting (FACS) Analysis—**L-cells expressing muPECAM-1 isoforms were non-enzymatically removed from tissue culture media of early mouse embryos. Poly(A)+ RNA, extracted by an oligo(dT) spin column (Micro Fast Tract, Invitrogen) was subsequently used as a template for reverse transcription (RT) in a reaction mixture of random and oligo(dT) primers and avian myeloblastosis virus reverse transcriptase as outlined in the manufacturer's instructions (cDNA Cycle Kit, Invitrogen). To polymerase chain reaction amplify the cytoplasmic domains of all possible muPECAM-1 isoforms from the reverse transcribed cDNA, the following primers were used: a sense primer (5'-CTCTAGTAC 9-1852CCAAGGCCAAACAGA1866-3') flanking the BstEII restriction site within the extracellular domain and an antisense primer (5'-CGATGCT9-1572AAGTTTAA1642-3') representing a region of muPECAM-1 homologous to exon 10; and antisense primer, 5'-AAGGGACCTCTGCC9-1840TCC4-3'

**Mixed Aggregation Assay—**To determine whether the muPECAM-1-
dependent L-cell aggregation was mediated by homophilic or heterophilic mechanisms, "mixed aggregation" assays were performed (Muller et al., 1992; DeLisser et al., 1993). In these experiments, L-cell aggregation was performed by mixing non-transfected and transfected cells, with one of the two cell types fluorescently labeled prior to mixing. After the cell line designated for labeling had been washed once with EDTA, it was resuspended in HBSS to a total volume of 1 ml. One ml of rhodamine-conjugated dye solution at a final concentration of 1 mM (Sigma), 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 of fetal bovine serum and by washing the cells with HBSS. The second EDTA wash and the two HBSS washes were then performed as described above. Each set of cells, one labeled, and the other unlabeled, were resuspended at 5–7 × 10⁵ cells/ml. Aliquots of 0.5 ml of each were combined in the wells of a 24-well non-tissue culture treated 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

Molecular Cloning and Identification of muPECAM-1 from Early Mouse Embryos—To isolate muPECAM-1 cytoplasmic domain isoforms for sequencing and functional analysis, we employed a shot-gun cloning strategy (detailed under "Experimental Procedures") in which a mixture of murine PECAM-1 isoforms isolated from an early mouse embryo was directly cloned into an expression vector. Clones were screened by polymerase chain reaction with primer pairs designed to amplify the entire cytoplasmic domain. The identities of candidate clones were then determined by dideoxy DNA sequencing. Comparison with the sequence of the human gene encoding PECAM-1 confirmed that these six isoforms, a full-length form (muPECAM-1) and five cytoplasmic domain variants (muPECAM-1D₁₂₁₅, muPECAM-1D₁₄₁₅, and muPECAM-1D₁₂₁₄₁₅), were the result of alternative splicing. Four isoforms (muPECAM-1D₁₅, muPECAM-1D₁₂₁₅, muPECAM-1D₁₄₁₅, and muPECAM-1D₁₂₁₄₁₅) were found to have an alternative exon 16, resulting from a frameshift caused by the insertion of an extra A or G residue before the first base of exon 16. These isoforms are unlikely to be the result of errors introduced during the reverse transcription, as these extra bases were found in 14 clones sequenced from four different isoforms. A seventh isoform missing exons 12 and 15 was previously isolated from an adult heart library (Baldwin et al., 1994).

Expression of muPECAM-1 Isoforms in L-cell Fibroblasts—Each isoform was expressed in L-cells. After selection in G418,
we have previously established that L-cell fibroblasts, transfected with full-length huPECAM-1 mediate calcium- and heparin-dependent, heterophilic aggregation (Albelda et al., 1991; Muller et al., 1992; DeLisser et al., 1993). Each isoform was therefore tested initially for its ability to support L-cell aggregation. From these studies, two patterns of reactivity were seen. Fig. 4A shows that muPECAM-1, muPECAM-1Δ12, muPECAM-1Δ15, and muPECAM-1Δ12,15 (Group 1) behaved like the human homologue in that they all mediated calcium-dependent aggregation, inhibitible by heparin (50 μg/ml). These properties, however, contrasted significantly with those of the cytoplasmic domain variants missing exon 14, muPECAM-1Δ14, muPECAM-1Δ14,15, and muPECAM-1Δ12,14,15 (Group 2). These muPECAM-1 isoforms were found to promote calcium-independent, heparin-insensitive aggregation (Fig. 4B). Sham transfectants demonstrated minimal aggregation in the absence or presence of calcium (Albelda et al., 1991).

Deletion of Exon 14 Changes Monoclonal Antibody Sensitivity—To confirm that the interactions studied were in fact PE-CAM-1-dependent, three monoclonal antibodies (390, EA-3, and Mec 13.3) to bind to L-cells expressing muPECAM-1 isomers was assayed by fluorescence-activated cell sorting analysis. The mean (log) fluorescence intensity for each antibody was determined for each cell type. The three monoclonal antibodies bound equally well to each isoform. A different pattern, however, was noted for muPECAM-1Δ14, muPECAM-1Δ15, and muPECAM-1Δ12,15, the control antibody appeared to inhibit aggregation; however, mAb 390 did not inhibit aggregation below the condition with no antibody. Failure of mAb 390 to inhibit aggregation was not due to a loss in ability to bind to these isoforms since each displayed roughly equal binding on FACS analysis (Table I). A non-bioactive anti-murine CD44

| Cell type       | Mean fluorescence intensity |
|-----------------|----------------------------|
| L-cell/Neo      | 41  39  32  29             |
| muPECAM-1       | 45  120  118  124           |
| muPECAM-1Δ12    | 35  112  106  116           |
| muPECAM-1Δ14    | 58  134  128  136           |
| muPECAM-1Δ15    | 52  142  131  142           |
| muPECAM-1Δ12,15 | 36  133  129  136           |
| muPECAM-1Δ14,15 | 37  127  127  131           |
| muPECAM-1Δ12,14,15 | 52  141  131  146          |

Deletion of Exon 14 Alters the Calcium and Heparin-Dependence of PE-CAM-1-mediated Aggregation—We have previously established that L-cell fibroblasts, transfected with full-length huPECAM-1 mediate calcium- and heparin-dependent, heterophilic aggregation (Albelda et al., 1991; Muller et al., 1992; DeLisser et al., 1993). Each isoform was therefore tested initially for its ability to support L-cell aggregation. From these studies, two patterns of reactivity were seen. Fig. 4A shows that muPECAM-1, muPECAM-1Δ12, muPECAM-1Δ15, and
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monoclonal antibody used as a control was not inhibitory. These findings confirm the involvement of PECAM-1-dependent interactions and support the conclusion that removal of exon 14 leads to differences in the binding characteristics of the different isoforms.

Deletion of Exon 14 Results in Homophilic Aggregation—The experiments described above suggest that the aggregation mediated by the isoforms missing exon 14 differed significantly from that of the isoforms of Group 1. To determine if the ligand binding specificity was heterophilic or homophilic for each of the isoforms, a mixed aggregation assay was used (Muller et al., 1992; DeLisser et al., 1993). In these studies, equal mixtures of non-transfected L-cells and L-cells transfected with muPECAM-1 were allowed to aggregate together in the presence of calcium, after fluorescent labeling of one of the cell lines. After 30 min, the composition of the aggregates of specific sizes was determined and a frequency distribution tabulated. Histographic analysis of the resulting aggregates can easily distinguish between a homophilic mechanism (PECAM-1 interacting with PECAM-1) and a heterophilic process (PECAM-1 interacting with a non-PECAM-1 ligand) (Albelda et al., 1991; Muller et al., 1992; DeLisser et al., 1993). In mixed aggregation assays with the isoforms of Group 1, the majority of the aggregates were composed of mixtures of transfected and non-transfected L-cell (heterophilic adhesion) (Fig. 6A). This pattern was consistent with a heterophilic interaction as noted for huPECAM-1 (Muller et al., 1992; DeLisser et al., 1993). Strikingly different results were obtained with variants missing exon 14 (Group 2) where aggregates consisted primarily of transfected cells (Fig. 6B). This is characteristic of a homophilic process and has been noted previously for cadherins (Jaffe et al., 1990; Muller et al., 1992) and for cytoplasmic domain mutants of huPECAM-1 in which exons 12–16 or 14–16 were deleted (DeLisser et al., 1994a). Similar results were obtained for 4- and 6-cell aggregates and when experiments were conducted in the absence of calcium (data not shown).

Phosphorylation of muPECAM-1—huPECAM-1 is constitutively phosphorylated to varying degrees in platelets (Newman et al., 1992; Zehnder et al., 1992), endothelial cells (Zehnder et al., 1992), and transfected L-cells (DeLisser et al., 1994a). We therefore studied the pattern of phosphorylation of muPECAM-1 and its cytoplasmic domain variants (Fig. 7). L-cell transfectants expressing each isoform were radiolabeled in parallel with sodium [32P]orthophosphate or [35S]methionine, ex-
Fig. 6. **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 muPECAM-1, muPECAM-1Δ12, and muPECAM-1Δ15, and muPECAM-1Δ12,15 L-cell transfectants (Group 1) 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., 1993). 

**B**, similar mixed aggregation experiments with muPECAM-1Δ14, muPECAM-1Δ14,15, and muPECAM-1Δ12,14,15 (Group 2) yielded aggregates that were composed primarily of transfected cells. The frequency distribution is shifted toward the right, reflecting a homophilic interaction.
tracted with nonionic detergents and the extracts immunoprecipitated with mAb 390. Like huPECAM-1, full-length muPECAM-1 was constitutively phosphorylated (Fig. 7B, lane 1) (DeLisser et al., 1994a). Interestingly, although exon 14 contains more serine and threonine residues (three threonine and one serine) than exon 12 (one serine) or exon 15 (one serine), the level of phosphorylation for muPECAM-1D14 was comparable to that of full-length muPECAM-1 (Fig. 7B, Lane 4), while the loss of exon 12 or 15 was associated with diminished incorporation of 32P label (Fig. 8B, lanes 2, 3, and 5–7). FACS analysis (see Fig. 2), as well as immunoprecipitation of parallel samples of transfected L-cells labeled with [35S]methionine (Fig. 7A), showed that the reduced 32P incorporation was not due to differences in the amount of PECAM-1 expressed by the transfected cells or to differences in the ability of the monoclonal antibody to immunoprecipitate each isoform. These data demonstrate that there is no simple correlation between gross changes in levels of phosphorylation and loss of exon 14.

Deletion of Exon 14 Alters the Dependence of PECAM-1-mediated Aggregation on Glycosylation but Does Not Change Its Binding Characteristics—Glycosylation is known to influence the function of a number of Ig-like cell adhesion molecules. Since PECAM-1 is heavily glycosylated (Newman et al., 1990), we postulated that changes in glycosylation might affect one or both types of PECAM-1 mediated binding or might convert one type of binding to the other type. We therefore investigated the effect of inhibitors of carbohydrate processing on each type of muPECAM-1-dependent aggregation. The inhibitors used were 1-deoxymannojirimycin, an early inhibitor of the synthesis of hybrid and complex-type N-linked oligosaccharides, swainsonine (an agent which disrupts N-linked oligosaccharides processing at a later step in the biosynthetic pathway), and neuraminidase (an agent which removes sialic acid residues). One representative variant from each group (muPECAM-1 from Group 1 and muPECAM-1D14 from Group 2) was exposed to each inhibitor. The activity of each agent was confirmed by an increase in mobility on SDS-polyacrylamide gel electrophoresis of extracts of cells treated with each agent (Fig. 8A). All three compounds were found to partially inhibit the aggregation of muPECAM-1 (Fig. 8B) which remained calcium-dependent and heterophilic in nature (data not shown). In contrast, none of the inhibitors affected the binding demonstrated by muPECAM-1D14 (Fig. 8B). Thus, the lack of sensitivity to removal of carbohydrates on PECAM-1 represents another difference between the binding characteristics of these two groups of muPECAM-1 isoforms.

**DISCUSSION**

To determine the functional consequences of alternative splicing of muPECAM-1, the behavior of L-cell transfectants expressing various isoforms was compared in an established adhesion assay (Albelda et al., 1991; Muller et al., 1992; DeLisser et al., 1993). Full-length muPECAM-1 and the three isoforms containing exon 14 (muPECAM-1D12, muPECAM-1D15, etc.) were expressed in L-cells. The activity of each agent was confirmed by an increase in mobility on SDS-polyacrylamide gel electrophoresis of extracts of cells treated with each agent (Fig. 8A). All three compounds were found to partially inhibit the aggregation of muPECAM-1 (Fig. 8B) which remained calcium-dependent and heterophilic in nature (data not shown). In contrast, none of the inhibitors affected the binding demonstrated by muPECAM-1D14 (Fig. 8B). Thus, the lack of sensitivity to removal of carbohydrates on PECAM-1 represents another difference between the binding characteristics of these two groups of muPECAM-1 isoforms.
and muPECAM-1,12,15) mediated calcium-dependent, hepa-
arin-sensitive, heterophilic aggregation that was inhibited by
the anti-murine PECAM-1 monoclonal antibody, mAb 390, and
was sensitive to deglycosylation. In contrast, all three of the
muPECAM-1 variants missing exon 14 (muPECAM-1,14, mu-
PECAM-1,14,15, and muPECAM-1,12,14,15) mediated ho-
mothophilic aggregation that was calcium-independent, heparin-
sensitive and not inhibited by mAb 390 or affected by
deglycosylation, as determined by the change in molecular size
after deglycosylation.2 Since there are 5 amino acids in exon 14 that
could potentially serve as phosphorylation sites, one potential mechanism
responsible for the changes in function resulting from loss of exon 14 could involve loss of a key phosphorylation site. PE-
CAM-1 is constitutively phosphorylated and there is evidence
to suggest that cellular activation is accompanied by changes in
phosphorylation at serine and threonine residues (Newman
et al. 1992; Zehnder et al., 1992). Our data (Fig. 8), however,
suggest that changes in the overall level of phosphorylation do
correlate with the deletion of exon 14. However, these data
do not rule out the possibility that the absence of one or
more of the specific phosphoratable residues in exon 14 may be
important. Since glycosylation of the extracellular domain has been
shown to play a crucial role in regulating the binding properties
of a number of Ig superfamily members, now including PE-
CAM-1, it is possible that loss of exon 14 could alter post-
translational processing and thus lead to a change in adhesive
function of PECAM-1. Although there may be changes in spe-
cific carbohydrate residues induced by loss of exon 14, we have
been unable to observe any gross changes in the overall level of
glycosylation, as determined by the change in molecular size
after deglycosylation.3

Another possibility, currently being examined, is that asso-
ciation of the cytoplasmic domain of PECAM-1 with another
membrane or cytoplasmic protein may be critical for its func-
tion. Exon 14 may be important in regulating this association.

Preliminary experiments using differential detergent extrac-
tions have not revealed differences in the ability of the various
isozymes to associate with the actin cytoskeleton.3 However, this
PECAM-1-associated molecule may be another PECAM-1
molecule, a molecule with enzymatic activity (such as the as-
ociation of the cytoplasmic domain of CD4 with the tyrosine
kinase p56
clck (Turner et al., 1990) or a novel protein.

Finally, it is theoretically possible that loss of exon 14 might
directly lead to a change in the overall conformation of the
molecule. A proline residue is the terminal amino acid of exon
14. Loss of this amino acid may induce an important confor-
mational change that alters the ability of the cytoplasmic domain
to fold normally. Clearly, this change must be rather subtle,
since all of the mAbs tested bind equally well to all isoforms.

### Table II

Comparison of muPECAM-1 isoforms

| Isoform          | Calcium dependence | Effect of heparin | Effect of mAb 390 | Effect of deglycosylation | Ligand interaction |
|------------------|--------------------|-------------------|-------------------|---------------------------|--------------------|
| Group 1          |                    |                   |                   |                           |                    |
| muPECAM-1        | Yes                | Inhibition        | Inhibition        | Inhibition                | Heterophilic       |
| muPECAM-1,12     |                    |                   |                   |                           |                    |
| muPECAM-1,15     |                    |                   |                   |                           |                    |
| muPECAM-1,12,15  |                    |                   |                   |                           |                    |
| Group 2          |                    |                   |                   |                           |                    |
| muPECAM-1,14     | No                 | None              | None              | None                      | Homophilic         |
| muPECAM-1,14,15  |                    |                   |                   |                           |                    |
| muPECAM-1,12,14,15 |                 |                   |                   |                           |                    |

2 H. M. DeLisser, J. Sun, H. Yan, and S. M. Albeda, unpublished data.
3 H. M. DeLisser, unpublished observations.
Regardless of the mechanism(s) involved in regulating PECAM-1 ligand interactions, the implications of these findings extend beyond their significance to the structure-function relationships of PECAM-1. First, it is possible that these sequences found within exon 14 may have importance in the function of other Ig superfamily members (Rojas et al., 1990). Second, our findings indicate that observations made with artificial mutations of huPECAM-1 (DeLisser et al., 1994a) are likely to have physiological relevance and thus advance the emerging concept of the cytoplasmic domain as a regulator of not only the strength but the mechanism of adhesion. Finally, these data also suggest that alternative splicing of cytoplasmic domain regions may represent a novel way a cell can alter its interactions with the environment during development, inflammation, and wound healing.

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