Cell Surface Glycosaminoglycans Do Not Serve as Ligands for PECAM-1

PECAM-1 IS NOT A HEPARIN-BINDING PROTEIN*

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Previous studies have suggested that PECAM-1 mediates cellular interactions via both homophilic and heterophilic adhesive mechanisms. Cell surface glycosaminoglycans have been implicated as one of the heterophilic ligands for PECAM-1. To determine whether PECAM-1 is capable of interacting directly with glycosaminoglycans, we examined the adhesive properties of multiple monovalent and multivalent forms of this adhesion molecule. We found that the binding of a bivalent PECAM-1/IgG chimeric protein or multivalent PECAM-1-containing proteoliposomes to multiple different cell lines was 1) strictly dependent upon cell surface expression of PECAM-1 and 2) unaffected by the presence of excess heparin or heparan sulfate. The extracellular domain of PECAM-1 failed to interact specifically with heparin-Sepharose, 3H-labeled heparin, or a heparin-bovine serum albumin conjugate. In addition, an amino acid sequence motif inadvertently created by the juxtaposition of PECAM-1 and IgG sequences within the hinge region of certain PECAM-1/IgG chimeric constructs was found to confer glycosaminoglycan binding properties not normally present within the extracellular domain of the native molecule. Together, these data suggest that the mechanism by which heparin is able to affect PECAM-1-dependent cell-cell adhesion is indirect and occurs via inhibition of events that occur downstream from PECAM-1 engagement.

PECAM-1 (CD31) is a 130-kDa member of the Ig gene superfamily that is constitutively expressed on the surface of circulating platelets, monocytes, neutrophils, and selected T cell subsets. It is also present at relatively high concentration at the cell junctions of all continuous endothelium, both in vitro and in vivo and in cell culture (for a recent review on the biology of PECAM-1, see Ref. 1). Because of its presence on these vascular cells, PECAM-1 has been implicated in mediating a number of cellular interactions, most notably those that take place between leukocytes and the vessel wall during the process of transendothelial migration (2–9) and between adjacent endothelial cells during the process of angiogenesis (10–12).

A number of different cell surface components have been implicated as counterreceptors or cellular targets for PECAM-1. Albelda et al. (13) found that PECAM-1 became concentrated at cell-cell borders only if both cells expressed PECAM-1, and they were the first to propose, based on this observation, that PECAM-1-mediated cellular interactions might operate homophilically, i.e. via PECAM-1/PECAM-1 intermolecular contacts. In support of this hypothesis, PECAM-1-containing proteoliposomes were recently shown to be able to self-assocaiate in a concentration-dependent, divalent cation-independent, manner (14), providing direct experimental support that PECAM-1 is capable of interacting with itself. Homophilic binding activity requires amino-terminal Ig homology domains 1 and 2 (14, 15), and specific residues within Ig domain 1 that participate in PECAM-1/PECAM-1 interactions have recently been identified by Newton et al. (16). The possibility that the amino-terminal region of the extracellular domain is physiologically relevant to PECAM-1-mediated cellular interactions is further supported by the findings that 1) anti-PECAM-1 monoclonal antibodies (mAbs) that inhibit leukocyte transendothelial migration almost without exception epitope map to Ig domain 1 or 2 (17, 18) and 2) soluble Ig domain 1 of PECAM-1 is sufficient to block transendothelial migration in vitro and in vivo (19).

A number of experimental observations have suggested that PECAM-1 may also be capable of interacting heterophilically with other components of the cell surface. Albelda et al. found that L cell fibroblasts transfected with recombinant PECAM-1 acquired the ability to aggregate with one another in a calcium-dependent manner and raised the possibility that cation-dependent, PECAM-1-mediated cellular interactions might involve additional ligands, such as integrins or proteoglycans (13). In this regard, these authors noted that PECAM-1 contains within Ig domain 2 the amino acid sequence LKRENK, which corresponds loosely to one of several possible consensus glycosaminoglycan recognition motifs (20). Shortly thereafter, PECAM-1-transfected L cells were found to bind as readily to nontransfected L cells as they did to each other (21), and the...
aggregation of PECAM-1-transfected L cells was shown to be
inhibitable by selected glycosaminoglycans, including chondroitin 6-sulfate, heparan sulfate, and heparin, as well as by the Ig domain 2-derived synthetic peptide, LKREKN (22). Taken together, these data have led to the widely held supposition that PECAM-1 is a heparin-binding protein.

Given the potential importance of PECAM-1 in regulating the interaction of vascular cells during the processes of inflammation, thrombosis, and angiogenesis, determining the range of potential cellular targets for PECAM-1 is crucial for understanding the mechanism by which it is able to initiate and respond to adhesive and signaling events. The purpose of the present investigation, therefore, was to specifcally determine whether PECAM-1 is capable of interacting directly with glycosaminoglycans. To accomplish this aim, we have examined the adhesive properties of multiple mono- and multivalent forms of this adhesion receptor, including full-length cellular soluble recombinant human PECAM-1 (srhuPECAM-1), PECAM-1-containing proteoliposomes, and a bivalent recombinant huPECAM-1-IgG chimeric protein.

EXPERIMENTAL PROCEDURES

Antibodies—PECAM-1.1 (specific for PECAM-1 Ig domain 5) and PECAM-1.3 (specific for PECAM-1 Ig domain 1) are murine anti-human PECAM-1 mAbs (characterized in Refs. 17 and 18). Fab fragments of PECAM-1.3 were generated using immobilized papain (Pierce) according to the manufacturer’s instructions, dialyzed against phosphate-buffered saline at 4°C overnight, and analyzed on SDS-polyacrylamide gel electrophoresis to confirm the absence of contaminating intact IgG in the preparations. Prior to their use, the reactivity PECAM-1.3 Fab fragments was determined by ELISA analysis using srhuPECAM-1 as the target antigen.

Cells and Cell Lines—Mouse L cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI medium with 10% fetal bovine serum. Cells were harvested with 10 mM EGTA in Hanks’ balanced salt solution (HBSS). PECAM-1-transfected L cells expressing full-length human PECAM-1 have been previously described (18). The transfected L cells were subcloned and grown in RPMI medium containing 10% fetal bovine serum and 0.5 mg/ml G418 (Life Technologies, Inc.). Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in RPMI medium containing 15% horse serum and 30 μg/ml endothelial cell growth factor. Raji, A375, U937, Dami, and HEL cell lines were obtained from the ATCC.

Flow Cytometric Evaluation of PECAM-1 Binding to Cells—PECAM-1- and GPIb-IIIa-containing proteoliposomes were prepared as described previously in detail by Sun et al. (14). Briefly, full-length glycoproteins comprising the extracellular, transmembrane, and cytoplasmic domains were affinity-purified from octyl glucoside-solubilized human platelet membranes, mixed with octyl glucoside-solubilized egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), and incorporated into liposomes by detergent dialysis (14). Proteoliposomes were made intrinsically fluorescent by incorporating 50 μg of the fluorescent phosphatidylcholine derivative, 7-nitrobenzo-2-oxa-1,3-diazole per mg of unlabeled phosphatidylcholine. Proteoliposomes were sized and purified by gel filtration chromatography before use. Association of the resulting 7-nitrobenzo-2-oxa-1,3-diazole-labeled proteoliposomes with cells was quantitatively determined by incubating 25 μl of proteoliposomes with 5 × 10⁵ of the indicated cells in a final volume of 250 μl for 90 min at 22°C and then performing analytical flow cytometry using a FACScan (Becton Dickinson Corp., San Jose, CA).

Chimeric proteins consisting of only the extracellular domain of human PECAM-1 (amino acid residues 1–557) fused to the C₃, C, and C₃, C, domains of human IgG, were purified from the culture media of CHO cells transfected with the IgG heavy chain and 2) by the presence of a polyhistidine tag at the COOH terminus of the molecule. The first chimeric protein, termed PECAM-IgG1, contains the amino acid sequence LAPWKKaaarrasvepkscd in the hinge region of human IgG1 and lacks a polyhistidine tag at the COOH terminus of the heavy chain. The second construct, termed PECAM-IgG1am, contains the sequence LAPWKKaaarrasvepkscd in the hinge region, and contains a His₆ sequence at the COOH terminus. Amino acids that distinguish the two in the hinge region of the chimera are underlined, and the two bivalent adhesion molecules are depicted schematically in Fig. 5A.

Soluble recombinant IgG chimeric protein was produced in large scale using hollow fiber capillary technology (Cellmax, Celico, Inc., Germantown, MD) and purified by Protein A-Sepharose chromatography using standard methods. Final purified products were examined by SDS-polyacrylamide gel electrophoresis and verified to be greater than 95% pure. Endotoxin levels of the purified proteins were determined using a limulus amebocyte assay and ranged from 40 to 400 ng/ml (14). Binding of proteoliposomes containing chimeric protein was evaluated by PECAM-1-IgG dissolved in HBSS to 5 × 10⁻⁵ cells such that the final concentration of PECAM-1-IgG equaled 40 μg/ml, and the final volume was 250 μl of HBSS, 10% fetal bovine serum. Following an incubation for 90 min at 22°C, the cells were washed, resuspended in 100 μl of HBSS containing 1.0 μl of fluorescein isothiocyanate-conjugated goat anti-human IgG γ-chain (Jackson ImmunoResearch Laboratories, West Grove, PA), and subjected to flow cytometric analysis as described previously (14). In selected experiments, cells were mixed with an equal volume of HBSS containing heparin, heparan sulfate (Sigma), or inhibitory mAbs before the addition of proteoliposomes or PECAM-1-IgG.

Interaction of srhuPECAM-1 with Heparin—srhuPECAM-1 has been previously described (23) and is comprised of all six extracellular Ig homology domains of human PECAM-1 (amino acids 1–574). Soluble recombinant srhuPECAM-1 was isolated from E. coli stably transfected Chinese hamster ovary cells and purified to homogeneity by mAb-based affinity chromatography. The interaction of srhuPECAM-1 with heparin was determined using three different, complementary methods. In the first, 50 μg each of srhuPECAM-1, thrombospondin (TSP), or platelet factor 4 (PF4), each dissolved in Tris- or Heps-buffered 100 mM saline, was loaded individually onto a 1–ml Hi-Trap FPLC Heparin-Sepharose column (Amersham Pharmacia Biotech). The column was washed with 0.1 M NaCl for 25 min at a flow rate of 0.1 ml/min, and bound proteins eluted in a 0.1–1.8 M NaCl gradient run at the same flow rate over 68 min. One-millimeter-calibrated fractions were collected and analyzed on a silver-stained 4–15% gradient polyacrylamide gel. In the second method, 50 μl each of TSP, PF4, or srhuPECAM-1 were mixed in solution with 30 μg/ml of [(3)H]heparin (NEN Life Science Products) in a final volume of 100 μl of phosphate-buffered saline containing 1% bovine serum albumin at 4°C overnight. The mixture was incubated overnight at 4°C with 100 μl of Protein A-Sepharose beads that had been preloaded with polyclonal antibodies specific for TSP, PF4, or srhuPECAM-1 and capture protein-heparin complexes that had formed. The beads were then washed six times, and the radioactivity that remained bound to the Protein A-Sepharose beads was determined in a β-scintillation counter. In the third method, 5-well plates (Immulon-2, Dynatech Laboratories, Chantilly, VA) were coated with 50 μg/ml of each of TSP, PF4, or srhuPECAM-1 in solution of purified human platelet PECAM-1, PECAM-1-IgG, srhuPECAM-1, or soluble recombinant rat PECAM-1 at 4°C overnight. After blocking the plates with 1% bovine serum albumin (BSA, fraction V), 50 μl of a 2 μg/ml solution of biotinylated heparin-BSA or biotinylated BSA (Sigma) was added and allowed to incubate for 2 h at 22°C. Binding was detected by the addition of an avidin-biotin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA), and color development was quantitated in an ELISA reader at 405 nm.

Evaluation of the Heparin Binding Properties of Synthetic Peptides by Solid Phase ELISA—Synthetic peptides corresponding to sequences present in the hinge region of PECAM-1-IgG and PECAM-1-IgGRR were synthesized using a model 9050 Pepsynthesizer (Millipore, Bedford, MA). The peptides, 10 mM trifluoroacetic acid, 0.1% trifluoroacetic acid (9-fluorobenzyloxycarbonyl) chemistry according to standard methods. Prior to cleavage from the resin, peptides were biotinylated at the amino terminus by incubation with a 1.5-fold molar excess of NHS-LC-biotin (Pierce) dissolved in dimethylacetamide containing 20% 4-dimethylaminopyridine to facilitate coupling. All peptides were >85% purity and were analyzed by electrospray mass spectroscopy (Protein Structure Facility, University of Michigan, Ann Arbor, MI). Peptide-aminoethylated molecular arrays of biotinylated peptides were finally dissolved in 10 mM sodium phosphate and 150 mM NaCl, pH 7.4. To assess the heparin binding properties of these biotinylated peptides, 96-well plates (Immulon-2, Dynatech Research Laboratories) were coated with 50 μl of 1 μg/ml BSA-heparin or BSA at 4°C overnight. After washing and blocking the wells with 1% BSA, 50 μl of the biotinylated peptide, ranging in concentration from 4 to 1000 μM, was added to the well and allowed to incubate for 60 min at 22°C. The
wells were rinsed, ABC reagent was added, and the binding of biotylinated peptide was quantitated in an ELISA reader at 405 nm.

RESULTS

Interaction of PECAM-1/IgG with Cells Correlates with Cell Surface Expression of PECAM-1—Previous studies have shown that phospholipid vesicles containing purified huPECAM-1 bind to huPECAM-1-transfected L cells but fail to interact with nontransfected L cells (14). Another high affinity PECAM-1 reagent, PECAM-1/IgG, exhibits the identical requirement for binding, in that cell surface PECAM-1 appears to be necessary to support binding (Fig. 1A). Together, these data suggest that L cells, by themselves, do not express a functional counter receptor for PECAM-1. To examine whether cellular targets for PECAM-1 might exist on cells of different lineages and having differing cell surface phenotypes, five other cell lines were tested for their ability to support binding of PECAM-1/IgG. Similar to what was observed in the L cell studies shown in Fig. 1A, U937, Dami, and HEL cells all supported PECAM-1/IgG binding in direct proportion to their endogenous cell surface expression of PECAM-1, while two Raji and A375 cells, which are both PECAM-1-negative, failed to support the binding of PECAM-1/IgG (Fig. 1B), even in the presence of Ca\(^{2+}\), Mg\(^{2+}\), or Mn\(^{2+}\) (not shown). These data support the notion that association of PECAM-1/IgG with the surface of multiple cell types is dependent on homophilic interactions with cell surface PECAM-1.

Cell Surface Glycosaminoglycans Are Incapable of Supporting PECAM-1 Binding—Previous studies have shown that both PECAM-1/IgG and PECAM-1-containing proteoliposomes associate with HUVECs in a PECAM-1 Ig domain 1- and 2-dependent manner (14). However, in addition to the nearly 1 \(\times\) 10\(^{6}\) molecules of PECAM-1 present on their cell surface (24), HUVECs also express 100,000 copies of the integrin \(\alpha_v\beta_3\) (25) as well as abundant levels of heparan sulfate and chondroitin sulfate (26, 27). \(\alpha_v\beta_3\) has previously been shown not to be involved in PECAM-1 proteoliposome (14) or PECAM-1/IgG (19) binding. To examine whether cell surface glycosaminoglycans contributed to the binding of PECAM-1 proteoliposomes or PECAM-1/IgG to HUVECs, each of these high affinity PECAM-1 reagents was added in the presence of exogenously added heparin or heparan sulfate. As shown in Fig. 3, srhuPECAM-1 failed to bind to the heparin-Sepharose beads only when the saline concentration was increased to 0.1 M NaCl (fractions 15–19) before the salt gradient was started. In contrast, TSP and PF4, two well characterized heparin-binding proteins, were retained by the same column and eluted in 0.6 and 1.8 M NaCl, respectively. Thus, even at salt concentrations below that of normal saline, the extracellular domain of PECAM-1 exhibits no capacity to bind heparin. In additional experiments (not shown), srhuPECAM-1 was incubated with heparin-Sepharose beads or Sepharose beads in 20 mM Tris buffer containing 150, 100, 50, 10, or 0 mM NaCl. Interaction with the beads was determined semiquantitatively by washing the beads in buffer having the same saline concentration as during the initial incubation, boiling the washed beads in SDS sample buffer, and analyzing the eluted proteins by SDS-polyacrylamide gel electrophoresis. We found that srhuPECAM-1 bound to the heparin-Sepharose beads only when the saline concentration dropped below 10 mM. Under these conditions, srhuPECAM-1 bound to native Sepharose beads as well. Finally, the association of srhuPECAM-1, TSP, and PF4 with
Heparin in solution was measured by incubating 50 μmol of the indicated protein with 30 μmol of 3H-labeled heparin, and the proteins were captured by immunoprecipitation using Protein A-Sepharose beads that had been presaturated with specific polyclonal antibodies. As shown in Fig. 4, [3H] heparin associated with both TSP and PF4 but failed to bind srhuPECAM-1. Together with the data shown in Fig. 3, these results suggest that there are no active or functional heparin-binding sites within the extracellular domain of PECAM-1.

Heparin Fails to Bind the Extracellular Domain of Immobilized Monovalent or Bivalent Human or Rat PECAM-1—The heparin binding properties of monovalent and bivalent recombinant forms of human PECAM-1, monovalent soluble recombinant rat PECAM-1, and monovalent, full-length PECAM-1 derived from human platelet membranes were compared in an ELISA assay by immobilizing these proteins in 96-well plates, adding biotinylated heparin-BSA, and detecting binding of heparin using avidin-biotin-conjugated horseradish peroxidase. As shown in Table I, heparin bound to immobilized PF4 but did not react with any of the immobilized forms of human or rat PECAM-1 that contained only the extracellular domain of PECAM-1. The addition of calcium to the wash or incubation buffers had no effect on heparin-BSA binding (not shown). Notably, heparin-BSA bound weakly but positively to full-length platelet PECAM-1, which differs from the other immo-
PECAM-1 Does Not Bind Heparin

The proteins indicated in the table headings were immobilized in 96-well plates (50 μl of a 1 μg/ml protein solution/well) at 4 °C overnight. After washing and blocking the wells with 1% BSA, biotinylated heparin-BSA or biotinylated BSA was added and allowed to incubate for 2 h at 22 °C. Binding was detected by the addition of the ABC reagent, and color development was quantitated in an ELISA reader at 405 nm. The relative efficiency of immobilization of bound target proteins was estimated by examining the binding of a biotinylated polyclonal rabbit antibody to rat PECAM-1, a murine mAb specific for human PECAM-1 (PECAM-1.1), and a mAb versus human IgG. The results shown represent the mean OD from six wells. Note that heparin-BSA fails to bind significantly to PECAM-1/IgG, soluble recombinant human or rat PECAM-1, or human platelet PECAM-1.

| Protein                | BSA  | Human platelet GP1b-IIIa | NHlgG | PF4 | Human PECAM-1/IgG | Human platelet PECAM-1 | srhuPECAM-1 | srRat PECAM-1 |
|------------------------|------|--------------------------|-------|-----|------------------|------------------------|-------------|--------------|
| Heparin-BSA            | 0.053| 0.045                    | 0.042 | 2.347| 0.059            | 0.139                  | 0.040       | 0.092        |
| BSA                    | 0.023| 0.024                    | 0.024 | 0.065| 0.024            | 0.027                  | 0.025       | 0.067        |
| Anti-rat PECAM-1       | 0.075| 0.052                    | 0.088 | 0.096| 0.029            | 0.439                  | 0.682       | 1.492        |
| Preimmune IgG          | 0.065| 0.038                    | 0.082 | 0.059| 0.073            | 0.039                  | 0.059       | 0.088        |
| mAb PECAM-1.1          | 0.032| 0.032                    | 0.060 | 0.035| 2.415            | 1.117                  | 1.941       | 0.059        |
| Anti-human IgG         | 0.030| 0.031                    | 2.065 | 0.043| 2.081            | 0.043                  | 0.038       | 0.014        |

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Artificially Created Short Amino Acid Sequences Present in Certain Chimeric PECAM-1/IgG Constructs, but Not in Native PECAM-1 Itself, Can Confer Artificial Heparin Binding Properties—During the course of our investigation, we created a number of PECAM-1/IgG constructs (shown schematically in Fig. 5A) using vectors that differed in the hinge region of IgG. One of these, designated PECAM-1/IgGRR, happens to juxtapose the COOH terminus of the PECAM-1 extracellular domain, LAPWKK, with the IgG Fc-derived hinge region sequence AAARASV, creating an artificial heparin-binding site. Thus, whereas both PECAM-1/IgG and PECAM-1/IgGHR bind PECAM-1-transfected L cells, PECAM-1/IgGRR also associates with nontransfected L cells (Fig. 5B). The interaction of PECAM-1/IgGRR with L cells appears to occur via cell surface glycosaminoglycans, since its binding can be totally abrogated with excess heparin (Fig. 5B). That the hinge region sequence of this chimeric protein is responsible for conferring glycosaminoglycan binding properties to the PECAM-1/IgGRR chimeric protein was demonstrated by examining the heparin binding properties of short biotinylated peptides corresponding to the hinge regions of PECAM-1/IgG or PECAM-1/IgGHR. As shown in Fig. 5C, a synthetic peptide containing the hinge region of PECAM-1/IgGHR was specifically captured by immobilized heparin-BSA, whereas a peptide of the same length corresponding to the hinge region of PECAM-1/IgG failed to interact with heparin-BSA. These data demonstrate that artifically created short amino acid sequences present in certain chimeric immunoadhesins are capable of conferring artificial heparin binding properties.

DISCUSSION

Although PECAM-1 was discovered as a novel membrane glycoprotein on the surface of platelets, leukocytes, and endothelial cells more than 10 years ago (28–30), its precise role in the biology of blood and vascular cells is still being defined. Recent studies have established a role for PECAM-1 in leukocyte transendothelial migration (2–9), in up-regulating integrin function (31–36), and in angiogenesis (10–12). Moreover, it has recently become apparent that, following cellular activation or engagement, the cytoplasmic domain of PECAM-1 may become tyrosine-phosphorylated (37–41), serving as a docking site for one or more cytosolic signaling molecules (42–45). In an effort to understand the molecular mechanisms underlying these events, we and others have been investigating the adhesive properties of PECAM-1 and have found that amino-termini of Ig domains 1 and 2 of PECAM-1 play a key role in mediating PECAM-1/PECAM-1 homophilic interactions (14–17, 19). In addition to PECAM-1 itself, however, a number of other cell surface components, including glycosaminoglycans (13, 21, 22), have been implicated as ligands for PECAM-1. The purpose of the present study, therefore, was to determine whether the extracellular domain of PECAM-1 contains one or more functional glycosaminoglycan-binding sites and whether cell surface glycosaminoglycans can serve as a biologically relevant target for PECAM-1-mediated cellular interactions.

To accomplish these aims, we examined the glycosaminoglycan binding properties of multiple mono- and multivalent forms of PECAM-1. Our results may be summarized as follows. First, the interaction with cells of an appropriately constructed bivalent PECAM-1/IgG chimeric protein is strictly dependent on cell surface expression of PECAM-1 (Fig. 1), and exogenously added glycosaminoglycans such as heparin and heparin sulfate have no effect on the ability of PECAM-1/IgG or PECAM-1 proteoliposomes to interact with the cell surface (Fig. 2A). Second, even in cells rich in cell surface glycosaminoglycans and integrins, PECAM-1 appears to be the only functional cellular target for PECAM-1, since the interaction of PECAM-1/IgG with the endothelial cell surface could be completely inhibited by preincubating the cells with small Fab fragments specific for PECAM-1 Ig homology domain 1 (Fig. 2B). Third, independent of either the source of PECAM-1 or the way in which heparin is presented, the extracellular domain of PECAM-1 is unable to interact directly with this glycosaminoglycan (Figs. 3 and 4, and Table I). Finally, we found that amino acid sequences contributed by certain Ig fusion vectors can, in some instances, result in the creation of artificial heparin-binding sites that are normally not present in the native molecule (Fig. 5).

Several other groups have examined the association of heparin with various cellular and chimeric forms of PECAM-1 and arrived at somewhat different conclusions from those drawn in the present work. Watt et al. (46) examined the retention on heparin-Sepharose beads of full-length PECAM-1 derived from detergent cell lysates and found that approximately 20% of the PECAM-1 remained bound to the beads after washing with normal saline. However, even the small proportion of PECAM-1 that remained bound to the heparin-Sepharose beads...
was found to be easily dissociable. Based upon their observations, they concluded that PECAM-1 exhibited weak, but measurable, low affinity interactions with heparin. To more specifically localize the site on full-length PECAM-1 responsible for this weak association, we compared the heparin binding properties of srhuPECAM-1, a recombinant protein containing only amino acid residues 1–574 (i.e. the entire extracellular domain of PECAM-1 containing Ig-homology domains 1–6), with that of full-length PECAM-1 derived from human platelet membranes. In physiological saline, srhuPECAM-1 exhibited no affinity for heparin immobilized on Sepharose beads (Fig. 3), for heparin in solution (Fig. 4), or for heparin conjugated to BSA (Table I). However, similar to Watt et al., we did observe a weak interaction of heparin with full-length PECAM-1 purified from cellular detergent lysates (Table I). These data suggest that the heparin-binding site on PECAM-1 lies not within the extracellular domain but rather within either the transmembrane or cytoplasmic domain of the molecule. In this regard, it is notable that there are a series of positively charged, basic amino acids immediately following the transmembrane domain of PECAM-1, having the sequence RKAKAK (residues 599–604), that confer weak heparin binding properties to the protein independent of those that might be present within the extracellular domain. In light of these findings, we suspect that the ability of full-length cell-derived PECAM-1 to bind to heparin-Sepharose (Ref. 46 and Table I) is probably due to the association of cytoplasmic domain residues 599–604 with heparin. This interaction is likely to be of little or no functional consequence in PECAM-1-mediated cellular interactions, since heparin-binding sequences not present in the extracellular domain of the molecule would obviously be unavailable to mediate heterophilic interactions between cells.

An important aspect of the present work is the finding that short amino acid sequences encoded by certain PECAM-1/IgG cDNA constructs, but not by others, can confer artifactual heparin binding properties to the chimeric protein. Margalit et al. (47) have recently shown, using three-dimensional computer graphic techniques, that heparin binding sites within proteins are dependent upon electrostatic interactions that result from topologically close basic amino acid residues. Thus, PECAM-1/IgG RR, but not PECAM-1/IgG, contains the sequence KKAARR within its hinge region (Fig. 5A), and this motif is by itself able to contribute significantly to the glycosaminoglycan-binding ability of proteins and polypeptides that contain it (Figs. 5, B and C). In fact, PECAM-1/IgG RR binds a variety of PECAM-1-positive and PECAM-1-negative cell lines in a heparin-inhibitable manner. By themselves, these observations would have led us to believe that PECAM-1 is a heparin-binding protein and that cell surface glycosaminoglycans represent a functional target for PECAM-1 on the cell surface. However, our discovery that simple deletion of the KKAARR sequence contributed by the COOH terminus of the extracellular domain of PECAM-1, creates an artificial glycosaminoglycan-binding motif having the sequence KKAARR, B, PECAM-1/IgG RR binds avidly non-transfected L cell fibroblasts, whereas PECAM-1/IgG, which lacks these amino acid residues, does not exhibit heterophilic binding properties. Note that the heterophilic binding of PECAM-1/IgG RR to L cells can be totally abrogated by the addition of exogenous heparin, demonstrating that interaction of PECAM-1/IgG RR with nontransfected L cell operates via cell surface glycosaminoglycans. C, ELISA quantitating the dose-dependent binding of biotinylated synthetic peptides corresponding to the hinge region of PECAM-1/IgG RR (peptide 1) or PECAM-1/IgG (peptide 2) to immobilized heparin-BSA. Note that the sequence motif KKAARR, present in both PECAM-1/IgG RR and in peptide 1, confers heparin binding properties not normally present in the native molecule.

Fig. 5. Artifactual binding of PECAM-1/IgG to cell surface glycosaminoglycans results from sequences uniquely present in certain IgG chimeric proteins. A, schematic representation of two variant constructs of PECAM-1/IgG. PECAM-1/IgG RR differs from PECAM-1/IgG in the hinge region such that it contains five additional amino acids, RRASV, which, together with the LAPWKR sequence contributed by the COOH terminus of the extracellular domain of PECAM-1, creates an artificial glycosaminoglycan-binding motif having the sequence KKAARR. B, PECAM-1/IgG RR binds avidly non-transfected L cell fibroblasts, whereas PECAM-1/IgG, which lacks these amino acid residues, does not exhibit heterophilic binding properties. Note that the heterophilic binding of PECAM-1/IgG RR to L cells can be totally abrogated by the addition of exogenous heparin, demonstrating that interaction of PECAM-1/IgG RR with nontransfected L cell operates via cell surface glycosaminoglycans. C, ELISA quantitating the dose-dependent binding of biotinylated synthetic peptides corresponding to the hinge region of PECAM-1/IgG RR (peptide 1) or PECAM-1/IgG (peptide 2) to immobilized heparin-BSA. Note that the sequence motif KKAARR, present in both PECAM-1/IgG RR and in peptide 1, confers heparin binding properties not normally present in the native molecule.
motif totally eliminates the ability of PECAM-1-IgG to bind to PECAM-1-negative cells argues strongly against this possibility. Thus, we speculate that the 6–30% observed binding to heparin of the particular PECAM-1-IgG construct used by Watt et al. (Fig. 8 of Ref. 46) may similarly be attributable, at least in part, to sequence motifs unknowingly introduced into the hinge region of their chimeric protein that are normally not present within in the extracellular domain of cellular PECAM-1. The presence of similar artificially juxtaposed amino acid residues might also explain the findings of Prager et al. (48), who observed heparin-inhibitable binding to T cells of their form of PECAM-1-IgG.

The artifactual binding of PECAM-1-IgGRR to cells, independent of their expression of the natural ligand for PECAM-1, may not be limited to PECAM-1-based immunoadhesins. There appears, in the immunoadhesin literature in general, to have been little consideration given to the possibility that the adhesive properties of IgG fusion proteins may be affected by a combination of sequence motifs, some of which are not normally present in the native molecule. For example, in addition to sequences that may be unknowingly introduced into the hinge regions of chimeric proteins, many immunoadhesin cDNA expression vectors encode a polyhistidine tag at the carboxyl terminus to simplify recovery and purification. Unfortunately, clusters of positively charged histidine residues are also able to contribute to the overall affinity of proteins for cell surface glycosaminoglycans (49). Thus, while hinge region sequences or polyhistidine tags by themselves may be unable to confer high affinity heparin binding characteristics, when expressed together with low affinity sites present within the native protein, they may be able to significantly alter the overall cell binding properties of the chimeric protein.

Previous results from our laboratory and others have led to the hypothesis that the six-amino acid sequence, LKREKN, of the native protein, they may be able to significantly alter the expressed together with low affinity sites present within the extracellular domain of cellular PECAM-1. The presence of similar artificially juxtaposed amino acid residues might also explain the findings of Prager et al. (48), who observed heparin-inhibitable binding to T cells of their form of PECAM-1-IgG.

The artifactual binding of PECAM-1-IgGRR to cells, independent of their expression of the natural ligand for PECAM-1, may not be limited to PECAM-1-based immunoadhesins. There appears, in the immunoadhesin literature in general, to have been little consideration given to the possibility that the adhesive properties of IgG fusion proteins may be affected by a combination of sequence motifs, some of which are not normally present in the native molecule. For example, in addition to sequences that may be unknowingly introduced into the hinge regions of chimeric proteins, many immunoadhesin cDNA expression vectors encode a polyhistidine tag at the carboxyl terminus to simplify recovery and purification. Unfortunately, clusters of positively charged histidine residues are also able to contribute to the overall affinity of proteins for cell surface glycosaminoglycans (49). Thus, while hinge region sequences or polyhistidine tags by themselves may be unable to confer high affinity heparin binding characteristics, when expressed together with low affinity sites present within the native protein, they may be able to significantly alter the overall cell binding properties of the chimeric protein.

Previous results from our laboratory and others have led to the hypothesis that the six-amino acid sequence, LKREKN, located at amino acid residues 150–155 within extracellular Ig domain 2, might function as a heparin binding site (13). This hypothesis was based on the similarity of this sequence with a known glycosaminoglycan binding motif having the sequence $X^2\text{X}^3\text{B}^2\text{B}^{-1}\text{X}^1\text{B}^1\text{X}^{-2}\text{X}^{-3}$, where $B$ is a basic amino acid and $X$ is hydropathic residue (20) and on the ability of both heparin and the LKREKN peptide to inhibit the in vitro association of PECAM-1-transfected L cells with nontransfected L cells (22). There are, however, a number of theoretical problems and recent observations that cast doubt on the ability of LKREKN to serve as a functional heparin binding domain. First, the reverse peptide, NKERKL, which does not contain the XBXXBX motif, was found to be equally inhibitory in the L cell aggregation assay, and neither the forward nor the reverse peptide exhibited significant inhibitory effects except at a relatively high concentration (>0.5 mM) (22). Second, as shown in Table II, it has only recently become appreciated that neither the LKREKN sequence nor the XBXXBX motif are particularly well conserved evolutionarily in PECAM-1; in rat PECAM-1, the −2-position is occupied by a serine rather than the obligatorily Lys or Arg residue, and the basic residue normally found at −1 is occupied in murine PECAM-1 by an isoleucine. In fact, a perfectly conserved glutamic acid residue occupies the normally hydrophobic +1-position in all PECAM-1 species examined to date, and negatively charged residues are specifically excluded from functional glycosaminoglycan-binding motifs because they have the potential to significantly alter the charge complementation of this region necessary to effect its interaction with negatively charged heparin (20). Third, Piali et al. (50) have recently found that the adhesion of murine lymphokine-activated killer cells to an immobilized chimeric protein composed of murine PECAM-1 Ig domains 1–3 fused to the mouse IgG constant region could be inhibited by anti-PECAM-1 antibodies but was not inhibitable by heparin at concentrations as high as 300 μg/ml (50). Fourth, neither heparin (100 μg/ml) nor the LKREKN peptide (1 mg/ml) had any effect on the adhesion of U937 cells to immobilized full-length PECAM-1/ IgG (51). Finally, neither leukocyte transendothelial migration nor endothelial cell tube formation, each of which are thought to involve PECAM-1, is inhibited by heparin (10, 17). Taken together with the fact that heparin has no affinity for synthetic peptides containing the LKREKN sequence, these data suggest that the mechanism by which heparin is able to inhibit the interaction of PECAM-1-transfected L cells would appear to be independent of the direct glycosaminoglycan binding properties of PECAM-1 itself and probably operates by inhibiting secondary adhesive interactions that occur downstream of PECAM-1 (1). Identification of the range of cellular and molecular events that emanate from PECAM-1 engagement remains an important avenue of future investigation.

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