Biosynthesis and Processing of the Cell Adhesion Molecule PECAM-1 Includes Production of a Soluble Form*

(Received for publication, December 23, 1993, and in revised form, April 1, 1994)

Amy Goldberger†, Kelly A. Middleton‡, Julie A. Oliver*, Cathy Paddock‡, Horng-Chin Yan$, Horace M. DeLisser$, Steven M. Albeida§, and Peter J. Newman¶

From the †Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, Wisconsin 53233, the §Department of Medicine, University of Pennsylvania Medical Center and The Wistar Institute, Philadelphia, Pennsylvania 19104, and the ¶Departments of Cellular Biology and Pharmacology, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226.

PECAM-1 (CD31) is a 130-kDa glycoprotein found on platelets, endothelial cells, granulocytes, and monocytes, as well as on certain myelomonocytic cell lines. Recent studies have shown that PECAM-1 may be involved in activation of leukocyte integrins and may also be involved in adhesive interactions of circulating leukocytes and the vessel wall. In spite of the important functional role that PECAM-1 plays in these processes, little is known about the biosynthesis, processing, and turnover of PECAM-1 on the cell surface. We have studied the biosynthesis of PECAM-1 in the promonocytic cell line U937, and in endothelial cells, by pulse-chase labeling and immunoprecipitation. PECAM-1 was synthesized as a 110-kDa precursor form, which was processed into the 130-kDa mature form within 1–3 h, during which time it began to move to the cell surface. The protein disappeared from the cell surface in both cell types about 48 h after labeling. A soluble form of PECAM-1, which is 5–10 kDa smaller than cell-associated PECAM-1 and contains the cytoplasmic tail, was observed in the culture media of HUVECs and phorbol ester-treated U937 cells. This form of soluble PECAM-1 is encoded by an alternatively spliced mRNA from which the exon containing the transmembrane domain has been removed. Soluble PECAM-1 was also detected in normal human plasma at levels of 10–25 ng/ml. Two isoforms of plasma PECAM-1, which differed in the presence of the cytoplasmic tail, were observed by Western blot analysis. In parallel with soluble forms of other cell adhesion molecules, soluble PECAM-1 may play a role in modulating the inflammatory response.

PECAM-1* (Platelet-Endothelial Cell Adhesion Molecule-1) is a 130-kDa member of the immunoglobulin gene superfamily

* This investigation was supported by Grant-in-Aid 91-GA-45 from the Wisconsin Affiliate of the American Heart Association (to A. G.), National Institutes of Health Grant HL-07902 (to J. A. O.), the Robert Wood Johnson Foundation Minority Faculty Program (to H. M. D.), and Grants HL-40926 (to J. P. N.) and HL-43611 (to S. M. A.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Established Investigators of the American Heart Association.

‡ The abbreviations used are: PECAM-1, platelet/endothelial cell adhesion molecule 1; sPECAM-1, soluble PECAM-1 produced by cultured cells; rPECAM-1, soluble recombinant PECAM-1 containing only the extracellular domain; ACT, polyclonal antibody against the cytoplasmic tail of PECAM-1; CHO cells, Chinese hamster ovary cells; D-PBS, Dulbecco’s phosphate-buffered saline; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing 0.05% Tween 20.
cell, its half-life on the cell surface, or potential alternative forms of the molecule that could modulate the process of leukocyte transmigration. Changes in the rate of synthesis or turnover, for example, could affect the stability or integrity of the endothelial cell barrier. In this report, we examine the biosynthesis and processing of PECAM-1 in a leukocyte model, as well as in endothelial cells. Our results provide evidence that the half-life of PECAM-1 on the endothelial cell surface is surprisingly short and demonstrate the existence of a circulating form of soluble PECAM-1 that may function to regulate PECAM-1-mediated cellular interactions.

**EXPERIMENTAL PROCEDURES**

**Cells**—The U937 and HEL cell lines were obtained from the American Type Culture Collection and were maintained in RPMI 1640 media (Life Technologies, Inc.) containing 10% heat-inactivated FCS (HyClone Laboratories) and 40 μg/ml gentamycin (Sigma). Human umbilical vein endothelial cells (HUVECs) were prepared and cultured as previously described (23).

**Pulse-chase Labeling**—Leukemia cell lines were treated with 16 μCi/ml [35S]methionine for 16 h, after which the culture media were removed and the cells were washed with cold D-PBS. The cells were then divided into two aliquots—one which was gel-permeated after 10-min boiling in 80 μl of SDS sample buffer containing 5% 2-mercaptoethanol, 100 mM DTT, 25 mM HEPES, pH 7.4, 10% glycerol, and 1% SDS, the other which was gel-permeated after boiling for 5 min in 10 pl of 0.5% SDS, 1% 2-mercaptoethanol. The cells were then washed with cold D-PBS/2 mM phenylmethylsulfonyl fluoride and lysed by sonication into the cold lysis buffer. The lysates were incubated for 20 min on ice with mixing and centrifuged at 100,000 × g for 15 min at 4 °C. All aliquots of the supernatants were precipitated with a cold solution of 1 M perchloric acid to remove any labeled inorganic phosphate and to effect a rapid removal of labeled AMP, GMP, or ATP. Following centrifugation, the supernatant was decanted and the precipitates were washed and resuspended in 100 mM Tris-HCl, pH 7.4, 10% glycerol, and 1% SDS. The samples were incubated for 10 min at 60 °C with mixing and were then added to the cold lysis buffer. The samples were then centrifuged at 100,000 × g for 1 h to remove any insoluble material. The supernatants were separated on 7% polyacrylamide gels followed by fluorography.

**Preparation of Cell Culture Media for Detection of Soluble PECAM-1**—Media were removed from the cells after the various chase times. Following low speed centrifugation, the media were concentrated 2–3-fold using a Centricron 30 (Amicon). The supernatants were processed through a 0.2-μm filter and aliquoted at 100,000 x g for 3 h at 4 °C. The aliquots were stored at −70 °C prior to immunoprecipitation analysis. HUVECs were labeled and processed as above.

**Immunoprecipitation of PECAM-1**—Equal volumes of lysate (about 10% of the total) and all of the culture media from each time point of a pulse/chase experiment were immunoprecipitated. Samples were pre-cleared by addition of 10 μg of normal rabbit IgG (Sigma) and 50 μl of a 10% slurry of ProteiNase (Calbiochem) at 4 °C for 1 h. After removal of the ProteiNase, the supernatants were incubated with 10 μg of polyclonal anti-PECAM-1 IgG or 0.02% NaN3 for 2 h at 4 °C. The cells were then washed extensively with ice-cold D-PBS containing 10% FCS and lysed as described above.

**Preparation of Cell Culture Media for Detection of Soluble PECAM-1**—Media were removed from the cells after the various times. Following low speed centrifugation, the media were concentrated 2–3-fold using a Centricron 30 (Amicon). The supernatants were processed through a 0.2-μm filter and aliquoted at 100,000 x g for 3 h at 4 °C. The aliquots were stored at −70 °C prior to immunoprecipitation analysis. HUVECs were labeled and processed as above.

**Immunoprecipitation of PECAM-1**—Equal volumes of lysate (about 10% of the total) and all of the culture media from each time point of a pulse/chase experiment were immunoprecipitated. Samples were pre-cleared by addition of 10 μg of normal rabbit IgG (Sigma) and 50 μl of a 10% slurry of ProteiNase (Calbiochem) at 4 °C for 1 h. After removal of the ProteiNase, the supernatants were incubated with 10 μg of polyclonal anti-PECAM-1 IgG or 0.02% NaN3 for 2 h at 4 °C. Immune complexes were collected by addition of 50 μl of a 50% slurry of protein A-Sepharose (Sigma) for 2 h at 4 °C, followed by centrifugation and careful washing. Lysates of cells which were incubated with anti-PECAM-1 antibodies prior to lysis to detect cell-surface PECAM-1 were immunoprecipitated by the addition of protein A-Sepharose without out pre-clearing. The samples were prepared for gel electrophoresis by boiling for 5 min in 80 μl of SDS sample buffer containing 5% 2-mercaptoethanol, and the supernatants were separated on 7% polyacrylamide gels followed by fluorography.

**Glycosidase Treatment of PECAM-1**—Samples were immunoprecipitated as described above. PECAM-1 was eluted from the beads and treated with several glycosidases essentially as described by Johnston et al. (24). PECAM-1 isolated from protein A-Sepharose beads was treated with 50 milliunits/ml of endo-β-N-acetylglucosaminidase (endo H; Boehringer Mannheim) for 16 h at 37 °C. For treatment with N-glycosidase F (N-glycanase; Genzyme), the beads were washed and boiled for 5 min in 10 μl of 0.5% SDS, 1% 2-mercaptoethanol. The volumes were adjusted to final detergent concentrations of 0.1% SDS and 1.25% Triton X-100 and incubated for 5 h at 37 °C in the presence of 15 units/ml N-glycanase. Samples treated with neuraminidase (Gen-}

17184 Biosynthesis and Processing of PECAM-1
recombinant protein was expressed in E. coli strain S13009 as previously described (29) and purified using a nickel-chelate affinity resin (Ni²⁺-NTA agarose, Qiagen) (30, 31), which bound tightly to the histidine residues. Purified recombinant protein was then injected subcutaneously into a rabbit at 14-day intervals, followed by bleeding 10 days after the third injection. Immunoprecipitation of [³⁵S]methionine-labeled, cell surface biotinylated protein extracts from platelets, endothelial cells, U937 cells, and 3T3 cell lines transfected with full-length PECAM-1 cDNA or with mutant constructs missing the entire cytoplasmic domain confirmed the specificity of the antiserum for the cytoplasmic tail of PECAM-1.

Identification of the Alternatively Spliced RNA Species Encoding Soluble PECAM-1—RNA was extracted from HUVECs and from both untreated and PMA-treated HEL cells by the method of Chomczynski and Sacchi (32). The synthesis of cDNA was primed from 1 μg of RNA using random hexamers (Pharmacia Biotech, Inc.) in the presence of Moloney murine leukemia virus reverse transcriptase (Life Technologies). The primary PCR was performed using the sense primer a (see Fig. 6A) from base 1754 to 1773 (5'-TCGTCAGGTGAAGACT-3'), and the antisense primer c (5'-TCGTCAGGTGAAGACT-3'), which extends from base 2077 to 2090. The primary PCR reaction was then diluted 1:10 and reamplified in a nested PCR using primers b and c (base 1865–1882; 5'-TCACACAGGGCGACACCG-3'). One-half of the PCR product after electrophoresis was separated on a gel, blotted to a nylon membrane (GeneScreen Plus, DuPont NEN), and UV-cross-linked. The blot was then hybridized to a [³²P]-labeled PECA-1 cDNA probe, washed to high stringency (0.1× SSC, 0.1% SDS, 68 °C), and exposed to Kodak XAR film at -70 °C with an intensifying screen. The 215-bp product was obtained by preparative agarose gel electrophoresis of the entire 1st PCR reaction, followed by isolation using GeneClean (Bio 101). The ends of the purified PCR product were flanked using the Klenow fragment of DNA polymerase, followed by ligation into pGEM-5Zf (Promega) that had been cut with EcoRV to yield blunt ends. Three clones generated from HUVEC RNA and 3 clones from HEL cell RNA were sequenced using (U. S. Biochemical Corp.).

Detection of PECAM-1 in Normal Human Plasma—Plasma from cells trated whole blood was ultracentrifuged at 100,000 × g for 3 h and processed through a 0.2-μm filter to remove debris and membrane fragments. Plasma PECAM-1 was absorbed out of some plasma samples using monoclonal and polyclonal anti-PECAM-1 antibodies which had been coupled at a ratio of 4 mg of IgG/ml to cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer's instructions. Parallel plasma samples were incubated with preimmune rabbit and mouse IgG-Sepharose beads, as well as an anti-GPllia antibody (AP-3) coupled to Sepharose. Complete absorption was achieved by incubating plasma samples with 25 μl of antibody-coupled Sepharose beads at 4 °C for 16 h with mixing.

PECAM-1 in plasma was measured using an antigen-capture ELISA. A rabbit polyclonal antibody directed against PECAM-1, diluted to 25 μg/ml as IgG in carbonate buffer, pH 9.5, was adsorbed onto flat-bottomed microtiter plates (Immulon 2, Dynatech Laboratories) for 4 h at 4 °C. The wells were rinsed with TBS containing 0.05% Tween 20 (TBS-T) and blocked with 2% bovine serum albumin in TBS-T for 1 h at 22 °C. Plasma samples (100 μl) processed as described above, were added to triplicate wells and incubated for 1 h at 37 °C. Plasma PECAM-1 was quantitated by comparison to a standard curve of purified sPECAM-1 serially diluted in plasma which had been depleted of anti-PECAM-1 antibodies, followed by goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson Laboratories). Finally, the blots were incubated with chemiluminescent substrate (ECL kit, Amersham) and exposed to film (Hyperfilm-ECL, Amersham).

Cell Aggregation Assay—Aggregation of PECAM-1-transfected L-cells was performed as previously described (9, 13). The ability of recombinant soluble PECAM-1 to block the interaction was determined by incubating aliquots of the cell suspension with the indicated amounts of FPLC-purified protein during the aggregation assay.

RESULTS

Kinetics of PECAM-1 Synthesis and Cell Surface Expression—The ability to induce de novo synthesis in PMA-treated U937 cells (6) allowed the development of an attractive model system for studying the biosynthesis and processing of PECAM-1 in leukocyte precursors. To determine the kinetics of PECAM-1 synthesis, cells were treated with PMA for 2 days to up-regulate PECAM-1 expression and were then metabolically labeled with [³⁵S]methionine for different pulse and chase times. The results of such an experiment are shown in Fig. 1. When U937 cells were labeled for 20 min and chased for 20 min, a pre-PECAM-1 species with Mᵣ = 110,000 became extensively labeled (Fig. 1A, left panel). At 60 min of chase, some label began to appear in the mature form of PECAM-1 (Mᵣ = 130,000). After a 3-h chase, most of the PECAM-1 detected was in the mature form. To determine the stability of the newly synthesized protein, the cells were pulsed for 1 h and then chased for longer periods of time (Fig. 1B, left panel). PECAM-1 remained present within the cell for 24 h, after which levels of labeled protein dropped significantly.

To detect the appearance of PECAM-1 on the cell surface, labeled U937 cells were incubated with anti-PECAM-1 antibodies prior to cell lysis. Antibody incubation was performed at 4 °C in the presence of sodium azide to prevent internalization. Following extensive washing, the cells were lysed and immune complexes were collected with protein A-Sepharose. As shown in Fig. 1A (right panel), mature PECAM-1 reaches the surface
of U937 cells about 3 h after a 20-min pulse, similar to the time required for its maturation within the cell. To observe the stability of PECAM-1 on the cell surface, cells were labeled for 1 h and chased for longer time periods. As shown in the right panel of Fig. 1B, PECAM-1 was strongly expressed on the cell surface up to 24 h postlabeling, but disappeared completely from the cell surface within 48 h.

The kinetics of PECAM-1 expression was also determined in vascular endothelial cells. Confluent monolayers of HUVECs were labeled with [35S]methionine for 20 min or 1 h and then chased for various times. Pre-PECAM-1 was detected in HUVECs within 20 min, and significant levels of the mature form appeared within 1 h after labeling (Fig. 2A). Mature PECAM-1 persisted in the cells for 48 h postsynthesis, after which most of the protein disappeared. Detection of PECAM-1 on the cell surface of HUVECs (Fig. 2B) indicated that most of the newly synthesized protein had disappeared from the cell surface within 2 days, similar to that observed in the myeloid U937 cells.

Analysis of PECAM-1 Glycosylation—Although there are 9 potential sites for N-linked carbohydrate modification in the amino acid sequence of PECAM-1 (1), the precise glycosylation pattern has not yet been determined. Similar to other membrane glycoproteins, however, the "pre-PECAM-1" protein detected early in its biogenesis (Figs. 1 and 2) likely represents a precursor containing high-mannose carbohydrate moieties which may be further processed into complex forms in the Golgi apparatus, resulting in the mature form of PECAM-1. To analyze the glycosylation pattern of PECAM-1, U937 cells were labeled for 20 min and chased for 1 h to obtain approximately equal amounts of pre-PECAM-1 and mature PECAM-1 (Fig. 3, lane 1). Detergent lysates were prepared, immunoprecipitated with anti-PECAM-1 antibody, and finally treated with several glycosidases. As shown in Fig. 3 (lane 2), only pre-PECAM-1 was sensitive to endo H, which selectively removes high mannose residues. When the immunoprecipitates were treated with N-glycanase, which cleaves most forms of N-linked carbohydrates, both pre- and mature PECAM-1 decreased in M, to about 100,000 (Fig. 3, lane 4). Further characterization of the complex carbohydrate moieties of PECAM-1 by evaluating sensitivity to neuraminidase treatment indicated that the mobility of mature PECAM-1, but not pre-PECAM-1, was increased by neuraminidase (Fig. 3, lanes 5 and 6). Treatment of PECAM-1 with neuraminidase followed by O-glycanase did not increase the mobility of PECAM-1 any more than neuraminidase alone (Fig. 3, lanes 5–8). This observation was confirmed by treatment with N-glycanase, neuraminidase, and O-glycanase (Fig. 3, lanes 9 and 10). PECAM-1 from HEL cells and HUVECs gave identical patterns of sensitivity to glycosidase treatment (not shown). Chemical deglycosylation of PECAM-1 with trifluoromethanesulfonic acid did not result in any further change in the mobility of PECAM-1 (not shown), suggesting that enzymatic deglycosylation had been complete.

Myeloid Cell Lines and HUVECs Secrete a Soluble Form of PECAM-1 Which Contains the Cytoplasmic Tail—Recently, several laboratories have reported the existence of soluble, biologically active, forms of L-selectin (33), ICAM-1 (34, 35), and P-selectin (36). In order to examine whether a soluble form of PECAM-1 might be synthesized by either endothelial or myeloid cells in culture, U937 cells were labeled with [35S]methionine for 1 h and chased for various lengths of time. Culture media from these cells were processed through a 0.2-μm filter and centrifuged at 100,000 × g for 3 h, and the top 1/2 to 3/4 of the supernatant was carefully removed for immunoprecipitation analysis using antibodies specific for the extracellular domain of PECAM-1. As shown in Fig. 4, soluble PECAM-1 could be detected at low levels in the culture media within 3 h after labeling and continued to accumulate in the media for 2 days. The soluble form migrated slightly more rapidly than the membrane-associated form. A soluble form of PECAM-1 was also observed in the culture media of HUVECs (not shown) and migrated slightly more rapidly than the corresponding cell-associated form. When the spun and filtered culture media were phase-extracted with Triton X-114 (37, 38), sPECAM-1 partitioned completely into the aqueous phase (data not shown), suggesting that the soluble form of PECAM-1 secreted into the media of cultured cells does not appear to contain a transmembrane domain.

Two possible mechanisms could lead to the appearance of a soluble form of PECAM-1 in the culture media (Fig. 5A). First, alternative splicing out of the exon encoding the transmem-
PECAM-1, collected at each time point, processed as described under “Experimental Procedures,” and immunoprecipitated with a PECAM-1-specific polyclonal antibody. The “culture media” lanes were exposed to x-ray film twice as long as the “cell lysates” lanes.

Cell Lysates
Cellular PECAM-1
Soluble PECAM-1

Culture Media

Fig. 4. Kinetics of sPECAM-1 secretion into the culture media of PMA-treated U937 cells. Cells were labeled for 1 h and chased for the indicated periods of time (in hours). The culture media were collected at each time point, processed as described under “Experimental Procedures,” and immunoprecipitated with a PECAM-1-specific polyclonal antibody. The “culture media” lanes were exposed to x-ray film twice as long as the “cell lysates” lanes.

Fig. 5. Soluble PECAM-1 retains the cytoplasmic tail. A, schematic representation of the predicted reactivity of the cytoplasmic tail-specific antibody, ACT, with different molecular species of PECAM-1. B, specificity of the ACT antibody. Cellular lysates prepared from 35S-labeled U937 cells (lanes 1 and 2) and cell culture media derived from CHO cells transfected with a PECAM-1 construct encoding residues 1–574 (srPECAM-1) (lanes 3 and 4) were immunoprecipitated with an anti-PECAM-1 polyclonal antibody (lanes 1 and 3) or with ACT (lanes 2 and 4). Note the specificity of ACT for PECAM-1 molecules that contain the cytoplasmic tail and its inability to react with the truncated recombinant form of PECAM-1. C, reactivity of soluble PECAM-1 with ACT. Culture media from 35S-labeled HUVECs was processed as described above and immunoprecipitated with normal rabbit IgG (lane 1), anti-PECAM-1 (lane 2), or with ACT (lane 3). The soluble cell culture isoform retains the cytoplasmic tail, as indicated by its strong reactivity with the ACT antibody.

bran domain would result in a soluble form of PECAM-1 which retains the 118-amino acid cytoplasmic tail. Second, proteolysis from the cell surface could cause release of a smaller, truncated form of PECAM-1 lacking both the transmembrane domain and the cytoplasmic tail. To distinguish between these 2 possibilities, we employed a polyclonal antibody that had been raised against the cytoplasmic domain of PECAM-1. This antibody, anti-cytoplasmic tail (ACT), should react with the membrane-bound as well as soluble transmembraneless PECAM-1, but not with truncated PECAM-1 (Fig. 5A). We found that ACT bound full-length PECAM-1 in detergent lysates of U937 cells (Fig. 5B, lane 2), but not a truncated form of PECAM-1 derived from the culture media of CHO cells transfected with a PECAM-1 construct encoding residues 1–574 (srPECAM-1) (Fig. 5B, lane 4), as predicted. None of the many other labeled proteins which are present in the CHO cell culture media were recognized by ACT, demonstrating its specificity. To determine whether the cytoplasmic tail is present on the soluble form of PECAM-1 found in conditioned media, culture media from HUVECs were immunoprecipitated with ACT. As shown in Fig. 5C, the soluble form of PECAM-1 secreted from cells was recognized as efficiently by ACT as by an anti-PECAM-1 polyclonal antibody directed against the extracellular domain of the molecule, indicating that the cytoplasmic tail is retained in the soluble form of PECAM-1. Similar results were obtained with culture media from PMA-treated U937 cells (not shown).

6A).

Fig. 6. Identification of an alternatively spliced PECAM-1 mRNA transcript. A, following reverse transcription of HUVEC mRNA, PCR amplification was performed using primer pair a + c (1' reaction) followed by primer pair b + c (2' reaction), which encompass the transmembrane domain (TM) of the PECAM-1 mRNA transcript. B, Southern blot analysis of the PCR products. Ten percent of the indicated PCR reactions were separated on a 1.9% agarose gel, blotted onto a nylon membrane, and hybridized with a 32P-labeled PECAM-1 cDNA probe. Note the presence of PCR products 108 bp smaller than the predicted size of the full-length product. C, sequencing of the alternatively spliced species. The 215-bp PCR product of primer pair a + c was eluted from a preparative agarose gel, subcloned, and subjected to nucleotide sequence analysis. The gel shows the sequence of the antisense strand of the cDNA synthesized from HUVEC RNA and demonstrates the deletion of exon 9, which encodes the transmembrane domain. The arrow indicates the splice junction between exons 8 and 10.

Detection of an Alternatively Spliced mRNA Species Locking the Exon Encoding the Transmembrane Domain—The presence in cell culture media of a soluble form of PECAM-1 containing an intact cytoplasmic tail is consistent with the possibility that alternative splicing of the exon encoding the transmembrane domain leads to secretion of a subpopulation of PECAM-1 from the cells. Characterization of the PECAM-1 gene in our laboratory2 has shown that the transmembrane domain is encoded by an exon of 108 base pairs. To determine whether an alternatively spliced species lacking the exon encoding the transmembrane domain might exist, RNA from HUVECs was reverse-transcribed with random hexamers and PCR-amplified using primer pairs which flank the exon encoding the transmembrane domain (Fig. 6A). DNA species of 323 bp for full-length and 215 bp for transmembraneless PECAM-1 were predicted for the primer pair a + c, while 212- and 104-bp species

2 N. Kirschbaum, R. J. Gumina, and P. J. Newman, submitted for publication.
were predicted for full-length and transmembraneless PECAM-1, respectively, from primer pair b + c. The PCR products were separated by agarose gel electrophoresis, Southern-blotted, and probed with a $^{32}$P-labeled PECAM-1 cDNA probe (Fig. 6B). All 4 predicted bands were detected, with the 215- and 104-bp species present at an estimated 1–5% of total transcript levels. To show conclusively that the exon encoding the transmembrane domain had been spliced out, the 215-bp fragment was gel-purified, subcloned, and sequenced. As shown in Fig. 6C, the sequence contained the predicted splice junction of exon 8 (..AGTCTC), encoding Ig loop 6, immediately followed by exon 10 (..GTTGTC), which encodes the first exon of the cytoplasmic tail. Several clones of the 215-bp PCR products derived from HUVECs and from PMA-treated HEL cells contained the same sequence. Thus, an alternatively spliced RNA species which encodes the soluble form of PECAM-1 is present in HUVECs and HEL cells.

**PECAM-1 Is Present in Normal Plasma**—Detection of a soluble form of PECAM-1 in the culture media of HUVECs and myeloid cell lines prompted us to look for soluble PECAM-1 in normal human plasma. Samples of plasma from several donors were ultracentrifuged and sterile-filtered to remove cell debris and then tested in an antigen-capture ELISA for the presence of PECAM-1. The assay was calibrated by a standard curve employing purified sPECAM-1, which indicated that PECAM-1 could be detected at levels as low as 5 ng/ml (Fig. 7A). Screening of several plasmas (Fig. 7B) demonstrated that a soluble form of PECAM-1 was present in plasma with approximately 10–25 ng/ml. The specificity of detection was verified by solid phase absorption of the plasmas with antibodies specific for either PECAM-1 or GPIIIa, as well as with control rabbit and mouse IgGs. Monoclonal as well as polyclonal anti-PECAM-1 IgGs were able to specifically remove PECA"1 from plasma samples from other normal individuals.

**Analysis of Plasma PECAM-1**—To assess the biochemical properties of human plasma PECAM-1, several samples were analyzed by immunoprecipitation using polyclonal anti-PECAM-1 or ACT antibodies after 10-fold dilution of plasma and ultracentrifugation at 100,000 x g for 3 h. The immuno-precipitated proteins were separated by SDS-PAGE, Western-blotted, and detected with an anti-PECAM-1 monoclonal antibody. As shown in Fig. 8A, soluble PECAM-1 from plasma samples is present as two distinct species with apparent molecular weights of 120,000 and 90,000, the latter being significantly smaller than the cell-associated form immuno-precipitated from a HUVEC lysate (cf. lane 2 with lanes 5, 8, 11, and 13). The 120-kDa soluble isoform was recognized by the ACT antibody (lanes 6, 9, 12, and 14), indicating that the cytoplasmic tail is present; however, the lower molecular weight species was not recognized by ACT (compare lanes 5, 8, and 11 with lanes 6, 9, and 12). To confirm that the 90-kDa band was indeed a form of PECAM-1 lacking the cytoplasmic tail, a sequential absorption experiment was performed. An aliquot of plasma was incubated with ACT-Sepharose, the beads were removed, and the sample was then incubated with anti-PECAM-1-Sepharose. As shown in Fig. 8A, lanes 13–15, ACT bound only the 120-kDa species of soluble PECAM-1, but failed to immunoprecipitate the lower band, which continued to be specifically recognized by the antiPECAM-1 antibody. Therefore, it would appear that circulating plasma PECAM-1 exists as at least 2 isoforms that differ by the presence of the cytoplasmic tail.

In order to determine the relationship between plasma PECAM-1 and that observed in cell culture media, immunoprecipitates from both sources were analyzed by Western blot analysis in the same gel. As shown in Fig. 8B, the 120-kDa isoform of sPECAM-1 is clearly the major form found in cell culture media (see Figs. 4, 5C, and 8B), although we occasionally observe traces of lower molecular mass products (Fig. 8B, second lane from the left). In contrast, plasma PECAM-1 is relatively evenly split between the 120- and 90-kDa isoforms. The results of immunoprecipitation of sPECAM-1 from both plasma and cell culture media with ACT (Fig. 8B, third and sixth lanes) indicate that only the 120-kDa form is derived by alternative splicing and thereby retains the cytoplasmic tail.

**Soluble PECAM-1 Can Block Adhesive Interactions of Membrane-bound PECAM-1**—Recent studies have shown that PECAM-1 mediates heterophilic, Ca$^{2+}$-dependent aggregation of transfected mouse L-cells (9, 10) and plays a key role in leukocyte transmigration through endothelial cell monolayers, both in cell culture (15) and in the intact vessel wall (16). To determine whether soluble forms of PECAM-1 similar to those in plasma might retain adhesive function and potentially modulate this process, we prepared large amounts of soluble recombinant PECAM-1 (see Fig. 5, panels A and B), which corresponds in size and composition to the 90-kDa form found in cell culture media and in plasma. Using the L-cell aggregation assay as a measure of functional activity (Fig. 9), we found that soluble PECAM-1 at a concentration of 10 g/ml was able to inhibit PECAM-1-specific cellular interactions almost completely, with moderate inhibition of intermediate concentrations. Thus, it would appear that soluble PECAM-1 is functional and may potentially be involved in modulation of the inflammatory process in vivo if high local concentrations of these PECAM-1 isoforms were present.

**DISCUSSION**

PECAM-1 is widely distributed on cells of the vascular system, as it is found on endothelial cells, platelets, neutrophils, monocytes, and some T-cell subsets. The protein localizes to the intercellular junctions of endothelial cells and transfected monolayer cells. In addition, PECAM-1 seems to serve as a “trigger” molecule in activating integrins on circulating leukocytes and may also be involved in the transendothelial migration of leukocytes during the inflammatory process. It is intriguing that the synthesis of PECAM-1 is so tightly regulated, as it is not found outside the vasculature. Little is known about the biochemical events involved in PECAM-1 synthesis and processing in those cell types that express it which may affect its subsequent functional activities.

Like other membrane glycoproteins, PECAM-1 has been shown to be synthesized as a smaller molecular weight precursor molecule that is further processed into a mature form (8). The kinetics of intracellular trafficking and turnover of PECAM-1 on the cell surface, however, are important parameters that have not yet been examined. Therefore, pulse-chase labeling of U937 cells and HUVECs was used to further characterize the synthesis and processing of PECAM-1. As shown in Figs. 1 and 2, pre-PECAM-1 could be detected in as little as 20 min following the addition of radiolabeled methionine, and mature PECAM-1 was expressed on the cell surface within 3 h. The newly synthesized protein was found to be only moderately stable, disappearing from the cell surface within 2–3 days and
Biosynthesis and Processing of PECAM-1

Fig. 7. Detection and quantitation of PECAM-1 in normal human plasma. A, standardization of the ELISA assay. Increasing amounts of FPLC-purified soluble recombinant PECAM-1 were diluted into PECAM-1-depleted human plasma. The curve shown in the large panel represents the linear end of the complete standard curve shown in the inset. B, detection and immunoabsorption of naturally occurring soluble PECAM-1 in plasma. Plasma samples (500 μl), prepared as described under "Experimental Procedures," were incubated overnight with the indicated antibody coupled to Sepharose. The beads were removed by centrifugation, and 100 μl of the immunoabsorbed plasma was added to triplicate wells of a microtiter plate precoated with PECAM-1. Bound PECAM-1 was detected using the biotinylated anti-PECAM-1 monoclonal antibody, PECAM 1.3 (see "Experimental Procedures"). Values are corrected for background absorbance (wells containing PECAM-1-depleted plasma) and represent the mean of triplicate readings ± S.D.

PECAM-1 from intracellular pools with approximately the same kinetics (Figs. 1 and 2).

Interestingly, the kinetics of PECAM-1 synthesis and down-regulation in HUVECs was similar to that observed in U937 cells, which seems to indicate that the homotypic interactions of PECAM-1 at endothelial cell junctions do not stabilize the turnover rate of the protein. We cannot rule out the possibility, however, that the rate of PECAM-1 synthesis and decay is slower in intact vessels, where endothelial cells are known to divide as slowly as once every 2 years.

Analysis of the glycosylation pattern of PECAM-1 indicated that the precursor form of PECAM-1 contains a number of high mannose carbohydrate residues, as demonstrated by its susceptibility to endo H digestion (Fig. 3). It would appear that all
of these are modified in the Golgi to complex carbohydrate residues, as the mature form of PECAM-1, while sensitive to N-glycanase and neuraminidase, was no longer affected by endo H. Treatment of either form of PECAM-1 with N-glycanase led to a decrease in apparent molecular weight to about 80 kDa; however, is about 80 kDa, suggesting that the remaining 20 kDa may be composed of O-linked carbohydrate moieties. Alternatively, PECAM-1 may simply bind serum carbohydrate (39). Notably, the 120-kDa soluble PECAM-1 isoforms derived from both cell culture media and normal human plasma contain the cytoplasmic tail, as evidenced by reactivity with ACT.

Discovery of a soluble form of PECAM-1 in culture media prompted us to look for soluble PECAM-1 in plasma, and the results indicate that PECAM-1 is present in normal human plasma at levels of 10–25 ng/ml. Although these levels are low, continuous production of soluble PECAM-1 from the minor transmembraneless PECAM-1 mRNA species by the 5,000 square meters of endothelial cells that are present in the body could easily account for the concentration detected. Interestingly, two isoforms of PECAM-1, which migrated at 120 kDa and 90 kDa on SDS-PAGE, were observed in plasma. The 120 kDa form appears to correspond to the major soluble PECAM-1 observed in HUVEC culture media and retains the cytoplasmic tail, while the 90-kDa form does not carry the cytoplasmic tail and appears to correspond to sPECAM-1 which is truncated at the transmembrane domain. It is not known whether 90-kDa soluble PECAM-1 is specifically cleaved from the cell surface or results from proteolytic cleavage of the 120-kDa form following its secretion from the cell.

Although the role of plasma PECAM-1 in vivo remains to be determined, we have shown that soluble PECAM-1 truncated at the transmembrane domain can inhibit the heterotypic adhesive interactions mediated by cell-surface PECAM-1 expressed on transfected L-cells. The recent studies of Muller et al. (15), which showed that PECAM-1 is required for transendothelial migration of leukocytes, presumably mediated through homotypic interactions between PECAM-1 on the leukocytes and endothelial cells, are also relevant. These authors were able to inhibit transendothelial migration of leukocytes.
with soluble recombinant PECAM-1 which is truncated within the sixth Ig domain. Thus, the presence of low levels of circulating PECAM-1 may help to counteract the tendency of leukocytes to leave the vasculature under normal conditions, whereas higher circulating levels present during inflammatory conditions, if found to exist, might be involved in limiting further transmigration as part of a negative feedback loop. The potential role of the cytoplasmic tail in modifying the functions of the two isoforms of soluble PECAM-1 is also of interest.

The widespread presence of PECAM-1 in the vascular system predicts that PECAM-1 has important functions in each of the cell types in which it is expressed. We have elucidated some of the events involved in the biosynthesis and processing of PECAM-1, including the synthesis of a soluble form. Further characterization of the cellular and molecular properties of PECAM-1 in each of these cell types should aid in our understanding of its role in inflammation and the immune response.

Acknowledgments—We are grateful to Glenn Larsen (Genetics Institute, Boston, MA) for the EMC-3 expression vector, to Lawrence Chasin (Columbia University, New York) for the CHO-DG44 cell line, and to Michael Berndt for helpful discussions on the PECAM-1 ELISA.

REFERENCES

1. Newman, P. J., Berndt, M. C., Gorski, J., White, G. C., III, Lyman, S., Peddock, C., and Muller, W. A. (1990) Science 247, 1219-1222
2. Ohno, H., Maeda, H., Shibata, Y., Chen, R.-P., Ozaki, Y., Higashihara, M., Takeuchi, A., and Tohyama, H. (1985) Blood 66, 873-881
3. Goyer, S. M., Ferrero, E. M., Seremetis, S. V., Winchester, R. J., Silver, J., and Mattison, A. C. (1986) J. Immunol. 137, 3909-3914
4. Ashman, A. C., Aylett, G. W., Cambareri, A. C., and Cole, S. R. (1989) Tissue Antigens 34, 199-207
5. Cabanas, C., Sanchez-Madrid, F., Bellon, T., Figdor, C. G., Te Velde, A. A., Fernandez, J. M., Acevedo, A., and Cole, S. R. (1991) Eur. J. Immunol. 21, 1373-1379
6. Lyons, A. B., Cooper, S. J., Cole, S. R., and Ashman, L. K. (1998) Pathology 30, 137-146
7. Albelda, S. M., Oliver, P. D., Romer, L. H., and Buck, C. B. (1990) J. Cell Biol. 110, 1227-1227
8. Muller, W. A., Ratti, C. M., McDonnell, S. L., and Cohn, Z. A. (1989) J. Exp. Med. 170, 899-915
9. Albelda, S. M., Muller, W. A., Buck, C. A., and Newman, P. J. (1991) J. Cell Biol. 114, 1059-1068
10. Muller, W. A., Berman, M. E., Newman, P. J., DeLisser, H. M., and Albelda, S. M. (1993) J. Exp. Med. 178, 1401-1404
11. Schimmenti, L. A., Yan, H.-C., Madri, J. A., and Albelda, S. M. (1992) J. Cell Physiol. 153, 417-428
12. Newman, P. J. (1994) Ann. N. Y. Acad. Sci., in press
13. Delisser, H. M., Yan, H. C., Newman, P. J., Muller, W. A., Buck, C. A., and Albelda, S. M. (1993) J. Biol. Chem. 268, 16927-16936
14. Bogen, S. A., Baldwin, H. S., Watkins, S. C., Albelda, S. M., and Abbas, A. K. (1992) Am. J. Pathol. 141, 843-854
15. Muller, W. A., Weigl, S. A., Deng, X., and Phillips, D. M. (1993) J. Exp. Med. 178, 449-460
16. Vapordeyan, A. A., Delisser, H. M., Yan, H., Mendiguch, I. L., Thom, S. R., Jones, M. L., Ward, P. A., and Albelda, S. M. (1993) Science 263, 1580-1582
17. Zehnder, J. J., Hirai, K., Shaterky, M., McGregor, J. L., Levits, L. J., and Leung, L. I. K. (1992) J. Biol. Chem. 267, 543-5429
18. Newman, P. J., Hillery, C. A., Albrecht, R., Parise, L. V., Berndt, M. C., Mazurow, A. V., Dunlop, L. C., Zhang, J., and Rottenhouse, S. E. (1992) J. Cell Biol. 119, 239-246
19. Tanaka, Y., Albelda, S. M., Horgan, K. J., van Seventer, G. A., Shimizu, Y., Newman, W., Hallam, J., Newman, P. J., Buck, C. A., and Shaw, S. (1992) J. Exp. Med. 176, 245-253
20. Fialli, L., Albelda, S. M., Baldwin, H. S., Hammel, P., Gisler, R. H., and Imhof, B. A. (1993) Eur. J. Immunol. 23, 2464-2471
21. Stockinger, H., Gadd, S. J., Eber, R., Magic, O., Schreiber, W., Kasinrerk, W., Strauss, B., Schnabl, E., and Knapp, W. (1960) J. Immunol. 145, 3889-3897
22. Takeuchi, A., Shimizu, A., Ohno, H., and Hashimoto, T. (1988) Clin. Immunol. Immunopathol. 49, 439-449
23. Newman, P. J., Kawai, Y., Montgomery, R. R., and Kunicki, T. J. (1986) J. Cell Biol. 103, 81-86
24. Johnston, G. I., Kurosky, A., and McEver, R. P. (1989) J. Biol. Chem. 264, 1616-1623
25. Kaufman, R. J., Davies, M. V., Pathak, V. K., and Hensley, J. W. (1989) Mol. Cell. Biol. 9, 946-958
26. Goldberger, A., Koldziej, M., Porco, M., Bennett, J. S., and Newman, P. J. (1989) Blood 73, 681-691
27. Abate, C., Luk, D., Gentz, R., Rauwerk, F. J., and Curran, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1032-1036
28. Bojard, H., Gentz, R., Lanzer, M., Steinber, D., Mueller, M., Ishibashi, I., Hasegawa, M., and Debderstein, B. (1987) Methods Enzymol. 155, 416-433
29. Gentz, R., Chen, C.-H., and Rosen, C. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 821-824
30. Gottesman, S., Halpern, B., and Trinder, P. (1981) J. Biol. Chem. 256, 265-273
31. Heinti, E., Bannwarth, W., Dobell, H., Genta, R., and Stuber, D. (1985) Bio/Technology 3, 1221-1225
32. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159
33. Schlieffenbaum, B., Spertino, O., and Tedder, T. F. (1993) J. Cell Biol. 119, 229-238
34. Rothlein, R., Mainolfi, E. A., Czajkowski, M., and Marlin, S. D. (1991) J. Immunol. 147, 3788-3793
35. Becker, J. C., Dummer, R., Hartmann, A. A., Burg, G., and Schmidt, R. E. (1991) J. Immunol. 147, 4598-4601
36. Dunlop, L. C., Skinner, M. P., Bendall, L. J., Favaloro, E. J., Castaldi, P. A., Gorman, J. J., Gamble, J. R., Vidas, M. A., and Berndt, M. C. (1992) J. Exp. Med. 175, 1147-1150
37. Bordier, C. (1981) J. Biol. Chem. 256, 1604-1607
38. Newman, P. J., Knapp, M. A., and Kahn, R. A. (1982) Thromb. Res. 27, 221-224
39. Umemoto, J., Bhavanandan, V. P., and Davidson, E. A. (1977) J. Biol. Chem. 252, 5603-5614

Biosynthesis and Processing of PECAM-1 17191