Activation-dependent Changes in Human Platelet PECAM-1: Phosphorylation, Cytoskeletal Association, and Surface Membrane Redistribution

Peter J. Newman,*‡ Cheryl A. Hillery,§ Ralph Albrecht,‖ Leslie V. Parise,§ Michael C. Berndt,¶ Alexey V. Mazurov,¶ Lindsay C. Dunlop,¶ Jin Zhang,** and Susan E. Rittenhouse**

*Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, Wisconsin; §The Department of Cellular Biology and Anatomy, The Medical College of Wisconsin, Milwaukee, Wisconsin; §The Department of Pharmacology, and Center for Thrombosis and Hemostasis, University of North Carolina, Chapel Hill, North Carolina; ‖Department of Animal Health and Biomedical Sciences, University of Wisconsin, Madison, Wisconsin; ¶Baker Medical Research Institute, Melbourne, Australia; and **The University of Vermont College of Medicine, Department of Biochemistry, Burlington, Vermont

Abstract. PECAM-1 is a recently described member of the immunoglobulin gene (Ig) superfamily that is expressed on the surface on platelets, several leukocyte subsets, and at the endothelial cell intercellular junction. Recent studies have shown that the extracellular domain of PECAM-1, which is comprised of 6 Ig-like homology units, participates in mediating cell-cell adhesion, plays a role in initiating endothelial cell contact, and may later serve to stabilize the endothelial cell monolayer. PECAM-1 also has a relatively large 108 amino acid cytoplasmic domain, with potential sites for phosphorylation, lipid modification, and other posttranslational events that could potentially modulate its adhesive function or regulate its subcellular distribution. Virtually nothing is known about the contribution of the intracellular region of the PECAM-1 molecule to either of these cellular processes. Using human platelets as a model, we now demonstrate that PECAM-1 becomes highly phosphorylated in response to cellular activation, and coincident with phosphorylation associates with the cytoskeleton of activated, but not resting, platelets. The engagement of PECAM-1 with the platelet cytoskeleton enables it to move large distances within the plane of the membrane of fully-spread, adherent platelets. This redistribution may similarly account for the ability of PECAM-1 to localize to the intracellular borders of endothelial cells once cell–cell contact has been achieved.

PLATELET endothelial cell adhesion molecule-1 (PECAM-1)[1] is a 130-kD membrane glycoprotein that is expressed on the surface of human platelets (32), both large and small vessel endothelium (2, 8, 30), monocytes and neutrophils (46), and on certain T-cell subsets (4, 47). mAb cross-reactivity studies (32) have shown that PECAM-1 is identical to the previously serologically defined CD31 differentiation antigen (20) which has been used for a number of years as a marker of myelomonocytic differentiation (6, 13, 25, 34). PECAM-1 has not been found outside the vasculature.

Molecular cloning of the cDNA encoding PECAM-1 (32, 46) has revealed that this molecule is a member of the broadly distributed Ig gene superfamily, and contains within its extracellular domain six Ig-like homology units of the C2 subclass that are typical of Ig-superfamily members that function as cell–cell adhesion molecules (CAMS). At least 40% of the molecular mass of PECAM-1 is composed of complex carbohydrate residues, all of which are predicted to lie outside the cell. On the other side of its 19-amino acid transmembrane domain, PECAM-1 is predicted to have a long cytoplasmic domain consisting of 108 amino acid residues, including numerous serine, threonine, and tyrosine residues that could potentially become phosphorylated after cellular activation.

Although the precise function of PECAM-1 is not known in all the cell types that express it, a number of recent investigations have shed light on its ability to function as a cellular CAM. Ohto and colleagues (34) produced two murine mAbs directed against an epitope on PECAM-1 that interfered with both monocyte and neutrophil chemotaxis toward endotoxin-activated serum, while Albelda et al. (1) were able to prevent the association of endothelial cells in monolayer culture into their typical cobblestone morphology using anti-endoCAM (the bovine equivalent of PECAM-1) antibodies. More recently, Albelda et al. (2) showed that PECAM-1 is capable of directly mediating aggregation of murine L-cells that have been transfected with PECAM-1 cDNA, and that the aggregation of the PECAM-1 transfectants can be speci-

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; PECAM-1, platelet endothelial cell adhesion molecule-1.

fically blocked by anti-PECAM-1 antibodies. At least two bioactive adhesive domains have been identified within the extracellular portion of the PECAM-1 molecule: one located at amino acid residues 150–155, which corresponds to a heparin-binding consensus sequence in Ig-loop 2, and the other defined by the epitopes of the murine mAbs PECAM-1.2 and 4G6, which bind within loop 6 of PECAM-1 (the Ig homology domain most proximal to the membrane) (DeLisser, H. M., P. J. Newman, W. A. Muller, L. Romer, C. A. Buck, and S. M. Albelda, submitted for publication).

An interesting aspect of PECAM-1 function is its ability to localize to the cell–cell borders of both endothelial cells (1, 27, 30, 43, 48) as well as PECAM-1–transfected COS-7 cells (2). Both of these cell types grow as monolayers of cells that continue to divide in culture until they reach confluence, at which point cell growth is arrested by an as yet incompletely understood contact inhibition process. The subcellular distribution of PECAM-1 in these two cell types is such that it remains diffusely distributed on the cell surface until cell–cell contact is achieved, at which point PECAM-1 becomes intensely localized to the intercellular junctions.

The mechanism by which PECAM-1 becomes concentrated at the intercellular junction is not known. It is possible that individual PECAM-1 molecules simply diffuse within the plane of the membrane until they contact a counter receptor or ligand on an adjacent cell, at which point an adhesive interaction is achieved that prevents further diffusion of PECAM-1. The net effect of such a mechanism would be a gradual accumulation of PECAM-1 at the borders of cells that contact each other. Alternatively, cell surface PECAM-1 might be actively transported to the intercellular junction when cell contact is made. Either mechanism could provide for the stabilization of the junctional complex, which could in turn prevent further cell migration or disruption of the cell monolayer. However, an understanding of the molecular events that regulate the subcellular distribution of this adhesive CAM would be expected to shed considerable light on the mechanisms that control cell–cell adhesion events that are important to such processes as endothelial cell migration, wound healing, metastasis, and angiogenesis.

Using a platelet model, we demonstrate in this manuscript that PECAM-1 is rapidly phosphorylated in response to cellular activation, and that coincident with the activation process, becomes associated with the platelet cytoskeleton. Finally, we provide evidence that PECAM-1 redistributes in a cytoskeleton-dependent fashion within the membrane of surface-activated human platelets except at points of intercellular contact, where it instead remains at the cell–cell border, reminiscent of its junctional localization in confluent human endothelial cell monolayers.

Materials and Methods

Antibodies

The following murine anti-human PECAM-1 mAbs were used: hec7, an IgG_{2A} antibody that has previously been shown to react with PECAM-1 at the endothelial cell borders (2, 30, 32), and PECAM-1.3, an IgG_1 antibody that binds PECAM-1 in both intact cells and on reduced or nonreduced Western blots (2). The rabbit polyclonal antibody, SEW-16, was raised by once-weekly subdermal injections of 100-μg doses of immunoaffinity-purified PECAM-1 antigen. The IgG fraction was purified using protein A–Sepharose chromatography, and had specificity for PECAM-1 that was equivalent to that of the mAb. Previously described anti–GMP-140 (P-selectin) and anti–GPIIb-IIIa polyclonal antibodies (44, 52) were affinity purified and stored in 10 mM Tris, 150 mM NaCl, pH 7.4, at −70°C before use.

Preparation of ^{32}P-labeled Platelets

Fresh human platelets were isolated from healthy aspirin-free adults as previously described (11) except that 50 ng/ml Prostaglandin I_2 (Sigma Chemical Co., St. Louis, MO) was included during the washing procedure and platelets were resuspended in a $Ca^{2+}/Mg^{2+}/PO_4$-free Tyrodes buffer (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 50 mM Hepes, pH 7.35) at a concentration of 1×10^9 platelets/ml. Washed platelets were incubated with 1.0 mCi of $^{32}P_i$ (carrier-free; ICN Biomedicals, Inc., Irvine, CA)/ml at 37°C for 4 h. ^{32}P-labeled platelets were sedimented by centrifugation and resuspended in $Ca^{2+}/Mg^{2+}/PO_4$-free Tyrodes buffer at a concentration of 1.5×10^8/ml. After incubation with 0.1 U/ml of thrombin (kindly supplied by John Fenton II, New York State Department of Health, Albany, NY), 3 min, 37°C, platelets were lysed by the addition of an equal volume of ice-cold lysis buffer (2% Triton X-100, 20 mM Tris, 2 mM sodium metavanadate, 40 mM molybdic acid, 80 mM sodium pyrophosphate, 40 mM KH_2PO_4, 4 mM EGTA, 0.2 mM trifluoperazine, 0.2 mM leupeptin, 2 mM PMSF, 0.2 U/ml hirudin [Sigma Chemical Co.], pH 7.1). After centrifugation (12,000 *g*, 10 min) to sediment the insoluble cytoskeletal proteins, the supernatant was passed through a 1-ml column of glycine/Affigel 10 (Bio-Rad Laboratories, Richmond, CA) to remove nonspecific binding proteins. The flow-through was then applied to a 0.2-ml column consisting of PECAM-1.3-Affigel 10 beads (coupled at 2.5 mg antibody per ml gel according to the manufacturer's directions) to isolate platelet PECAM-1. After washing (150 mM NaCl, 0.1% Triton X-100, 0.02% NaN_3, 20 mM Tris, pH 7.4, 25 ml) PECAM-1 was eluted in 0.5-ml fractions with 0.1% Triton X-100, 100 mM glycine, pH 12.5. Peak fractions were identified by Cerenkov counting and precipitated by the addition of TCA (15% final concentration) with 20 μg of BSA/tube added as a carrier. The TCA insoluble material was dissolved in Laemmli SDS sample buffer containing 10% β-mercaptoethanol (100°C, 5 min). ^{32}P-labeled PECAM-1 was resolved by electrophoresis on 7.5% SDS-polyacrylamide gels and visualized by autoradiography.

Phosphoaminoacid Analysis

^{32}P-labeled PECAM-1 was excised from SDS-polyacrylamide gels and homogenized in 1 ml of 0.5 M ammonium bicarbonate, 0.1% (wt/vol) SDS, and 1% (vol/vol) β-mercaptoethanol. The homogenate was centrifuged, and the protein in the supernatant was precipitated by the addition of TCA to a final concentration of 15%, with 30 μg of BSA added as a carrier. The TCA-insoluble material was washed once with ethanol/ether (1:1) and incubated in 5.7 M HCl for 1 h at 110°C. The sample was lyophilized to remove the HCl and dissolved in H_2O containing 1 mg/ml each of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine. The sample was spotted onto cellulose TLC plates (Eastman Kodak Co., Rochester, NY) and separated by electrophoresis and chromatography, as described by Cooper et al. (7) In brief, the samples were electrophoresed at 500 V for 75 min in 88% formic acid/acetic acid/H_2O, pH 1.9 (50:156:1794 vol/vol). The plates were dried at room temperature and chromatographed (isobutyric acid: 0.5 M ammonium hydroxide, 5:3 vol/vol). After autoradiography, the ^{32}P-labeled samples were identified by their comigration with standards stained with ninhydrin.

Preparation of Platelet Membranes, Cytosols, and Cytoskeletons

Fresh human platelets were prepared by gel filtration, and resuspended in gel filtration buffer (GFB; 123 mM NaCl, 12.2 mM NaCitrate, 9.9 mM Tris, 5.6 mM Dextrose, 2 mM Hepes, 2.8 mM KCl, 8.9 mM NaH_2CO_3, 0.86 mM $CaCl_2$, pH 7.3) at a concentration of $2–4 \times 10^9$/ml, as previously described (18, 40). Platelet suspensions were preincubated for 5 min at 37°C, and then incubated 45 s further with or without 5 U/ml thrombin. Incubations were terminated by addition of an equal volume of lysis buffer containing 2% Triton X-100, 100 mM Tris-Cl, 10 mM EGTA, 0.4 mM leupeptin, and 0.2 mM sodium vanadate, pH 7.4. This procedure resulted in complete platelet lysis, as gauged by the release of lactate dehydrogenase to the 100,000 *g* supernatant (>98%). Two Triton X-100–insoluble fractions and a soluble fraction were separated by successive centrifugations at 4°C: 4 min at 15,460 *g* (15-kD pellet), followed by 100,000 *g* for 3 h (100-

kD pellet), as previously described (11, 12, 53). Pellets were washed four times in GFB and finally resuspended in GFB containing 0.2 mM leupeptin, 1 mM Na_3VO_4 in a volume equal to that of the supernatant. Resuspension was achieved with the aid of sonication on ice. Fractions were aliquoted and stored at −70°C.

Platelet membrane and cytosol were prepared by suspending washed platelets (10^{10}/ml) in a hypotonic buffer composed of 10 mM Hepes, pH 7.0, 1 mM EGTA, 1 mM NaH_2PO_4, 1 mM Na_3VO_4, 0.2 mM leupeptin, 5 μg/ml antipain, and 50 μM DTT, and sonicating on ice (3 × 10 s). Unbroken platelets were removed by centrifugation at 4500 rpm for 5 min at 4°C. The membrane-rich supernatant was subsequently centrifuged at 100,000 *g* for 90 min at 4°C. The membrane pellet was washed four times with hypotonic buffer and resuspended to the original volume in this buffer with the aid of sonication on ice.

Aliquots from equal numbers of platelets were mixed with Laemmli buffer, boiled, and resolved on 7.5% SDS-polyacrylamide gels. After electrophoretic transfer to nitrocellulose, membranes were blocked and then blotted with 25 μg/ml of SEW-16 anti-PECAM-1 polyclonal antibody or 50 μg/ml of PECAM-1.3 mAb. Bound antibodies were visualized and quantitated with ^{125}I-protein A (for SEW-16) (19) or rabbit anti-mouse IgG followed by ^{125}I-protein A (PECAM-1.3), followed by autoradiography and gamma counting of excised bands. Quantitation was performed in a range where ^{125}I-protein A binding was determined to be linear with respect to platelet protein.

Immunoelectron Microscopy

Colloidal gold granules were prepared and conjugated to anti-PECAM-1 mAbs using methods that have been previously described (23). Platelets obtained from normal, healthy volunteers were collected into 10 mM EGTA and purified by differential centrifugation and gel filtration through a Sepharose CL-2B column into Ca^{2+}-free Tyrodes buffer. Platelets were then deposited onto Formvar filmed Ni maxiform grids and allowed to settle and spread for 15 min at 37°C. The grids were then either fixed in 1% glutaraldehyde, 0.2% tannic acid and incubated with anti-PECAM-1-gold for an additional 10 min at 37°C, or incubated with anti-PECAM-1 gold and then fixed. Samples were then postfixed, subjected to critical-point drying, and coated with a thin layer of carbon before examination with the Hitachi S900 high-resolution field emission scanning electron microscope (Hitachi Scientific Instruments, Mountain View, CA) of the Madison High Voltage Electron Microscopy facility (Madison, WI) (23).

Results

Phosphorylation of PECAM-1 in Resting and Thrombin-activated Platelets

Since phosphorylation of the cytoplasmic domain is one of several ways to modify the cellular associations made by a membrane glycoprotein, experiments were performed to evaluate whether PECAM-1 is phosphorylated under resting conditions, and whether the degree of phosphorylation changes after cellular activation. As shown in Fig. 1, platelet PECAM-1 is indeed phosphorylated to a limited extent in resting, control platelets. This finding is qualified by the acknowledged uncertainty that platelet preparation and washing could have resulted in a small degree of inadvertent activation, in spite of the presence of PGI_2 and the absence of added calcium or magnesium ions. Upon platelet activation, however, a significantly increased amount of ^{32}P becomes incorporated into PECAM-1 (lane *T*). Quantitation of ^{32}P incorporation by densitometric scanning of lanes *R* (resting) and *T* (thrombin activated), as well as by excision and counting of the labeled, immunoprecipitated PECAM-1 bands from the gel, revealed that the level of PECAM-1 phosphorylation increases four- to fivefold after exposure of platelets to thrombin (Fig. 1) and increases two- to threefold in response to phorbol myristate acetate (not shown).

Molecular cloning of PECAM-1 cDNA has previously shown that the 108-amino acid cytoplasmic domain of PECAM-1 contains 5 tyrosine, 5 threonine, and 12 serine residues (32), all of which potentially could serve as sites of phosphate modification. To determine which of these might become phosphorylated upon platelet activation, the radioactive band shown in lane *T* of Fig. 1 was excised from the gel, homogenized, hydrolyzed in 5.7 M HCl, and subjected to phosphoamino analysis using thin-layer electrophoresis/chromatography. All of the detectable ^{32}P was found to be incorporated into serine residues. (Fig. 2).

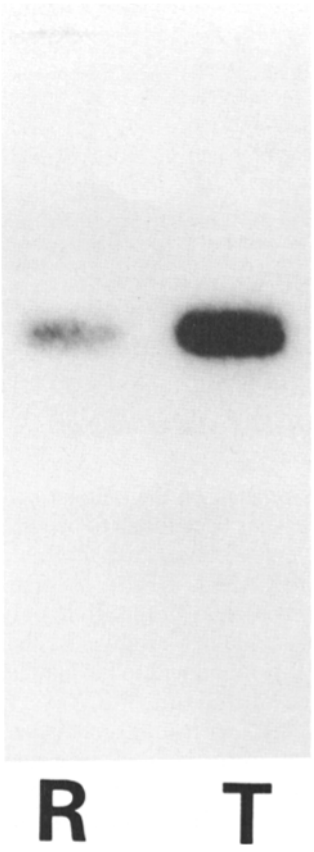

Figure 1. Phosphorylation of PECAM-1 in resting and thrombin-activated platelets. Washed human platelets were resuspended in a $Ca^{2+}/Mg^{2+}/PO_4$-free Tyrodes buffer and labeled with $^{32}PO_4$ for 4 h at 37°C. Aliquots of 2.5 × 10^8 platelets were incubated with 0.1 U/ml thrombin or control buffer for 3 min under nonstirring, nonaggregating conditions. Reactions were stopped by the addition of lysis buffer containing inhibitors of phosphatases, kinases, and proteases, as described in Materials and Methods. PECAM-1 was affinity isolated from Triton lysates with PECAM-1.3/Affigel 10 beads, eluted, separated by SDS-PAGE, and exposed to x-ray film for autoradiography. (lane *R*) PECAM-1 from resting platelets. (lane *T*) PECAM-1 from thrombin-stimulated platelets.

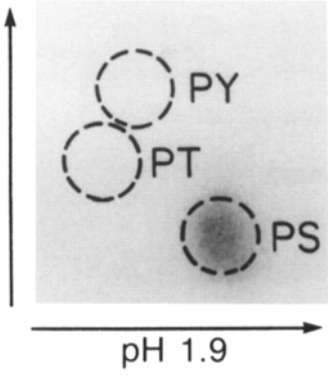

Figure 2. Phosphoamino acid analysis of human platelet PECAM-1. ^{32}P-PECAM-1 was immunoaffinity isolated from thrombin-activated platelets, acid hydrolyzed, and analyzed by two-dimensional electrophoresis as described in Materials and Methods. The circles show the positions of phosphoserine (*PS*), phosphothreonine (*PT*), and phosphotyrosine (*PY*) standards that were run simultaneously with the radiolabeled sample. The standards were detected with ninhydrin, and the ^{32}P phosphoamino acids were subsequently revealed by autoradiography of the same cellulose plates.

Table I. Percent Distribution of PECAM-1 in Resting Human Platelets

Platelet Fraction	Determined with SEW-16	Determined with PECAM-1.3	Percent PECAM-1 of total protein
	%	%	%
Membrane	90.6	93.1	1.9
Cytosol	9.4	6.9	0.18

Membranes and cytosols were prepared by hypotonic lysis and sonication of washed human platelets, and separated by centrifugation at 100,000 *g* for 90 min at 4°C. After SDS-PAGE and Western transfer, PECAM-1 was detected using anti-PECAM-1 antibodies SEW-16 (polyclonal) or PECAM-1.3 (monoclonal). Bound antibody was visualized and then quantitated using ^{125}I protein A (for SEW-16) or rabbit anti-mouse IgG followed by ^{125}I protein A (for PECAM-1.3), autoradiography, and gamma counting of excised bands (19).

Association of PECAM-1 with the Cytoskeleton of Thrombin-activated Platelets

Of the numerous glycoproteins present on the platelet surface, only the GPIIb–IIIa complex has been found to become specifically associated with the cytoskeleton after thrombin activation (36, 38, 49). Like PECAM-1, GPIIIa becomes phosphorylated upon platelet activation (37), though to only a limited extent (15). Preliminary studies established that >90% of total platelet PECAM-1 is found within the platelet membrane (Table I). To examine whether this PECAM-1 is associated with the cytoskeleton in either resting or activated platelets, Triton-insoluble residues were prepared and examined for PECAM-1 content by both immunoprecipitation and Western blot analysis. As shown in Fig. 3, most of the PECAM-1 (~90%) was found in the soluble fraction of resting platelets, with little detectable PECAM-1 in the 15-kD cytoskeleton, and ~10% associated with the membrane

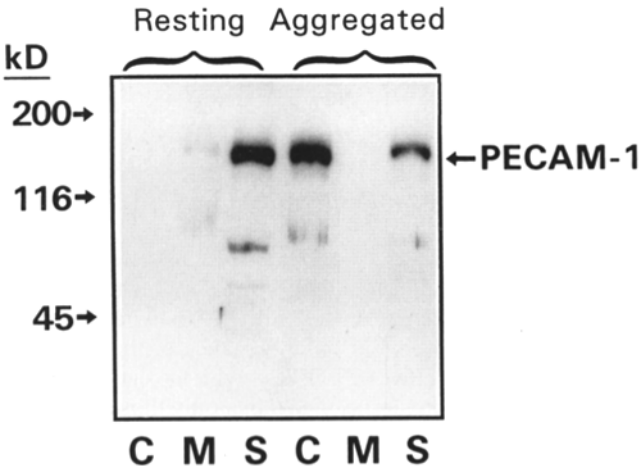

Figure 3. Association of PECAM-1 with the cytoskeleton of thrombin-aggregated platelets. Resting or thrombin-aggregated platelets were lysed in 1% Triton X-100, and the two Triton-insoluble fractions, as well as the soluble supernatant, were isolated by differential centrifugation as described in Materials and Methods. Proteins from the indicated subcellular fractions were electrophoresed, immunoblotted with the polyclonal anti-PECAM-1 antibody SEW-16, detected with ^{125}I protein A, and visualized by autoradiography. (*C*) 15,000 *g* cytoskeletal pellet; (*M*) 100,000 *g* membrane skeleton; and (*S*) Triton-soluble supernatants. Note the profound shift of PECAM-1 from the soluble fraction of resting platelets into the 15-kD cytoskeleton of thrombin-activated cells.

Table II. Distribution of PECAM-1 in Triton-lysed Platelet Fractions

	Resting platelets	Thrombin-activated platelets
Sub-cellular fraction	PECAM-1 (protein)	PECAM-1 (protein)
	(%)	(%)
15-kD cytoskeleton	0.9 ± 0.6 (19)	64.0 ± 1.6 (38)
100-kD membrane skeleton	10.9 ± 1.0 (15)	1.6 ± 0.6 (9)
Triton-soluble fraction	88.2 ± 0.4 (66)	34.4 ± 2.2 (53)

Platelet suspensions were incubated at 37°C with (activated) or without (resting) thrombin, and then lysed with an equal volume of lysis buffer containing 2% Triton X-100 and 10 mM EGTA. Two Triton-insoluble residues as well as the Triton-soluble fraction were isolated by differential centrifugation as described in Materials and Methods. The amount of PECAM-1 in each of these subcellular fractions was determined as described in Table I. The percentage of total protein (in parenthesis) in each fraction was determined using a standard colorometric protein assay.

skeleton (10). However, when the platelets were aggregated with thrombin, a dramatic increase in the amount of cytoskeletally associated PECAM-1 was found (Fig. 3). Excision of the radiolabeled bands identifying PECAM-1 allowed an estimation of the distribution of PECAM-1 among the various subcellular fractions in both resting and aggregated conditions (Table II). From these experiments we determined that ~64% of membrane-bound PECAM-1 becomes associated with the 15-kD Triton-insoluble cytoskeleton upon platelet aggregation. Control experiments were also carried out that examined the subcellular distribution of GPIIb–IIIa and GMP-140 (P-selectin) in parallel with PECAM-1. As shown in Fig. 4, although both the GPIIb–IIIa complex and PECAM-1 cosedimented with the 15-kD cytoskeleton of activated, but not resting platelets, GMP-140, which is also a transmembrane glycoprotein (17, 28, 45), did not cosediment with the cytoskeleton. These results demonstrate that the 15-kD pellet containing PECAM-1 did not result from nonspecific trapping of this membrane glycoprotein or from incompletely solubilized and pelleted membrane fragments.

Functional Redistribution of PECAM-1 with the Platelet Cytoskeleton

To see whether the biochemically demonstrated linkage with the platelet cytoskeleton represents an actual functional association, we next investigated the whole-cell distribution of PECAM-1 on the surface of the human platelets that had become activated by allowing them to spread on formvar surfaces. High-resolution scanning EM was used to visualize directly gold-conjugated anti-PECAM-1 mAbs that had been incubated with either glutaraldehyde-fixed or unfixed, fully spread platelets. This method has previously been exploited to show sequential changes in the three-dimensional organization of the platelet cytoskeleton (24), and to show ligand- or antibody-triggered, cytoskeletally directed, centralization of GPIIb–IIIa. (23). Preliminary studies (not shown) established that pseudopodial platelets in early stages of activation and spreading were labeled evenly across their surfaces with anti-PECAM-1 immunogold particles. As shown in Fig. 5, PECAM-1 remains evenly distributed on the surface of fully spread, fixed platelets (*A*), but is actively transported in the plane of the membrane toward the center of unfixed cells once the cytoskeleton has redistributed after

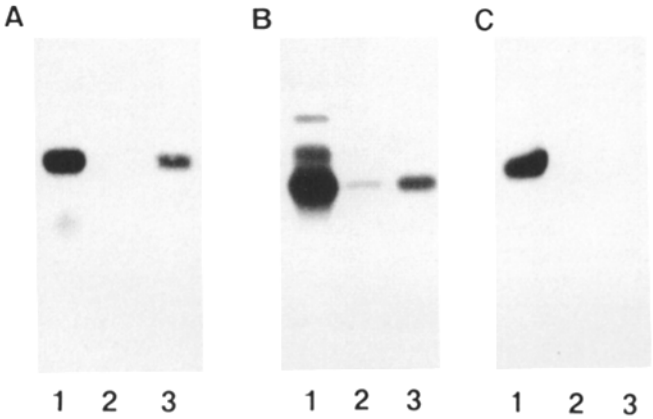

Figure 4. Specificity of PECAM-1–cytoskeletal interactions. Triton-insoluble cytoskeletons from resting and aggregated platelets were prepared from 10 ml of washed platelets (2.5–5 × 10^8/ml) which had been incubated in the presence or absence of 0.1 U/ml of thrombin for 5 min at 37°C with constant stirring. An equal volume of cold Triton lysis buffer (0.1 M Tris, 0.01 M EGTA, 2% [wt/vol] Triton X-100, pH 7.4, containing 400 μg/ml of leupeptin and 1 mM PMSF) was then added and the samples processed for electrophoresis as described in Materials and Methods. 50 μg of protein was loaded per lane. Protein bands were revealed using either affinity-purified anti-PECAM-1 (*A*), anti-GP IIb-IIIa (*B*), or anti-GMP-140 (*C*) polyclonal antibodies (5 μg/ml, final concentration), and ^{125}I-labeled goat anti-mouse IgG (1 μg/ml; 10^6 dpm/ml) with subsequent autoradiography. Control experiments confirmed that glycoprotein quantitation was linear over the range evaluated by this technique in these experiments. Whole platelets (lane *1*), resting platelet cytoskeletons (lane *2*), and thrombin aggregated platelet cytoskeletons (lane *3*).

surface activation (*B*). Interestingly, although the majority of the anti-PECAM-1 gold particles moved away from the periphery of the cell, some remained localized at sites of platelet–platelet contact (Fig. 5, *C* and *D*), reminiscent of its enriched distribution at the intercellular junctions of adjacent endothelial cells (2, 30, 32). Whether or not the subpopulation of PECAM-1 that remains at the site of interplatelet contact functions to stabilize the platelet aggregate is not known.

Discussion

The purpose of the present investigation was to determine whether PECAM-1 is capable of forming transmembrane connections with the underlying cytoskeleton in response to a cellular activation event, and whether PECAM-1 can be found to redistribute within the plasma membrane in a cytoskeleton-directed manner. Both of these issues are difficult to address experimentally in monolayer cells because they remain tightly attached to an extracellular matrix and are themselves difficult to activate and then solubilize effectively. Therefore, we exploited the fact that PECAM-1 is also expressed on the surface of human platelets, a cell type that is extremely amenable to studies of activation-dependent events, as they can be easily transformed from resting, nonadherent single cells into active, adhesive aggregates. Moreover, the cytoskeletal elements of platelets are well defined and are relatively easy to obtain in purified form. Our approach was based in part on that adopted by Phillips et al. (38), who showed more than 12 years ago that while most cellular proteins are solubilized by the addition of nonionic detergents, the platelet cytoskeleton remains Triton X-100 insoluble. Membrane proteins that participate in cell–cell adhesion can likewise be rendered detergent-insoluble providing that they become associated with cytoskeletal structures within the cell.

Phosphorylation is one of several potential posttranslational modifications that could modulate the subcellular distribution and adhesive activity of PECAM-1, and thus it was of considerable interest that PECAM-1 was found to become highly phosphorylated after platelet stimulation. Although PECAM-1 shares considerable sequence identity with the PDGF receptor in the cytoplasmic tail (32), and also contains a number of tyrosine residues located in a favorable environment for phosphorylation, phosphate was found to be incorporated only on serine residues. A two- to threefold increase in phosphorylation of cytoplasmic serine residues was found after activation with PMA, while a four- to fivefold increase was achieved using thrombin as the agonist. It seems likely that protein kinase C (PKC) mediates, at least in part, the phosphorylation of PECAM-1, since both thrombin and PMA are known to induce PKC activation in platelets (37, 41), and PKC is a serine- or threonine-specific enzyme (33). The differences in degree of phosphorylation induced by thrombin versus PMA suggest that other kinases, such as CA^{2+}/calmodulin kinase (26) may also be involved. Further studies will be required to determine the complement of cellular kinases that may be responsible for phosphorylating PECAM-1.

In resting platelets, a majority of the GPIb/V/IX complex, as well as the GPIa–IIa collagen receptor, exist preassociated with a membrane skeleton (9, 10) that is largely composed of actin, actin-binding protein, and spectrin (14). In contrast, <5% of the GPIIb–IIIa complex (49), and only 1% of PECAM-1 (this study) are linked to the cytoskeleton in resting cells. These differences may reflect the role of GPIb/V/IX and GPIa–IIa in mediating cell–matrix, versus cell–cell interactions, as platelet–collagen interactions occur initially with resting cells, while cell–cell contact develops only after cellular activation of some type. After aggregation of platelets with thrombin, however, >60% of platelet PECAM-1 became associated with the 15-kD cytoskeleton (Fig. 3, Table II). This is proportionally ~2–3 times the amount of GPIIb–IIIa that becomes cytoskeletally associated after thrombin- (20%) (38) or arachidonic acid- (28%) (49) induced platelet aggregation. The physiological significance of the association of either GPIIb–IIIa or PECAM-1 with the cytoskeleton is unclear, but it has been speculated that only those proteins directly mediating the interactions between cells become incorporated into the cytoskeleton in such a manner (38) (discussed further below). The fact that none of the other 20 or more platelet membrane glycoproteins have been found to cosediment with the 15-kD cytoskeleton of aggregated platelets speaks to the functional specificity and potential significance of this event.

Whether PECAM-1 actually becomes associated with the platelet cytoskeleton as a result of its phosphorylation remains to be determined, as incorporation of radiolabeled phosphate into PECAM-1, and PECAM-1's parallel incorporation into the cytoskeleton are merely correlative events at this time. Preliminary studies suggest that the stoichiometry

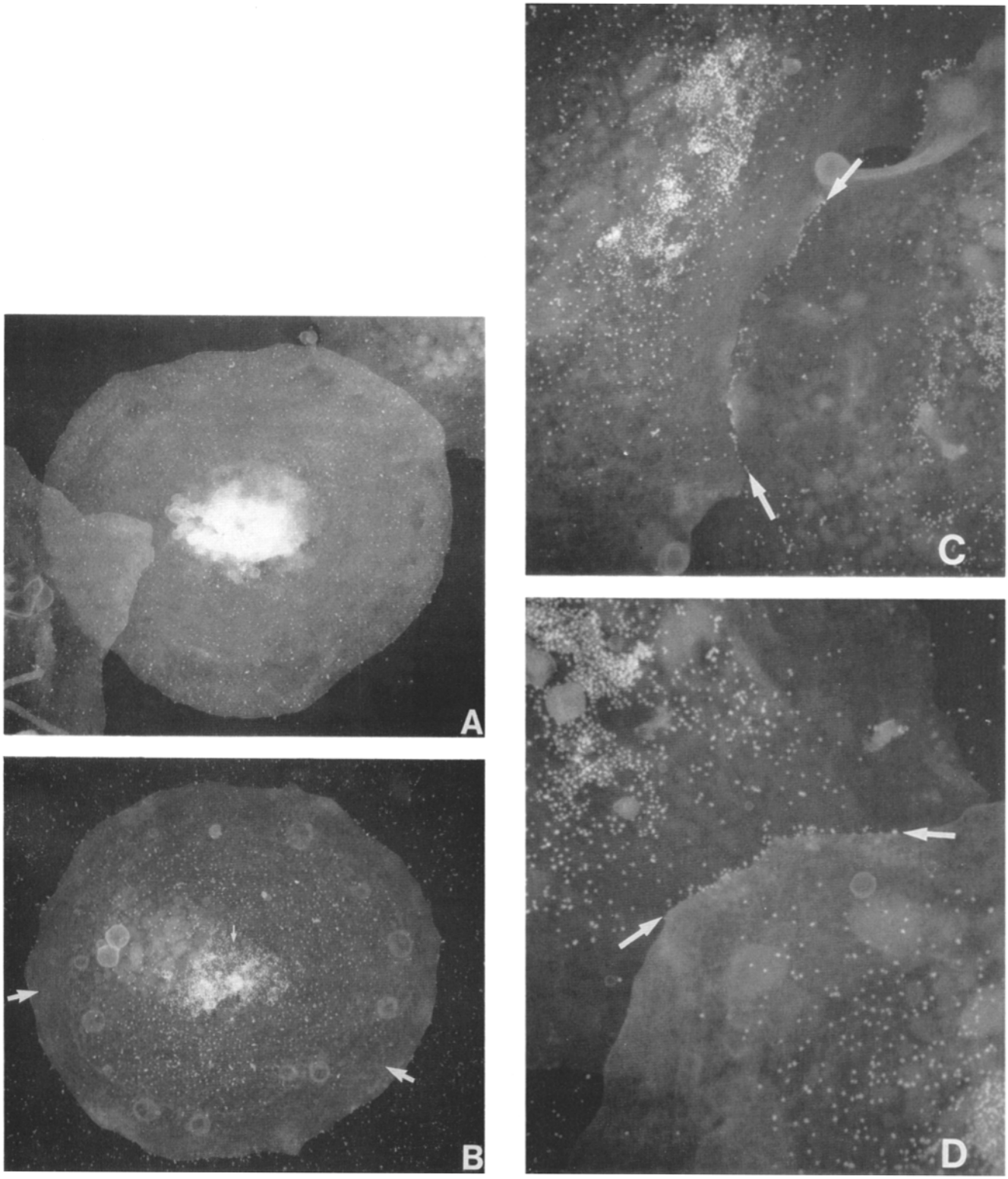

Figure 5. (*A* and *B*) Redistribution of PECAM-1 with the platelet cytoskeleton of fully spread platelets. (*A*) Platelets were allowed to spread onto a Formvar grid for 15 min at 37°C, and then were fixed with 1% glutaraldehyde, 0.2% tannic acid. Anti-PECAM-1 gold particles were then used to visualize PECAM-1 on the platelet surface. Note the even distribution of gold particles. The light region at the center is due to upward protrusion of the platelet granulomere, and is not antibody enriched. (*B*) Fully spread, unfixed platelets were labeled with anti-PECAM-1/gold and incubated for an additional 10 min at 37°C. Note the large concentration of gold particles at the center (*small arrows*) and the relative absence of particles at the periphery (*large arrows*). (*C* and *D*) Localization of platelet PECAM-1 to sites of intercellular contact. Fields illustrate overlapping regions of adjacent platelets at sites of interaction. Arrows indicate zones of concentrated anti-PECAM 1/18-nm gold particles that are representative of multiple fields observed. Antibodies to GPIIb-IIIa showed similar distribution, while control antibodies to GPIb-IX complex did not become similarly localized. (*C*) × 20,000; (*D*) × 30,000.

of PO_4/PECAM-1 incorporation may be >1 (Hillery, C. A., and P. J. Newman, manuscript in preparation), suggesting that all PECAM-1 molecules that associate with the cytoskeleton may in fact be phosphorylated first. Future studies aimed at identification of the precise serine residue(s) within the cytoplasmic domain that become phosphorylated, as well as site-specific prevention of its phosphorylation, will be helpful in establishing the role, if any, of phosphoserine residues in mediating the linkage of PECAM-1 with specific cytoskeletal elements.

In addition to the biochemical evidence of PECAM-1/cytoskeletal association, we also used a morphological approach to show that PECAM-1 is functionally linked to the platelet cytoskeleton. As previously pointed out by Fox et al. (10), protein–protein associations that are detected after detergent lysis could conceivably have occurred during the solubilization process, highlighting the importance of performing correlative biochemical and morphological studies before drawing conclusions regarding the association of a membrane glycoprotein with the underlying cytoskeleton. At least three different types of movement of glycoprotein receptors within the plane of the platelet plasma membrane have been previously described: receptor clustering (16, 39, 42, 51), translocation of receptors into the open canalicular system (OCS) (22), and centripetal movement of receptor/ligand or receptor/antireceptor complexes towards the center of fully spread platelets (23). Both clustering and translocation of receptors to the OCS are thought to be driven by the underlying membrane skeleton, and occur independently of reorganization of the cytoplasmic cytoskeleton (16, 21, 22), while centripetal redistribution of receptors from the platelet periphery into the central zone of fully spread platelets has been shown to be critically dependent on the presence of an intact, contractile actin cytoskeleton, and is abolished in the presence of both cytochalasin B (50), as well as cytochalasins D and E (35). Therefore, the spreading platelet system was used to investigate whether or not PECAM-1 formed a functional link with the cytoskeleton in intact cells. PECAM-1 was found to be evenly distributed over the platelet surface in fully spread cells that had been fixed to prevent receptor mobility (Fig. 5 *A*). Anti-PECAM-1 antibodies, however, triggered a rapid, cytoskeletally directed redistribution of PECAM-1 from the periphery of unfixed, fully spread platelets into the central region (Fig. 5 *B*). Thus, PECAM-1, like the fibrinogen receptor, GPIIb–IIIa, appears to establish a link with the underlying cytoplasmic actin cytoskeleton that results in its movement within the plane of the membrane. We postulate that PECAM-1, like GPIIb–IIIa, may function in this manner to direct incoming platelets towards the center of a developing platelet thrombus, thereby organizing and consolidating the platelet clot (3).

Although the majority of PECAM-1 was found to redistribute toward the platelet granulomere, a subset of PECAM-1 molecules was found to remain at points of platelet–platelet contact (Fig. 5, *C* and *D*), suggesting that they may function to stabilize platelet–platelet interactions. That these peripherally localized PECAM-1 molecules do not play a major role in mediating platelet cohesion is evidenced by the fact that (*a*) platelets from patients with Glanzmann thrombasthenia, which lack functional fibrinogen receptors (31) but express the full complement of PECAM-1 (5), are not capable of aggregating in response to any normal physiologic stimulus, and (*b*) anti-PECAM-1 polyclonal or monoclonal antibodies do not interfere significantly with the platelet aggregation process (27, 29, 34) (P. J. Newman, unpublished observations). Future studies will be required to determine the function of the subpopulation of PECAM-1 that remains at the site of interplatelet contact.

In summary, the major findings of the study are that: (*a*) PECAM-1 becomes phosphorylated on serine residues upon cellular activation; (*b*) coincident with the activation process, PECAM-1 becomes specifically associated with the Triton-insoluble cytoskeleton; and (*c*) the association of PECAM-1 with contractile elements of the platelet cytoskeleton results in its centralization in response to surface activation events that take place during the platelet spreading process. Based upon these results, it would be of considerable interest to examine the effect of deleting the cytoplasmic domain of PECAM-1 on its ability to localize to the intercellular junctions of transfected fibroblasts using the system that we have previously described (2). Serine to alanine mutagenesis within the cytoplasmic domain could also be used to localize precisely which serine(s) are necessary to become phosphorylated for cytoskeletal association to occur. Finally, although PECAM-1 appears to make connections with the cytoskeleton, the specific cytoskeletal elements with which it interacts remain to be identified. It is expected that further elucidation of the cellular and molecular properties of PECAM-1 will be important in helping us understand its role in vascular biology, inflammation, wound healing, and the immune response.

The authors thank Dr. Gilbert C. White for helpful comments on PECAM-1/cytoskeletal associations, and Dr. William A. Muller for supplying the hec7 antibody. This work was supported by Public Health Services grants HL-40926 (P. J. Newman) and HL-38622 (S. E. Rittenhouse) from the National Institutes of Health.

Received for publication 10 April 1992 and in revised form 16 June 1992.

References

1. Albelda, S. M., P. D. Oliver, L. H. Romer, and C. A. Buck. 1990. EndoCAM: a novel endothelial cell-cell adhesion molecule. *J. Cell Biol.* 110:1227–1237.
2. Albelda, S. M., W. A. Muller, C. A. Buck, and P. J. Newman. 1991. Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. *J. Cell Biol.* 114:1059–1068.
3. Albrecht, R. M., S. L. Goodman, and S. R. Simmons. 1989. Distribution and movement of membrane-associated platelet glycoproteins: use of colloidal gold with correlative video-enhanced light microscopy, and high-voltage transmission electron microscopy. *Am. J. Anat.* 185:149–164.
4. Ashman, L. K., and G. W. Aylett. 1991. Expression of CD31-epitopes on human lymphocytes – CD31-monoclonal antibodies differentiate between naive (CD45RA+) and memory (CD45RA-) CD4-positive T-cells. *Tissue Antigens.* 38:208–212.
5. Burk, C. D., P. J. Newman, S. Lyman, J. Gill, B. S. Coller, and M. Poncz. 1991. A deletion in the gene for glycoprotein IIb associated with Glanzmann's thrombasthenia. *J. Clin. Invest.* 87:270–276.
6. Cabanas, C., F. Sanchez-Madrid, T. Bellon, C. G. Figdor, A. A. Te Velde, J. M. Fernandez, A. Acevedo, and C. Bernabeu. 1989. Characterization of a novel myeloid antigen regulated during differentiation of monocytic cells. *Eur. J. Immunol.* 19:1373–1378.
7. Cooper, J. A., B. M. Sefton, and T. Hunter. 1983. Detection and quantification of phosphotyrosine in proteins. *Methods Enzymol.* 99: 387–402.
8. Fawcett, J., A. L. Harris, and R. Bicknell. 1991. Isolation and properties in culture of human adrenal capillary endothelial cells. *Biochem. Biophys. Res. Commun.* 174:903–908.
9. Fox, J. E. B. 1985. Linkage of a membrane skeleton to integral membrane glycoproteins in human platelets. Identification of one of the glycoproteins as glycoprotein Ib. *J. Clin. Invest.* 76:1673–1683.

10. Fox, J. E. B. 1985. Identification of actin-binding protein as the protein linking the membrane skeleton to glycoproteins on platelet plasma membranes. *J. Biol. Chem.* 260:11970–11977.
11. Fox, J. E., and D. R. Phillips. 1982. Role of phosphorylation in mediating the association of myosin with the cytoskeletal structures of human platelets. *J. Biol. Chem.* 257:4120–4126.
12. Fox, J. E. B., C. C. Reynolds, and J. K. Boyles. 1992. Methods for studying the platelet cytoskeleton in Triton X-100 lysates. *Methods Enzymol.* In press.
13. Goyert, S. M., E. M. Ferrero, S. V. Seremetis, R. J. Winchester, J. Silver, and A. C. Mattison. 1986. Biochemistry and expression of myelomonocytic antigens. *J. Immunol.* 137:3909–3914.
14. Hartwig, J. H., and M. DeSisto. 1991. The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments. *J. Cell Biol.* 112:407–425.
15. Hillery, C. A., S. S. Smyth, and L. V. Parise. 1991. Phosphorylation of human platelet glycoprotein IIIa (GPIIIa). Dissociation from fibrinogen receptor activation and phosphorylation of GPIIIa in vitro. *J. Biol. Chem.* 266:14663–14669.
16. Isenberg, W. M., R. P. McEver, D. R. Phillips, M. A. Shuman, and D. F. Bainton. 1987. The platelet fibrinogen receptor: an immunogold-surface replica study of agonist-induced ligand binding and receptor clustering. *J. Cell Biol.* 104:1655–1663.
17. Johnston, G. I., R. G. Cook, and R. P. McEver. 1989. Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. *Cell.* 56:1033–1044.
18. King, W. G., and S. E. Rittenhouse. 1989. Inhibition of protein kinase C by staurosporine promotes elevated accumulations of inositol triphosphates and tetrakisphosphotate in human platelets exposed to thrombin. *J. Biol. Chem.* 264:6070–6074.
19. King, W. G., G. L. Kucera, A. Sorisky, J. Zhang, and S. E. Rittenhouse. 1991. Protein kinase C regulates the stimulated accumulation of 3-phosphorylated phosphoinositides in platelets. *Biochem. J.* 278:475–480.
20. Knapp, W., B. Dorken, W. R. Gilks, E. P. Rieber, R. E. Schmidt, H. Stein, and A. E. G. Kr. von dem Borne. 1989. Leucocyte Typing IV. White Cell Differentiation Antigens. Oxford University Press, Oxford. 985–995.
21. Kouns, W. C., C. F. Fox, W. J. Lamoreaux, L. B. Coons, and L. K. Jennings. 1991. The effect of glycoprotein IIb-IIIa receptor occupancy on the cytoskeleton of resting and activated platelets. *J. Biol. Chem.* 266:13891–13900.
22. Leistikow, E. A., M. I. Barnhart, G. Escolar, and J. G. White. 1990. Receptor-ligand complexes are cleared to the open canalicular system of surface-activated platelets. *Br. J. Haematol.* 74:93–100.
23. Loftus, J. C., and R. M. Albrecht. 1984. Redistribution of the fibrinogen receptor of human platelets after surface activation. *J. Cell Biol.* 99:822–829.
24. Loftus, J. C., J. Choate, and R. M. Albrecht. 1984. Platelet activation and cytoskeletal reorganization: high voltage electron microscopic examination of intact and Triton-extracted whole mounts. *J. Cell Biol.* 98:2019–2025.
25. Lyons, A. B., S. J. Cooper, S. R. Cole, and L. K. Ashman. 1988. Human myeloid differentiation antigens identified by monoclonal antibodies to the myelomonocytic leukemia cell line RC-2A. *Pathology.* 20:137–146.
26. MacNicol, M., A. B. Jefferson, and H. Schulman. 1990. Ca^{2+}/Calmodulin kinase is activated by the phosphatidylinositol signaling pathway and becomes Ca^{2+}-independent in PC12 cells. *J. Biol. Chem.* 265:18055–18058.
27. Mazurov, A. V., D. V. Vinogradov, N. V. Kabaeva, A. N. Antonova, Y. A. Romanov, T. N. Vlasik, A. S. Antonov, and V. N. Smirnov. 1991. A monoclonal antibody, VM64, reacts with a 130 kDa glycoprotein common to platelets and endothelial cells: heterogeneity in antibody binding to human aortic endothelial cells. *Thromb. Haemost.* 66:494–499.
28. McEver, R. P., J. H. Beckstead, K. L. Moore, L. Marshall-Carlson, and D. F. Bainton. 1989. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest.* 84:92–99.
29. Metzelaar, M. J., J. Korteweg, J. J. Sixma, and H. K. Nieuwenhuis. 1991. Biochemical characterization of PECAM-1 (CD31 antigen) on human platelets. *Thromb. Haemost.* 66:700–707.
30. Muller, W. A., C. M. Ratti, S. L. McDonnell, and Z. A. Cohn. 1989. A human endothelial cell-restricted externally disposed plasmalemmal protein enriched in intercellular junctions. *J. Exp. Med.* 170:399–414.
31. Newman, P. J. 1991. Platelet GPIIb-IIIa: molecular variations and alloantigens. *Thromb. Haemost.* 66:111–118.
32. Newman, P. J., M. C. Berndt, J. Gorski, G. C. White, S. Lyman, C. Paddock, and W. A. Muller. 1990. PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science (Wash. DC).* 247:1219–1222.
33. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.).* 308:693–695.
34. Ohto, H., H. Maeda, Y. Shibata, R-F. Chen, Y. Qzaki, M. Higashihara, A. Takeuchi, and H. Tohyama. 1985. A novel leukocyte differentiation antigen: two monoclonal antibodies TM2 and TM3 define a 120-kd molecule present on neutrophils, monocytes, platelets, and activated lymphoblasts. *Blood.* 66:873–881.
35. Olorundare, O. E., S. R. Simmons, and R. M. Albrecht. 1992. Cytochalasin-D and Cytochalasin-E—effects on fibrinogen receptor movement and cytoskeletal reorganization in fully spread, surface-activated platelets—a correlative light and electron microscopic investigation. *Blood.* 79:99–109.
36. Painter, R. G., and M. Ginsberg. 1982. Concanavalin A induces interactions between surface glycoproteins and the platelet cytoskeleton. *J. Cell Biol.* 92:565–573.
37. Parise, L. V., A. B. Criss, L. Nannizzi, and M. R. Wardell. 1990. Glycoprotein IIIa is phosphorylated in intact human platelets. *Blood.* 75: 2363–2368.
38. Phillips, D. R., L. K. Jennings, and H. H. Edwards. 1980. Identification of membrane proteins mediating the interaction of human platelets. *J. Cell Biol.* 86:77–86.
39. Polley, M. J., L. L. K. Leung, F. Y. Clark, and R. L. Nachman. 1981. Thrombin-induced platelet membrane glycoprotein IIb and IIIa complex formation. An electron microscope study. *J. Exp. Med.* 154:1058–1068.
40. Rittenhouse, S. E. 1984. Activation of human platelet phospholipase C by ionophore A23187 is totally dependent upon cyclooxygenase products and ADP. *Biochem. J.* 222:103–110.
41. Sano, K., Y. Takai, J. Yamanishi, and Y. Nishizuka. 1983. A role of calcium-activated phospholipid-dependent protein kinase in human platelet activation. Comparison of thrombin and collagen actions. *J. Biol. Chem.* 258:2010–2013.
42. Santoso, S., U. Zimmermann, J. Neppert, and C. Mueller-Eckhardt. 1986. Receptor patching and capping of platelet membranes induced by monoclonal antibodies. *Blood.* 67:343–349.
43. Simons, D. L., C. Walker, C. Power, and R. Pigott. 1990. Molecular cloning of CD31, a putative intercellular adhesion molecule closely related to carcinoembryonic antigen. *J. Exp. Med.* 171:2147–2152.
44. Skinner, M. P., C. M. Lucas, G. F. Burns, C. N. Chesterman, and M. C. Berndt. 1991. GMP-140 binding to neutrophils is inhibited by sulfated glycans. *J. Biol. Chem.* 266:5371–5374.
45. Stenberg, P. E., R. P. McEver, M. A. Shuman, Y. V. Jacques, and D. F. Bainton. 1985. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J. Cell Biol.* 101: 880–886.
46. Stockinger, H., S. J. Gadd, R. Eher, O. Majdic, W. Schreiber, W. Kasinrerk, B. Strass, E. Schnabl, and W. Knapp. 1990. Molecular characterization and functional analysis of the leukocyte surface protein CD31. *J. Immunol.* 145:3889–3897.
47. Torimoto, Y., D. M. Rothstein, N. H. Dang, S. F. Schlossman, and C. Morimoto. 1992. CD31, a novel cell surface marker for CD4 cells of suppressor lineage, unaltered by state of activation. *J. Immunol.* 148:388–396.
48. van Mourik, J. A., O. C. Leeksma, J. H. Reinders, P. G. de Groot, and J. Zandbergen-Spaargaren. 1985. Vascular endothelial cells synthesize a plasma membrane protein indistinguishable from platelet membrane glycoprotein IIa. *J. Biol. Chem.* 260:11300–11306.
49. Wheeler, M. E., A. C. Cox, and R. C. Carroll. 1984. Retention of the glycoprotein IIb-IIIa complex in the isolated platelet cytoskeleton. Effects of separable assembly of platelet pseudopodal and contractile cytoskeletons. *J. Clin. Invest.* 74:1080–1089.
50. White, J. G. 1990. Induction of patching and its reversal on surface-activated human platelets. *Br. J. Haematol.* 76:108–115.
51. White, J. G., and G. Escolar. 1990. Induction of receptor clustering, patching, and capping on surface-activated platelets. *Lab. Invest.* 63:332–340.
52. Wong, C. S., J. R. Gamble, M. P. Skinner, C. M. Lucas, M. C. Berndt, and M. A. Vadas. 1991. Adhesion protein GMP140 inhibits superoxide anion release by human neutrophils. *Proc. Natl. Acad. Sci. USA.* 88: 2397–2401.
53. Zhang, J., M. J. Fry, M. D. Waterfield, S. Jaken, L. Liao, J. E. B. Fox, and S. E. Rittenhouse. 1992. Activated phosphoinositide 3-kinase associates with membrane skeleton in thrombin-exposed platelets. *J. Biol. Chem.* 267:4686–4692.