Platelet Endothelial Aggregation Receptor 1 (PEAR1), a Novel Epidermal Growth Factor Repeat-containing Transmembrane Receptor, Participates in Platelet Contact-induced Activation*

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The present study was designed to identify novel membrane proteins that signal during platelet aggregation. Because one putative mechanism for signaling by a membrane protein involves phosphorylation, we used oligonucleotide-based microarray analyses and mass spectrometric proteomics techniques to specifically discover membrane proteins and also identify those proteins that become phosphorylated on tyrosine, threonine, or serine residues upon platelet aggregation. Surprisingly, both techniques converged to identify a novel membrane protein we have termed PEAR1 (platelet endothelial aggregation receptor 1). Sequence analysis of PEAR1 predicts a type-1 membrane protein, 15 extracellular epidermal growth factor-like repeats, and multiple cytoplasmic tyrosines. Analysis of the tissue distribution of PEAR1 showed that it was most highly expressed in platelets and endothelial cells. Upon platelet aggregation induced by physiological agonists, PEAR1 became phosphorylated on tyrosine (Tyr-925), and serine (Ser-953 and Ser-1029) residues. PEAR1 tyrosine phosphorylation was blocked by eptifibatide, an IIb/IIIa antagonist, which inhibits platelet aggregation. Immune clustering of PEAR1 resulted in PEAR1 phosphorylation. Aggregation-induced PEAR1 tyrosine phosphorylation lead to the subsequent association with the SheB adaptor protein. Platelet proximity induced by centrifugation also induced PEAR1 tyrosine phosphorylation, a reaction not inhibited by eptifibatide. These data suggest that PEAR1 is a novel platelet receptor that signals secondary to αIIbβ3-mediated platelet-platelet contacts.

Platelet aggregation during arterial thrombosis results in ischemic complications precipitating in acute myocardial infarction and stroke. Platelet aggregation is known to be mediated by signaling events initiated by primary platelet agonists such as thrombin, ADP, and collagen, which induce a conformational change in the platelet integrin αIIbβ3, allowing it to bind soluble fibrinogen and von Willebrand factor, resulting in platelet cross-linking. Platelet-platelet contacts during aggregation subsequently initiate secondary signaling events. Aggregation-induced signaling can result in multiple platelet secondary signaling events such as calcium mobilization, protein tyrosine phosphorylations, cytoskeletal rearrangements, and the release of platelet-dense bodies and α-granules. Aggregation-induced signaling is key to the formation of stable aggregates, particularly when aggregation is induced by low concentrations of one or more primary agonists. Platelet activation also causes the release of ADP from dense bodies and the generation of thromboxane A2, both of which induce further platelet stimulation.

Several mediators of aggregation-induced signals have been identified. One is αIIbβ3 itself, which becomes tyrosine-phosphorylated and also associates with numerous signaling and cytoskeletal proteins following platelet activation, allowing fibrinogen and/or von Willebrand factor binding and platelet aggregation. The importance of αIIbβ3 “outside-in” signaling in the enhancement of platelet aggregation was demonstrated by the generation of knock-in mice where tyrosine residues Tyr-747 and Tyr-759 were mutated to phenyalanine (1). These so-called DiYF mice displayed selective impairment of outside-in signaling, resulting in the formation of unstable aggregates. Other mediators are released from the activated platelets. One is the soluble CD40 ligand, a hydrolytic product produced by metalloprotease cleavage of the CD40 ligand on activated platelets that subsequently binds to αIIbβ3. Another is GAS5, a protein released from α-granules that is involved in the stabilization of platelet-rich thrombi (2, 3).

Although the importance of each of the platelet secondary signaling reactions described above is well documented, these reactions are dependent upon platelet activation. It has also been well documented, however, that platelet stimulation can be induced by platelet-platelet contact. Signaling of platelet receptors induced by platelet-platelet proximity, independent of platelet aggregation, have not been described. One exception may be the Eph kinases and ephrins, specifically EphA4 and ephrinB1, which, through receptor-ligand interactions on the platelet surface, enhance the binding of αIIbβ3 to immobilized fibrinogen in the presence of physiological agonists (4, 5); however, the importance of this mechanism in platelet-platelet signaling on unstimulated platelets is unknown.

The present study was designed to identify novel platelet proteins involved in platelet proximity-induced activation. Reasoning that many signaling receptors become tyrosine-phosphorylated during signaling, we sought not only to identify novel receptors on platelets but to specifically identify those that become phosphorylated upon platelet-platelet interac-

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Tyrosine-phosphorylated PEAR1

1034-amino acid protein we termed PEAR1 (platelet-endothelial aggregation receptor 1). Bioinformatic analyses revealed that PEAR1 is a type 1 membrane protein containing fifteen EGF-like repeats in its extracellular domain. The intracellular domain contains five proline rich domains, which may interact with Src homology 3 domain-containing proteins, as well as four potential tyrosine phosphorylation sites (Tyr-804, Tyr-925, Tyr-943, Tyr-979). The LC/MS/MS data demonstrated that PEAR1 is, in fact, tyrosine-phosphorylated at Tyr-925. We further demonstrated that PEAR1 becomes tyrosine phosphorylated in response to receptor clustering and during platelet aggregation. Finally, we showed that the PEAR1 signaling is induced through platelet-platelet contacts independent of platelet activation. These data are the first demonstration of a platelet receptor to signal through platelet-platelet contacts independent of platelet activation.

MATERIALS AND METHODS

Reagents—The following reagents were purchased from the suppliers listed in parentheses: anti-Myc (9E10) monoclonal IgG (Covance, Princeton, NJ); goat anti-rabbit HRP and goat anti-mouse HRP (Bio-Rad); anti-PY99 monoclonal IgG (Santa Cruz Technologies, Santa Cruz, CA); anti-phospho-Src (Tyr-416) polyclonal IgG, anti-Phc monoclonal IgG, and anti-S-Sch monoclonal IgG (Cell Signaling Technology, Beverly, MA); 4G10 anti-pY monoclonal antibody (Upstate Cell Signaling Solutions); anti-CD62-P and anti-PY20 monoclonal antibodies and isotype-specific anti-mouse and anti-rabbit antibodies (BD Biosciences); IV.3 monoclonal antibody hybridoma (American Type Culture Collection, Manassas, VA); thrombin (Hematologic Technology, Essex Junction, VT); Collagen (Chromolog Corp., Havertown, PA); TRAP (SynPep Corp., Dublin, CA); epitifibatide, (Millennium Pharmaceuticals, Cambridge, MA); Easy-IFIT kit (Pierce); Superscript II reverse transcriptase, platinum Pfx DNA polymerase, and pCR-BluntII-Topo vector (Invitrogen); goat anti-rabbit/mouse IgG HRP (Bio-Rad); ECL chemiluminescence detection kit and protein G-Sepharose (Amerham Biosciences); Restore stripping buffer (Pierce); and Immobilon-P polyvinylidene difluoride membranes (Millipore).

Purification of Platelet RNA—Platelets were collected from a healthy volunteer by apheresis, including a filtration step that removed lym- phocytes and platelet contaminants following an Institutional Review Board-approved protocol. This procedure yielded 108 platelets and immediately vortexed to facilitate platelet lysis. The supernatant was removed, and the platelet pellet was re-

100 rpm in a Beckman Coulter Allegra 6 centrifuge at room temper-

ature. The supernatant was removed, and the platelet pellet was re-

2200 rpm in a Beckman Coulter Allegra 6 centrifuge at room temper-

ature.

Cell Culture and Transfection—COS-7 cells were cultured in Dul-

becco’s modified Eagle’s media supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in 6-well dishes (4 × 10^6 cells/well). After 24 h, transfections were performed using FuGENE 6 (2 μg of DNA per well) for 24 h. The transfection efficiency was determined using a luciferase reporter assay. For Western analysis, cells were harvested and total cellular RNA was extracted using TRIzol (Invitrogen). Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen). RT-PCR was performed using Platinum Taq DNA polymerase (Invitrogen) and oligonucleotide primers for PEAR1. PCR products were analyzed on 1% agarose gels.

Immunoprecipitations and Western Blotting from COS-7 Cells—Transfected cells were lysed with 0.4 ml of lysis buffer (40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 100 μM sodium orthovanadate, 10 mM NaF, 1 mM Pefabloc, 10 μM leupeptin, and 10 μg/ml aprotinin). For immunoprecipitations, lysates were incubated with 2 μg of the indicated antibodies. For Western analysis, polyvinylidene difluoride membranes were incubated with the indicated primary antibodies (1 μg/ml) for 2 h at 4 °C. Membranes were then incubated with 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG for 1 h at room temperature and developed with the ECL™ chemiluminescence detection kit. Methodologies used for SDS-PAGE, Western blotting, and immunoprecipitations have been described previously (8).

Phosphopeptide Identification by In-gel Digestion and Mass Spectrometry—Washed human platelets were aggregated with 5 μM human (Affymetrix) and Rosetta Resolver (Rosetta Biosoftware). cRNA hybridized to probe sets with intensity p values of ≤0.01 were identified. Platelet cRNA hybridized to a probe set for FLJ00193, and through bioinformatic analyses it was determined that the FLJ00193 cDNA encoded an incomplete open reading frame that contained two EGF repeats and a potential transmembrane domain. A proprietary in-house DNA sequence and a publicly available DNA sequence were used to assemble a 3.6-kb predicted cDNA. Platelet cDNA was synthesized by using Superscript II reverse transcriptase. PEAR1 cDNA was amplified using Platinum Pfx DNA polymerase and the primers 5’-CCGCTCGAGACGGTCCTGGCGTCGAAGTGG-3’ (forward) and 5’-GCTCTAGATGGGATCCGAGTCCTGGCAGAGGTGATG-3’ (reverse). The resulting 3.2-kb cDNA was cloned into pCR-BluntII-Topo vector.

Generation and Purification of Anti-peptide Antibodies—Anti-Pear1 rabbit polyclonal antibodies were generated by BIOSOURCE using keyhole limpet hemocyanin-conjugated peptides derived from the N-terminal extracellular domain (aa 72–88; YRTVYQVYKT-DHQRRL) and the intracellular domain (as 856–874, QHGDHTTL-PADWKRHRREP). Serum was affinity-purified using the immunizing peptide coupled to a thiol reactive gel. The antibodies raised against peptide 71–85 are referred to as α-extracellular domain (α-ECD) PEAR1 IgGs. The antibodies raised against peptide 864–874 are referred to as α-intracellular domain (α-ICD) PEAR1 IgGs.

In situ Localization of PEAR1 mRNA Using Sensitized Oligonucleotide Probes Detected by ABC Peroxidase—Formalin-fixed, paraffin-embedded human and rodent tissues were used in this study. Digoxi- genin-labeled riboprobes were used to localize PEAR1 mRNA. The details of the non-isotopic in situ hybridization and the tyramide-mediated signal amplification, as well as the methods used for acquiring digital images, have been described earlier (6, 7). Following the manu-

factured protocol (Roche Applied Science), digoxigenin-labeled anti- sense and sense riboprobes were generated from a DNA template con- taining nucleotides 2369–2896.

TaqMan Analysis—TaqMan experiments were performed using an ABI PRISM 7700 system (Applied Biosystems). Primers were designed using Primer Express software. 50 ng of RNA from a variety of human tissues and primary cell lines were used for analysis. PEAR1 and glyceraldehyde-3-phosphate dehydrogenase PCR products were labeled with different fluorophores as per the manufacturer’s protocol. β-Macroglobulin was used as an endogenous control to allow for normalization of the amount of RNA added to each reaction. The differential labeling of the target gene (PEAR1) and the reference gene (β-macroglobulin) occurred in the same well. hPEAR1 Constructs, FLAG- and Myc-tagged—The primers 5’-CGGAATTCACCCAGGTACTTCAATACCCTGC-3’ (forward) and 5’-GCTCTAGATTTACGCTTGGCTCAGAGGTGATG-3’ (reverse) were used to PCR-amplify a PEAR1 fragment that coded for amino acids 23–1037. The resulting PCR fragment was digested with EcoR1 and BamH1 and subcloned into pcDNA3-v-Src. A C-terminally Myc-tagged PEAR1 was constructed by PCR amplification using the primers 5’-CCGCTCGAGACGGTCCTGGCGTCGAAGTGG-3’ (forward) and 5’-GCTCTAGATTTACGCTTGGCTCAGAGGTGATG-3’ (reverse) and the reference gene (β-macroglobulin) occurred in the same well. hPEAR1 Constructs, FLAG- and Myc-tagged—The primers 5’-CGGAATTCACCCAGGTACTTCAATACCCTGC-3’ (forward) and 5’-GCTCTAGATTTACGCTTGGCTCAGAGGTGATG-3’ (reverse) were used to PCR-amplify a PEAR1 fragment that coded for amino acids 23–1037. The resulting PCR fragment was digested with EcoRI and Xhol and subcloned into pFLAG-CMV1 (Sigma) to express a mem-

brane-localized, terminal-FLAG-tagged PEAR1. A C-terminally Myc-tagged PEAR1 was constructed by PCR amplification using the primers 5’-CCGCTCGAGACGGTCCTGGCGTCGAAGGTGATG-3’ (forward) and 5’-GCTCTAGATTTACGCTTGGCTCAGAGGTGATG-3’ (reverse) and the reference gene (β-macroglobulin) occurred in the same well.

1 The abbreviations used are: LC/MS/MS, liquid chromatography tandem mass spectrometry; aa, amino acid(s); ECD, extracellular domain; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; HUVEC, human umbilical vein endothelial cell; HRP, horseradish peroxidase; ICD, intracellular domain; PEAR1, platelet endothelial aggregation receptor 1; iPPEAR1, human PEAR1; iPPEAR1, mouse PEAR1; FTB, phosphoryrosine binding; SREC, scavenger receptor expressed by endothelial cells; TRAP, thrombin receptor activating peptide.
Tyrosine-phosphorylated PEAR1

TRAP as described below and solubilized in 2× lysis buffer (20 mM Tris-HCl, pH 8, 2% Triton X-100, 4 mM EDTA, 250 mM NaCl, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). Solubilized platelet-aggregated proteins from 6 × 10^6 platelets were incubated with α-phosphotyrosine IgG beads (PY99; Santa Cruz Biotechnology) overnight at 4 °C. The phosphopeptides were eluted with 100 mM phenyl phosphate, resolved by SDS-PAGE, and visualized by Coomassie staining. All Coomassie-stained bands were excised and subjected to in-gel proteolytic digestion with trypsin and analyzed by LC/MS/MS (9). Data-dependent LC/MS/MS was performed using electrospray ionization on an LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan). An aliquot of each digest mixture was introduced to the mass spectrometer by reversed-phase chromatographic separation with a 75-μm inner diameter capillary column flowing at a rate of ~350 nl/min and eluted using a 30-min acetoniitride, 0.1% formic acid gradient. Chromatographic separation yielded ~30-peak widths, and mass spectra were acquired in 9-s cycles. Each cycle was of the form consisting of one full mass spectrometry scan followed by four MS/MS scans on the most abundant precursor ions subjected to dynamic exclusion for a period of 1.5 min. The identity of each peptide sequence was determined by interpreting the MS/MS spectra using the SpectrumMill software (Agilent Technologies).

**Platelet Preparation**—Human venous blood from healthy drug-free donors was drawn into ACDF (85 mM citrate, 111 mM glucose, 714 mM citric acid) supplemented with 50 ng/ml prostacyclin (Sigma) and washed as described previously (10). Washed platelets were resuspended in Tyrode’s solution-Hepes buffer with 1 mM CaCl2 and MgCl2 at a cell density of ~1–3 × 10^7 platelets/ml.

**Platelet Lysate Preparation**—Aggregates were measured in a Chrono-Log Lumiaggregometer. Human washed platelets (3 × 10^7 cells/ml) were initiated with 0.5 units/ml thrombin (Hematologic Technologies). Human venous blood from healthy drug-free donors was drawn into ACDF (85 mM citrate, 111 mM glucose, 714 mM citric acid) supplemented with 50 ng/ml prostacyclin (Sigma) and washed as described previously (10). Washed platelets were resuspended in Tyrode’s solution-Hepes buffer with 1 mM CaCl2 and MgCl2 at a cell density of ~1–3 × 10^7 platelets/ml.

**Immunoprecipitation and Cross-linking of Surface PEAR-1**—Immunoprecipitations were performed with Protein G beads and 2.5 μg/ml α-ICD PEAR1 IgG or isotype control overnight at 4 °C. Immunoblotting of the complexes were performed with α-pTyr-4G10 (Upstate Cell Signaling) and α-pTyr-PY20 (Santa Cruz Biotechnology) mouse antibodies. For cross-linking experiments, 3 × 10^6 platelets/ml were incubated with 5 μg/ml α-ECF PEAR1 IgG in the presence of 25 μg/ml IV.3 monoclonal antibody for 30 min on ice followed by cross-linking the bound IgG with α-rabbit secondary antibody for 10 min at 37 °C. Post cross-linking, the reactions were stopped by the addition of ice-cold 2× lysis buffer. Immunoprecipitation of PEAR1 was performed as described above.

**Flow Cytometry Analysis**—α-ECF PEAR1 IgG was FITC-labeled using the EASY-FITC kit. Washed human platelets (1 × 10^7) were stained or were activated with 5 μM TRAP for 5 min at 37 °C without stirring. Platelets were fixed with 2% (v/v) paraformaldehyde for 30 min at room temperature and stained with nonspecific rabbit-IgG-FITC, α-ECF PEAR1-FITC, or α-CD62-P (P-selectin)-phycoerythrin. Platelets were analyzed on a FACSsort flow cytometer (BD Biosciences).

**RESULTS**

**Identification of PEAR1 through Gene Profiling**—In an effort to identify novel receptors in human platelets, high throughput molecular profiling of RNA isolated from double-phoresed platelets was performed on a custom array that contained probe sets representing 25,000 genes. The sequences for the probe sets represented on the custom arrays were derived both from sequencing of platelet and HUVEC cDNA libraries as well as all the non-overlapping human genes present in the public gene data base. The protein coding sequences of the platelet cRNAs, which hybridized to the microarrays, were then classified based on PROSITE, Pfam, and SMART (11) sequence-motif searches. The selected probe sets were assigned struc-
presented in Fig. 2A. The chromosomal localizations and the degree of identities between hPEAR1 and mPEAR1, KIAA1780, KIAA1781, CED-1, and SREC-I are summarized in Table I. The domain structures present in PEAR1 and related proteins are shown in Fig. 2B. Putative signaling motifs present within the ICD of PEAR1 were identified by the Scansite program (14). Five potential Src homology 3-binding, proline-rich domains (Fig. 3) were identified in hPEAR1 and mPEAR1 but not in KIAA1780 or KIAA1781. Human PEAR1, mPEAR1, and KIAA1780 contain an NPXY motif (Fig. 3) that is known to act as interaction sites for PTB domain-containing proteins (15). The Scansite program also predicted that the tyrosine residues 804, 925, 943, and 979 present in hPEAR1 may be tyrosine-phosphorylated (Fig. 3). Similar tyrosine residues are also present in mPEAR1, KIAA1780, and KIAA1781. Based on overall homology, it is likely that PEAR1, KIAA1780, and KIAA1781 represent a novel family of EGF repeat-containing proteins. Because of the EGF repeats and their homology to SREC and CED-1, possible functions for this novel family of proteins include cell adhesion (16), the uptake of acetylated low density lipoprotein (13), and phagocytosis of apoptotic bodies (12).

Identification of hPEAR1 through Proteomics—We also employed a proteomics approach to identify novel membrane receptors and specifically targeted proteins that become tyrosine-phosphorylated during aggregation. Platelet aggregates were detergent-solubilized, and phosphotyrosine proteins were captured on an antibody phosphotyrosine affinity column (PY99). The bound proteins were eluted with phenylphosphate and subjected to SDS-PAGE. Eight bands were detected post Coomassie staining (Fig. 4A). The identities of these eluted proteins are as follows: band 1, Dep-1; band 2, Ship-1; band 3, CD84; band 4, Lyn; band 5, SLP-76; bands 6 and 7, Lyn and Lck; and band 8, Dok-2. Many of the proteins identified are well known signaling proteins in platelets. The in-gel digestion of band 2 (Fig. 4A) migrating at \( \pm 150-180 \text{ kDa} \) revealed seven peptides whose sequences co-
responded to that of human PEAR1. The locations of the seven peptides are shown in Fig. 1. Most importantly, the mass spectra of three of these seven peptides were phosphorylated at Tyr-925, Ser-953, and Ser-1029 (Fig. 4B). The locations of the phosphorylated residues are shown in Figs. 1 and 3.

**Tissue Distribution**—To investigate the expression pattern of PEAR1, real time quantitative PCR (TaqMan) was performed on a variety of human tissues and primary cell lines (Fig. 5A). PEAR1 is most highly expressed in HUVECs, followed by megakaryocytes, osteoblasts, coronary smooth muscle cells, and erythroid cells. We also found low or no expression in peripheral blood leukocytes or macrophages. In normal human tissues, TaqMan analysis revealed expression in heart, kidney, skeletal muscle, pancreas, ovary, breast, lung, brain cortex, hypothalamus, spinal cord, and dorsal root ganglion. No expression was detected in liver, small intestine, and colon. A 5-kb transcript was detected by probing a Northern blot containing poly(A)-enriched mRNA from numerous human tissues; an expression pattern similar to that observed by TaqMan analysis was obtained (data not shown).

**In situ** hybridizations revealed that PEAR1 is expressed in megakaryocytes present in rat adult femur marrow sections (Fig. 5B, section A), as well as in platelet aggregates present in human peripheral blood (Fig. 5B, section B). PEAR1 expression was also detected in endothelial cells of human umbilical cord artery and vein tissue sections (Fig. 5B, sections C and D) and also in microcapillaries and larger vessels of 16-day-old mouse embryo lung and heart tissues (Fig. 5B, sections E and F).

Western blotting with the α-ICD PEAR1 IgG detected a protein of ~150 kDa in COS-7 cells transfected with the full-length hPEAR1 cDNA. The α-ICD IgG also recognized a 150-kDa protein in HUVEC and platelet lysates (Fig. 5C, lanes 2 and 5) along with an additional weaker band, migrating at ~180 kDa, cross-reacting in platelet lysates (lane 5). Only the 150-kDa band was immunoprecipitated from both HUVECs and platelets (Fig. 5C, lanes 4 and 7). The α-ICD PEAR1 IgG was also used to probe the expression of PEAR1 in sections of human umbilical cord, revealing expression in endothelial cells and aggregated platelets within a blood clot (data not shown).

**Platelet Distribution of PEAR1**—The expression of PEAR1 on the surface of control and stimulated platelets was detected by staining unpermeabilized, paraformaldehyde-fixed resting and TRAP-activated platelets with an α-ICD PEAR1, ECD PEAR1 IgG. The α-ICD PEAR1 IgG was also used to probe the expression of PEAR1 in sections of human umbilical cord, revealing expression in endothelial cells and aggregated platelets within a blood clot (data not shown).

**Table I**

| Chromosome | Message | Amino acids | ECD aa | ICD aa | EGF repeats | Homology of α-ICD PEAR1
|------------|---------|-------------|--------|--------|-------------|---------------------------|
| hPEAR1     | 1q23.1  | 3282        | 1037   | 754    | 259         | 100                      |
| mPEAR1     | 3       | 4290        | 1034   | 753    | 257         | 76/77                    |
| hKIAA1780  | 5q33    | 7522        | 1140   | 885    | 261         | 43/44                    |
| hKIAA1781  | 15q22.2 | 5702        | 969    | 773    | 172         | 48/25                    |
| CED-1      | AF332568| Unknown     | 3784   | 1111   | 910         | 38/0                     |
| SREC       | NM_003893| 17P13.3     | 3457   | 830    | 420         | 38/0                     |

*GenBank™ accession number.

1 First number is ECD percentage, second number is ICD percentage.

**Fig. 3.** Alignment of the intracellular domains of human PEAR1, mPEAR1, KIAA1780, and KIAA1781 and identification of potential tyrosine phosphorylation sites and interaction motifs. The intracellular domains were aligned based on a ClustalW analysis. Amino acid identities are highlighted as boxed regions. The Scansite program (14) was used to identify potential tyrosine phosphorylation and protein-protein interaction motifs. Predicted tyrosine phosphorylation sites are marked by black stars. NPXY motifs are boxed by dashed lines, and proline-rich domains present in hPEAR1 are outlined by thick black boxes. Experimentally identified phosphorylation sites are marked by gray stars. The locations of the seven sequenced peptides are indicated by the dashed lines above the hPEAR1 sequence.
Fluorescence-activated cell sorter analyses indicated PEAR1 expression on the surface in resting platelets, and, interestingly, there was no increase in H9251-ECD PEAR1 IgG binding upon agonist stimulation (Fig. 6A, top section). Platelet activation was confirmed by the increase in surface expression of P-selectin post TRAP activation (Fig. 6A, bottom section).

**PEAR1 Tyrosine Phosphorylation**—To determine the condition required for PEAR1 tyrosine phosphorylation, H9251-ECD PEAR1 immunocomplexes from resting or aggregated platelets were probed with H9251-phosphotyrosine-specific IgGs (Fig. 6B, top section). Phosphorylated PEAR1 was not detected in H9251-ECD PEAR1 immunocomplexes from resting platelets; however, upon platelet aggregation stimulated by collagen, TRAP, and thrombin, robust tyrosine-phosphorylation was observed. This phosphorylation was markedly reduced in the presence of the platelet αIIbβ3-specific antagonist eptifibatide, which blocks platelet aggregation but not platelet activation (17). The data show that PEAR1 tyrosine phosphorylation is an aggregation-induced event.

We next examined the effect of immune clustering on PEAR1 tyrosine phosphorylation. Ligation of platelet-bound α-ECD PEAR1 IgG, but not nonspecific IgG crosslinked by a secondary IgG, resulted in tyrosine phosphorylation of PEAR1 (Fig. 6C, top section). The α-ECD PEAR1 IgG clustering-induced phosphorylation was not inhibited in the presence of the FcRyRI/IIa antibody IV.3, indicating that signaling was induced through PEAR1 and not through the platelet Fc receptor.

**Non-receptor Tyrosine Kinase-induced Tyrosine Phosphorylation of PEAR1 and PEAR1 Association with the Shc Adaptor Protein**—Emerging data suggest that many signaling proteins in platelets are tyrosine-phosphorylated by Src kinases. PEAR1 contains four predicted tyrosine phosphorylation sites and an NPxY motif that may serve as an interaction site for PTB domain-containing proteins such as Shc (15). To determine whether Src kinases can phosphorylate PEAR1, Myc-tagged PEAR1 was co-expressed with v-Src in COS-7 cells. Panel 1 of Fig. 7A demonstrates that equal amounts of Myc-PEAR1 were co-expressed with v-Src in COS-7 cells. Panel 1 of Fig. 7A demonstrates that equal amounts of Myc-PEAR1 were co-expressed with v-Src in COS-7 cells.

**Identification of PEAR1 in platelets.** A, Coomassie-stained SDS-PAGE gel of proteins eluted from anti-phosphotyrosine affinity column. 5 μM TRAP-aggregated platelets were solubilized, and tyrosine phosphoproteins were isolated using α-phosphotyrosine IgG beads (PY99). The eluted proteins were resolved by SDS-PAGE and detected by Coomassie staining. See “Results” for a discussion of bands 1–8 (second paragraph). B, ion trap LC/MS/MS spectra of PEAR1 tryptic peptides establishing phosphorylation sites. Spectra are labeled to indicate the mass differences between consecutive y ions consistent with each amino acid. The precursor masses are consistent with one phosphate group in each peptide. Top, phosphotyrosine 925 established in the peptide GLISEEGLGAVSLSSENPyATIR by the y6 ion. Absence of a P(\(\mathrm{m}/\zeta\)H3PO4) ion excludes phosphothreonine 927. Middle, phosphoserine 953 established in the peptide GPPSsPFRQPQQQFDWSQR by the y13\(^+\), y15\(^+\) ions. Bottom, phosphoserine 1029 established in the peptide HPPsFPLRR by the y3, y5, and y6\(^+\) ions. The phospho-amino acids are identified by lowercase letters in the above sequences.
next determined if Shc associated with tyrosine-phosphorylated PEAR1 in platelets. Equal amounts of PEAR1 were immunoprecipitated from unstimulated and aggregated platelet lysates (Fig. 7B, Panel 1). As shown in Fig. 7B, Panel 2, ShcB only coimmunoprecipitated with PEAR1 upon TRAP-induced platelet aggregation (Fig. 7B, Panel 2, lane 6).

Platelet-Platelet Contacts Induce PEAR1 Tyrosine Phosphorylation—Because the data above indicated that PEAR1 was tyrosine-phosphorylated upon platelet aggregation and that its surface expression was not increased by platelet activation, we investigated whether platelet-platelet contacts were sufficient in the absence of platelet stimulation to induce tyrosine phosphorylation of PEAR1. Platelet-platelet contacts were induced by centrifugation of human washed platelets; PEAR1 tyrosine phosphorylation (Fig. 8, Panel 1) was increased as compared with that occurring in resting platelets. The tyrosine phosphorylation occurred rapidly and was as apparent in samples solubilized immediately after centrifugation as in samples incubated for an additional 5 min (Fig. 8). Unlike the PEAR1 tyrosine phosphorylation observed in aggregation reactions, phosphorylation during centrifugation was not inhibited by the αIIbβ3 antagonist epifibatide (Fig. 8, Panel 1).

DISCUSSION

It is well established that the platelet integrin αIIbβ3 is responsible for the primary interaction of platelets during thrombosis and hemostasis and that soluble stimuli (e.g. sCD40L, thromboxane A2, ADP, GAG6, and serotonin) are released from activated platelets to support this process. It is also established that aggregation-induced signaling also occurs. Numerous secondary receptors have been shown to be involved in this process, including outside-in signaling through αIIbβ3, a reaction dependent in part upon tyrosine phosphorylation of β3 (1, 18). Signaling reactions induced directly by platelet-platelet contact have not been previously described. Because aggregation-induced signaling is directly involved in aggregate stability, we sought to identify novel platelet receptors involved in platelet proximity-induced signaling.

The present study identified a novel platelet protein, termed PEAR1, that signals upon the formation of platelet-platelet contacts induced both by platelet aggregations or by platelet centrifugation. PEAR1 was identified using two independent techniques. First, by profiling platelet RNA on oligonucleotide-based microarrays, we identified a transcript encoding PEAR1. Second, experiments performed in parallel using a proteomics approach designed to identify platelet proteins phosphorylated upon platelet aggregation also led to the identification of PEAR1. In platelets, PEAR1 was shown to be a surface-expressed protein that, upon aggregation, was tyrosine-phosphorylated. This phosphorylation event was inhibited by the αIIbβ3 antagonist epifibatide and, thus, demonstrated that PEAR1 tyrosine phosphorylation is dependent on surface contacts between activated platelets. Interestingly, we also demonstrated that, unlike other secondary signaling molecules that require platelet activation to signal, platelet-platelet contacts induced by centrifugation of washed platelets resulted in PEAR1 tyrosine phosphorylation. This phosphorylation was not inhibited by epifibatide, implying that platelet-platelet contacts independent of platelet activation can induce PEAR1 phosphorylation.

Induction of PEAR1 phosphorylation by clustering through
immune complexes also provided evidence that PEAR1 phosphorylation was an oligomerization-dependent event. Oligomerization of integrins and growth factor receptors is a well-established mechanism for the promotion cell signaling (19, 20). EGF and neuregulin induce oligomerization and signaling of ErbB receptors (21, 22). In platelets, the forced clustering of integrins and growth factor receptors is a well-established mechanism for the promotion cell signaling (19, 20). EGF and neuregulin induce oligomerization and signaling of ErbB receptors (21, 22).

**PEAR1 displays extensive homology with KIAA1780 and KIAA1781 in the extracellular and intracellular domains; it is likely these proteins belong to a unique family of EGF repeat-containing transmembrane proteins. The extracellular domain of PEAR1 is also highly similar to CED-1, SREC-I, and SREC-II. CED-I is a Caenorhabditis elegans gene required for engulfment of apoptotic bodies (12). SREC-I was originally identified based on its ability to bind to and mediate uptake of anionic ligands such as acetylated low density lipoprotein and oxidized low density lipoprotein (13). SREC-II, which is 52% identical to SREC-I in the extracellular domain, does not mediate uptake of oxidized low density lipoprotein but appears to form transheterotypic interactions with SREC-I to promote cell-cell aggregation (23). By analogy, the activities of CED-1, SREC-I, and SREC-II suggest possible ligands for PEAR1.

PEAR1 can be phosphorylated in an α1β3 integrin-dependent manner on tyrosine (Tyr-925) and serine residues (Ser-953 and Ser-1029) and, potentially, at Tyr-804, Tyr-943, and Tyr-979. Src family kinases are known to transmit integrin-dependent signals that lead to platelet adhesion and aggregation (24, 25). Co-transfection studies of v-Src with PEAR1 in COS-7 cells results in Src-promoted tyrosine phosphorylation of PEAR1. An INPXY motif, an interaction site for PTB domain-containing proteins, and five potential Src homology 3-binding domains were also identified in PEAR1. The Shc adaptors proteins ShcA, ShcB, and ShcC each contain a PTB domain as well as a phosphotyrosine-binding Src homology 2 domain (26).

**Fig. 6. PEAR1 tyrosine phosphorylation upon platelet aggregation or immune clustering.** A, surface expression of platelet PEAR1. Fixed and un-permeabilized resting or TRAP (5 μM)-activated human washed platelets (1 × 10⁷) were analyzed by fluorescence-activated cell sorter analysis. Top, resting platelets were stained with FITC-labeled nonspecific (solid line) or α-ECD PEAR1 (dashed line) IgG, and activated platelets were stained with FITC-labeled α-ECD PEAR1 IgG (dotted line). Bottom, platelet activation was confirmed by staining resting (solid line) and activated (dashed line) platelets with phycocerythrin-labeled α-CD62P mouse IgM. B, agonist-induced tyrosine phosphorylation of platelet PEAR1. Washed human platelets (3 × 10⁴) were incubated with either 5 μg/ml nonspecific (lane 1) or α-PEAR1-ICD-IgG followed by Western blotting with α-phosphotyrosine antibodies PY20 and 4G10 (Panel A) or α-PEAR1-ICD (Panel B) polyclonal IgG. C, immune clustering induced tyrosine phosphorylation of PEAR1. Washed human platelets (1.5 × 10⁶) were incubated with either 5 μg/ml nonspecific (lane 1) or α-ECD PEAR1 (lanes 2, 3, 4, and 5) IgG for 30 min at room temperature in the presence (lane 4) or absence (lanes 1, 2, and 3) of 25 μg/ml IV.3 IgG. IgG signaling was induced with 10 μg/ml α-rabbit secondary whole IgG for 30 min at 37 °C. Stained platelets were lysed and immunoprecipitated with α-PEAR1-ICD-IgG followed by Western blotting with α-phosphotyrosine antibodies PY20 and 4G10 (Panel A) or α-ICD PEAR1 (Panel B) IgG.
sine phosphorylation of PEAR1 enhanced the association of ShcA and ShcB with PEAR1. ShcB is the only Shc isoform that is expressed in platelets (8) and, as demonstrated in this paper, associates with PEAR1 upon TRAP-induced platelet aggregation. The association of ShcB with PEAR1 may provide a mechanism, in addition to its interaction with αIIbβ3, to localize Shc to the plasma membrane where it may further enhance signaling pathways such as the activation of Ras.

In this study we have described the use of two techniques, genomics and proteomics, to identify a novel single transmembrane

FIG. 7. Δ-Src induced tyrosine phosphorylation of PEAR1 and association with the Shc adaptor proteins. A, COS cells were transfected with vector control (lane 1), PEAR1-Myc (lane 2), Δ-Src (lane 3), or PEAR1-Myc and Δ-Src (lane 4) DNAs. Lysates were prepared, incubated with anti-Myc (9E10) monoclonal antibody, precipitated with protein G-Sepharose, and separated on 10% SDS-PAGE. Myc-PEAR immunoprecipitations (IP) were blotted with α-Myc monoclonal antibody and goat anti-mouse HRP (Panel 1). The level of tyrosine-phosphorylated PEAR1-Myc was determined by Western analysis using anti-PY99 monoclonal antibody and goat anti-mouse HRP (Panel 2). Shc adaptor proteins, co-immunoprecipitated with PEAR1-Myc, were detected by immunoblotting with anti-Shc polyclonal antibody and goat anti-rabbit HRP (Panel 3).

B, agonist-induced association of ShcB with platelet PEAR1. Washed human platelets (1.5 × 10^8) were left resting or aggregated with the 5 μM TRAP by stirring (1000 rpm) for 3 min at 37 °C. Platelets were lysed and immunoprecipitated with either control rabbit IgG (lanes 3 and 5) or α-ICD PEAR1 IgG (lanes 4 and 5) followed by Western blotting with an anti-Shc monoclonal antibody and goat anti-mouse HRP (Panel 2). Lanes 1 and 2 contain 2.5 μg of lysates from resting and aggregated platelets. PEAR1 immunoprecipitates were blotted with α-ICD PEAR1 IgG and goat anti-rabbit HRP (Panel 1).

FIG. 8. Platelet contact-induced PEAR1 phosphorylation. A, washed human platelets (3 × 10^8) were left resting (lanes 2 and 3) or spun for 5 min at 13,000 rpm (lanes 1, 4, 5, 6, and 7) in the absence (lanes 1, 2, 3, 4, and 5) or presence (lanes 6 and 7) of 2 μM epifibatide. Resting or spun platelets were lysed 0 or 5 min post spinning and immunoprecipitated (IP) with α-PEAR1-ICD-IgG followed by Western blotting with α-phosphotyrosine (α-PY) antibodies PY20 and 4G10 (Panel 1) or α-PEAR1-ICD (panel 2) polyclonal IgG.
brane receptor expressed in platelets and endothelial cells that is designated PEAR1. This combination of these techniques may be applicable not only to characterizing proteins that signal during platelet aggregation but also proteins involved in other reactions important to platelet biology. Included in this list might be proteins involved in α-granule release and dense body secretion, proteins regulating cytoskeletal assembly and rearrangement, and proteins that signal in response to specific platelet agonists. We have also shown for the first time a platelet receptor that is activated directly by platelet-platelet contacts independent of platelet activation and secondary to platelet aggregation. Cell-cell contact-dependent mechanisms are responsible for a multitude of cellular processes. The cadherin/catenin adhesion system becomes activated upon cell-cell contact and has been implicated in contact inhibition of growth. Loss of these molecules has been implicated in tumorigenesis (27). Cell-contact dependent signaling events are also responsible for the immune synapse formation, where primary cell-cell interactions are mediated by integrins, but secondary signaling by additional receptors is initiated by cell proximity. Although the function of PEAR1 signaling during platelet aggregation is extensively studied, the multitude of signaling events triggered by platelet aggregations is extensive and includes α-granule and dense body release, thromboxane A2 generation, cytoskeletal rearrangements, αIIbβ3 activation, and increase in cytosolic Ca2+ concentration. Understanding the relationship of PEAR1 as a secondary adhesion receptor mediated by platelet cohesion in these processes is an important area for future studies.

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Platelet Endothelial Aggregation Receptor 1 (PEAR1), a Novel Epidermal Growth Factor Repeat-containing Transmembrane Receptor, Participates in Platelet Contact-induced Activation

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