Wheat Germ Agglutinin-Induced Platelet Activation via Platelet Endothelial Cell Adhesion Molecule-1: Involvement of Rapid Phospholipase Cγ2 Activation by Src Family Kinases[†]

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ABSTRACT: Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is a 130K transmembrane glycoprotein that belongs to the immunoglobulin gene superfamily and is expressed on the surface of hematological or vascular cells, including platelets and endothelial cells. Although the importance of this adhesion molecule in various cell−cell interactions is established, its function in platelets remains ill-defined. In the process of clarifying the mechanism by which the lectin wheat germ agglutinin (WGA) activates platelets, we unexpectedly discovered that PECAM-1 is involved in signal transduction pathways elicited by this *N*-acetyl-D-glucosamine (NAGlu)-reactive lectin. WGA, which is a very potent platelet stimulator, elicited a rapid surge in Syk and phospholipase C (PLC)-γ2 tyrosine phosphorylation and the resultant intracellular Ca^{2+} mobilization; collagen, as reported, induced these responses, but in a much slower and weaker manner. WGA strongly induced tyrosine phosphorylation of a 130−140K protein, which was confirmed to be PECAM-1 by immunoprecipitation and immunodepletion studies. WGA-induced PECAM-1 tyrosine phosphorylation occurred rapidly, strongly and in a manner independent of platelet aggregation or cell−cell contact; these characteristics of PECAM-1 phosphorylation were not mimicked at all by receptor-mediated platelet agonists. In addition, WGA was found to associate with PECAM-1 itself, and anti-PECAM-1 antibody, as well as NAGlu, specifically inhibited WGA-induced platelet aggregation. In PECAM-1 immunoprecipitates, Src family tyrosine kinases existed, and a kinase activity was detected, which increased upon WGA stimulation. Furthermore, the Src family kinase inhibitor PP2 inhibited WGA-induced platelet aggregation, Ca^{2+} mobilization, and PLC-γ2 tyrosine phosphorylation. Finally, WGA induced PECAM-1 tyrosine phosphorylation and cytoskeletal reorganization in vascular endothelial cells. Our results suggest that (i) PECAM-1 is involved in WGA-induced platelet activation, (ii) PECAM-1 clustering by WGA activates unique and strong platelet signaling pathways, leading to a rapid PLC activation via Src family kinases, and (iii) WGA is a useful tool for elucidating PECAM-1-mediated signaling with wide implications not confined to platelets.

Platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31)[1] is a member of the immunoglobulin (Ig) gene superfamily and is expressed on the surface of platelets, leukocytes, and endothelial cells, where it accumulates at cell−cell borders (*1*, *2*). PECAM-1 is able to mediate cell−cell adhesion through interaction both with itself (homophilic interaction) or with other non-PECAM-1 molecules (heterophilic interaction), a feature that has also been observed for other cell adhesion proteins of the Ig gene superfamily (*1*−*4*). Due to its cellular distribution and ability to mediate adhesive phenomena, it was suggested that PECAM-1 is a multifunctional vascular cell adhesion molecule involved in leukocyte-endothelial and endothelial-endothelial interactions (*1*−*4*). In fact, recent studies have implicated the involvement of PECAM-1 in the inflammatory process, in the endothelial cell migration, and in the formation of blood vessels (*5*−*9*).

In addition to mediating cell−cell adhesion, a variety of studies have revealed that PECAM-1 may transduce transmembrane signals that modulate the integrin functions. Homophilic engagement of PECAM-1, including antibody-induced dimerization, reportedly results in the upregulation of integrin affinity; antibody-induced cross-linking of PECAM-1 on specific subpopulations of T cells increased the adhesive function of β1 integrins (*10*). Furthermore, PECAM-1-mediated integrin activation was also observed for β1 integrins on $CD34^+$ hematopoietic progenitor cells (*11*) and β2 integrins on lymphokine-activated killer cells, monocytes, neutrophils, and natural killer cells (*12*−*14*). On the other hand, PECAM-1 not only modulates integrin function, but is itself affected by integrins; integrin-mediated cellular adhesion led to phosphorylation of the PECAM-1 cytoplas-

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[1] Abbreviations: PECAM-1, platelet endothelial cell adhesion molecule-1; Ig, immunoglobulin; GP, glycoprotein; MoAb, monoclonal antibody; WGA, wheat germ agglutinin; NAGlu, *N*-acetyl-D-glucosamine; TRITC, tetramethyl rhodamine isothiocyanate; PBS, phosphate-buffered saline; PLC, phospholipase C; FcR, Fc receptor; HUVECs, human umbilical vein endothelial cells; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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mic domain (*2*). Platelet PECAM-1 tyrosine phosphorylation and its involvement in cellular activation pathways (including those for integrins) have been repeatedly reported (*15*, *16*), and platelets may provide a good and unique model system to elucidate the interaction between PECAM-1 and integrins. Thrombin-induced PECAM-1 tyrosine phosphorylation in these anucleate cells is mostly dependent on integrin αIIbβ3, i.e., glycoprotein (GP) IIb/IIIa, suggesting that this platelet-specific integrin modulates PECAM-1 tyrosine phosphorylation (*15*). Furthermore, F(ab′)2 fragments of a monoclonal antibody (MoAb) against the sixth Ig-like domain of PECAM-1 activate integrin αIIbβ3 and augment platelet adhesion and aggregation (*16*).

Wheat germ agglutinin (WGA), a tetravalent plant lectin with high specificity for *N*-acetyl-D-glucosamine (NAGlu) (*17*, *18*), is a potent platelet stimulant and has been used in the study of platelet signal transduction (*19*−*23*). Although protein-tyrosine phosphorylation and phospholipase C (PLC) activation leading to intracellular Ca^{2+} mobilization seem to play important role(s) in platelet activation induced by WGA (*19*, *20*, *23*), as is the case with most of the receptor-mediated agonists, the precise mechanism by which WGA transduces signals into platelets has remained ill-defined. In the process of analyzing WGA-induced platelet activation, we unexpectedly discovered that PECAM-1 is involved in signal transduction pathways elicited by this lectin, which we report in this study. Furthermore, we describe that WGA induces PLCγ2 tyrosine phosphorylation and the resultant Ca^{2+} mobilization through Src family tyrosine kinase activation following PECAM-1 cross-linking, although involvement of other cell surface molecule(s) cannot be ruled out. WGA-induced PECAM-1 tyrosine phosphorylation can be reproduced in vascular endothelial cells, and this lectin seems to be a useful tool for elucidating PECAM-1-mediated signaling with wide implications not limited to platelets.

EXPERIMENTAL PROCEDURES

Materials. Anti-GPIb MoAb (TM60) (*24*) and anti-GPIIb/IIIa MoAb (NNKY2−11) (*25*) were kindly provided by Dr. M. Yamamoto (Tokyo Metropolitan Medical Institute, Tokyo, Japan) and Dr. S. Nomura (Kansai Medical University, Osaka, Japan), respectively. Anti-Src MoAb (clone 327) was kindly provided by Dr. G. Katoh (Yamanashi Medical University, Yamanashi, Japan). Convulxin, a GPVI-selective agonist (*26*), was a gift from Dr. T. Morita (Meiji Pharmaceutical University, Tokyo, Japan). Fc receptor (FcR) γ-chain-deficient C57BL/6 mice and wild-type C57BL/6 mice were kindly provided by Dr. T. Takai (Tohoku University, Miyagi, Japan).

The following materials were obtained from the indicated suppliers: thrombin (Green Cross, Osaka, Japan); collagen (Hormon-Chemie, Munich, Germany); GRGDS peptide (Peptide Institute, Osaka, Japan); anti-FcR γ-chain polyclonal antibody, anti-phosphotyrosine MoAb (4G10), and anti-GPIa MoAb (Upstate Biotechnology, Lake Placid, NY); anti-Syk MoAb and anti-Lyn MoAb (Wako Pure Chemical Industries, Tokyo, Japan); anti-Fyn MoAb and anti-PLCγ2 polyclonal antibody (Santa Cruz Biotec., Santa Cruz, CA); anti-GPIIa MoAb and anti-phosphotyrosine MoAb (PY20) (Transduction Laboratories, Lexington, KY); PP2 (Calbiochem-Novabiochem Co., San Diego, CA); WGA, peroxidase-labeled WGA, fibrinogen, enolase, and tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin (Sigma Chemical Co., St. Louis, MO); staurosporine (Kyowa Medex, Tokyo, Japan); protein A-Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden); anti-PECAM-1 MoAb (clone JC/70A) (Dako A/S, Glostrup, Denmark); [γ-^{32}P]ATP (111 TBq/mmol) (Du Pont-New England Nuclear, MA).

Cell Preparation. Human platelet-rich plasma was prepared as previously described (*27*), and incubated with 1 mM acetylsalicylic acid for 30 min to exclude any secondary effects of thromboxane A_2. Washed platelets were prepared as previously described (*27*). The final platelet suspensions were adjusted to 1.0×10^9 cells/mL and supplemented with 1 mM $CaCl_2$, unless otherwise stated. Human umbilical vein endothelial cells (HUVECs) were prepared as described previously (*28*). Murine blood was taken by cardiac puncture immediately after death, and washed platelets were prepared as described above.

Measurement of the Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ measurement was performed using Ca^{2+}-sensitive fluorophore fura2 as described previously (*29*).

Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assays. Platelets were lysed in equal amounts of 2 × ice-cold lysis buffer (100 mM Tris (pH 7.4), 2% Triton X-100, 20 mM NaF, 100 mM NaCl, 6 mM EDTA, 1 mM Na_3VO_4, 0.5 mM PMSF, and 25 μg/mL of leupeptin), while HUVECs were lysed in 1 × ice-cold lysis buffer with the aid of a cell scraper. A part of each lysate was used as a whole cell lysate. The subsequent immunoprecipitation, immunoblotting, and in vitro kinase assays were performed as described previously (*27*). The procedures for repeated immunodepletion using a specific antibody were described previously (*30*).

When indicated, WGA-interacting platelet proteins were examined by incubation of the blots with 0.1 μg/mL of peroxidase-labeled WGA for 30 min.

Platelet Aggregation. Platelet aggregation was examined with the use of washed platelet suspensions adjusted to 3×10^8 cells/mL. Except in Figure 6, the response was measured with the conventional method based on changes in light transmission (*31*). The instrument was calibrated with a platelet suspension for zero light transmission and with a buffer for 100% transmission.

In Figure 6, platelet aggregation was measured simultaneously via two methods, i.e., light transmission and light scattering intensities, with an AG-10 (Mebanix Co., Ltd., Tokyo, Japan). The instrument was calibrated for light transmission, as described above. A new light scattering method based on particle counting was previously described by us (*32*). Briefly, a diode laser light beam (40 μm width, wavelength 675 nm) was passed through PRP in a cylindrical glass cuvette. An optical device was focused on a limited area of the platelet suspension, and the intensity of light scattered by particles passing through the area was measured. The light-scattering intensities detected by this device provides information on the number and size of aggregates in the suspension. It has been demonstrated that this device is particularly sensitive in detecting small platelet aggregates (*32*). The quantitative data for small platelet aggregate formation were presented as the total light intensity (V) per minute, as described previously (*32*).

Unless otherwise stated, platelets were stimulated at 37 °C under continuous stirring at 1000 rpm. To inhibit platelet

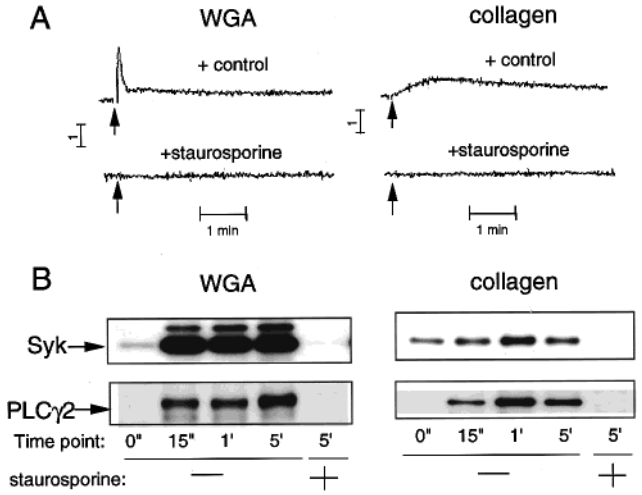

FIGURE 1: Effects of staurosporine on WGA- or collagen-induced intracellular Ca^{2+} mobilization and tyrosine phosphorylation of Syk and PLCγ2 in human platelets. In panel A, fura2-loaded platelets pretreated without or with 1 μM staurosporine for 5 min were challenged with 50 μg/mL of WGA (left) or collagen (right). Intracellular $[Ca^{2+}]_i$ changes were monitored by the ratio of fura2 fluorescence (340/380 nm). In panel B, platelets pretreated without or with 1 μM staurosporine for 5 min were challenged with 50 μg/mL of WGA (left) or collagen (right) for the indicated durations. The platelet protein lysates were immunoprecipitated with anti-Syk MoAb (upper) or anti-PLCγ2 antibody (lower), resolved on an 8% SDS−PAGE, and then immunoblotted with anti-phosphotyrosine MoAb.

aggregation, platelets were preincubated with 500 μM GRGDS (a competitive antagonist to inhibit fibrinogen-integrin αIIbβ3 interaction) for 5 min and not stirred; platelet aggregates were not formed under these conditions (data not shown).

Actin Staining. For actin staining, HUVECs were fixed with 3% paraformaldehyde in PBS for 40 min, then permeabilized with 0.2% Triton X-100 for 8 min. Actin filaments were detected by staining with 0.1 μg/mL of TRITC-conjugated phalloidin. Actin staining was observed and photographed using a confocal microscope.

Data Presentation. The data shown are representative of at least three independent experiments, unless otherwise stated.

RESULTS

WGA-Induced Intracellular Ca^{2+} Mobilization and Protein-Tyrosine Phosphorylation in Platelets. Although it is established that WGA is a potent platelet stimulant (*19*, *20*), the mode of intracellular events triggered by this lectin seems unique compared with that triggered by ordinary receptor-mediated agonists. WGA induced a prompt and sharp increase in $[Ca^{2+}]_i$ (Figure 1A), which was indistinguishable from that induced by G protein-coupled receptor agonists such as thrombin, platelet-activating factor, and arginine vasopressin (data not shown), as we previously described (*19*). However, the intracellular Ca^{2+} mobilization induced by WGA was completely inhibited by staurosporine, a nonspecific inhibitor of protein kinases (including tyrosine kinases) (*33*) (Figure 1A), while that by G protein-coupled receptor agonists was insensitive to this inhibitor (data not shown) (*19*). In this context, WGA-induced Ca^{2+} mobilization is similar to the collagen-induced one in its sensitivity

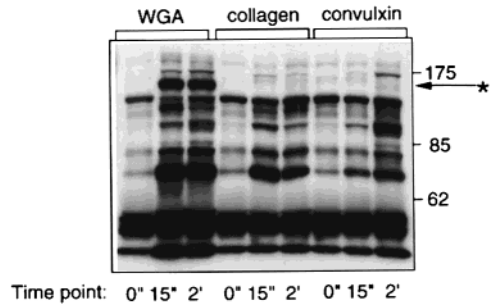

FIGURE 2: Comparison of platelet protein-tyrosine phosphorylation induced by WGA, collagen, and convulxin. Platelets were challenged with 50 μg/mL of WGA (left), 50 μg/mL of collagen (middle), or 50 ng/mL of convulxin (right) for the indicated durations. The platelet lysates were resolved on an 8% SDS−PAGE, and then immunoblotted with anti-phosphotyrosine MoAb. On the right, the locations of molecular weight markers are shown. Note that a 130−140Kprotein is heavily tyrosine-phosphorylated specifically upon WGA stimulation (indicated by an asterisk).

to staurosporine, although the latter is apparently slower compared with the former (Figure 1A). Protein-tyrosine phosphorylation plays an important role in phosphoinositide turnover induced by collagen; collagen activates Src family tyrosine kinases through interaction with the Ig gene superfamily molecule GPVI, leading to Syk and PLCγ2 activation (*34*). Since WGA-induced Ca^{2+} mobilization was completely inhibited by staurosporine, as was the case with collagen (Figure 1A), we examined the tyrosine phosphorylation of Syk and PLCγ2 in platelets stimulated with WGA. As well as collagen, WGA also elicited tyrosine phosphorylation of Syk and PLCγ2 (Figure 1B). Consistent with the data on intracellular Ca^{2+} mobilization, Syk and PLCγ2 phosphorylation induced by WGA, as well as collagen, was inhibited by staurosporine, and the WGA-induced response was much faster than the collagen response (Figure 1B).

We next compared whole platelet protein-tyrosine phosphorylation induced by WGA, collagen, and convulxin, a GPVI agonist (*26*). The phosphorylation patterns induced by these stimuli were basically the same, but a protein of 130−140K protein was strongly tyrosine-phosphorylated by WGA (Figure 2). This WGA-induced phosphorylation occurred very rapidly and intensely (as was the case with PLCγ2 phosphorylation) and seemed important in WGA-induced platelet activation.

Identification of the 130−140K Protein (tyrosine-phosphorylated by WGA) as PECAM-1. To identify the 130−140K protein which was strongly tyrosine-phosphorylated by WGA (indicated by an asterisk in Figure 2), we performed immunoprecipitation with antibodies against various candidate molecules. WGA was found to heavily phosphorylate the Ig gene superfamily adhesion molecule PECAM-1, the location of which corresponded to the 130−140K tyrosine-phosphorylated protein, when PECAM-1 immunoprecipitates were probed with anti-phosphotyrosine MoAb (Figure 3A). Collagen and convulxin did induce PECAM-1 tyrosine phosphorylation, but very faintly compared with WGA; sometimes collagen-induced PECAM-1 tyrosine phosphorylation could not be detected under the conditions in which the WGA-induced response was observed (Figure 3B). Similar immunoprecipitation studies were performed with FAK, Cas, Pyk2, JAK1, JAK2, Cbl, GPIIb/IIIa, GPIa,

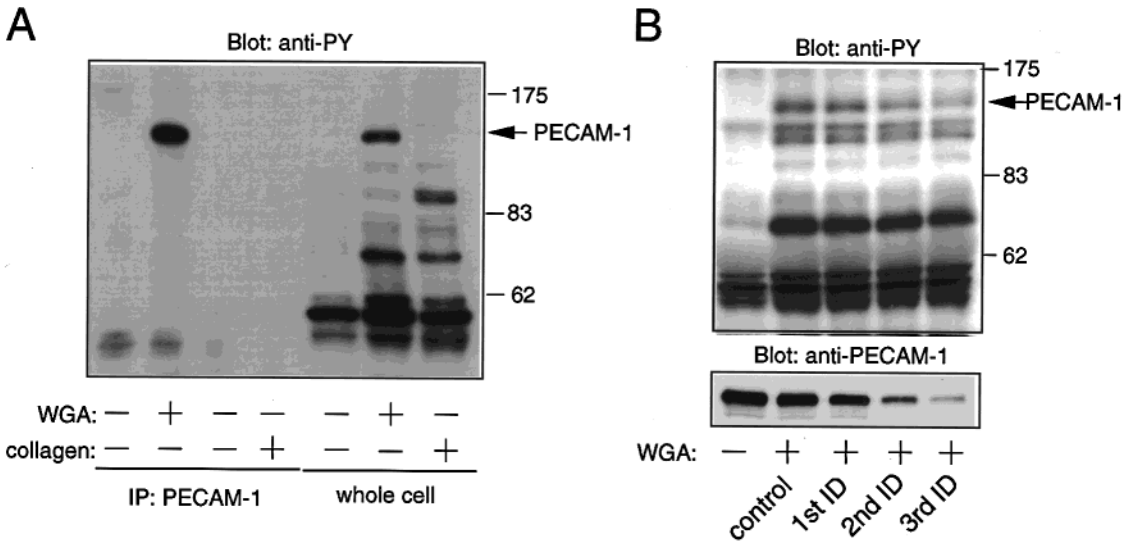

FIGURE 3: Identification of the 130−140K protein tyrosine-phosphorylated by WGA as PECAM-1. In panel A, platelets were stimulated without or with 50 μg/mL of WGA or collagen for 30 s. Platelet lysates were immunoprecipitated with anti-PECAM-1 MoAb. The immunoprecipitates, along with whole platelet lysates, were resolved on an 8% SDS−PAGE, and then immunoblotted with anti-phosphotyrosine (PY) MoAb. In panel B, PECAM-1 was repeatedly immunodepleted from cell lysates by immunoprecipitation with anti-PECAM-1 MoAb. The platelet lysates without (control) or with (first to third ID) PECAM-1 immunodepletion were subjected to an 8% SDS−PAGE and immunoblotted with anti-phosphotyrosine (PY) MoAb (upper) or anti-PECAM-1 MoAb (lower). On the right, the locations of molecular weight markers and PECAM-1 are shown.

PLCγ1, PLCγ2, GPIIa, GPIb, and vinculin, the molecular weights of which range from 120 to 140K. However, the possibility of any of these molecules being the 130−140K tyrosine-phosphorylated protein was ruled out (data not shown). Furthermore, repeated immunodepletion with anti-PECAM-1 MoAb resulted in a specific reduction of the 130−140K tyrosine-phosphorylated protein, as well as PECAM-1 itself, from the cell lysates obtained from platelets stimulated with WGA (Figure 3B). Since the amounts of PECAM-1 remaining after the third immunodepletion were negligible compared with control samples (Figure 3B), it was concluded that the 130−140K phosphoprotein consists mainly of PECAM-1.

Aggregation-Independent PECAM-1 Tyrosine Phosphorylation Induced by WGA. Platelet aggregation mediated by integrin αIIbβ3 is one of the most important functional responses in these anucleate cells and has been reported to affect a variety of platelet signaling events involving protein-tyrosine phosphorylation (*35*, *36*). PECAM-1 tyrosine phosphorylation is not an exception (*15*). We pretreated platelets with a GRGDS peptide and omitted stirring of the samples to block platelet aggregation. As previously reported (*15*, *37*), both thrombin (Figure 4B) and collagen (Figure 4C)-induced PECAM-1 tyrosine phosphorylation, although much more slowly and weakly when compared with WGA (Figure 4A), in a manner dependent on integrin αIIbβ3-mediated platelet aggregation. In contrast, WGA-induced PECAM-1 tyrosine phosphorylation was not affected by elimination of platelet aggregation (Figure 4A). Recently, it was suggested that cell−cell contact, rather than aggregation, may play an important role in agonist-induced PECAM-1 tyrosine phosphorylation in platelets (*37*). However, aggregation or cell−cell contact is not required for WGA-induced PECAM-1 tyrosine phosphorylation, since this response was observed similarly with or without stirring platelets (Figure 4A). It is strongly suggested that WGA can transduce very strong and efficient signal(s) leading to PECAM-1 tyrosine phospho-

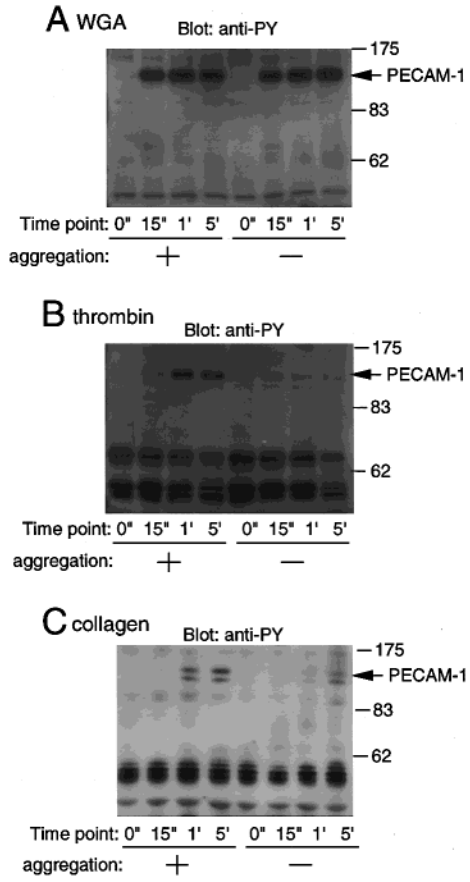

FIGURE 4: Aggregation-independent tyrosine phosphorylation of PECAM-1 elicited by WGA. Platelets were stimulated with 50 μg/mL of WGA (A), 0.1 unit/mL of thrombin (B), or 50 μg/mL of collagen (C) for various durations under the conditions in which platelets formed aggregates (+) or did not (−); platelet aggregation was inhibited by preincubation with 500 μM GRGDS for 5 min and by omission of stirring. PECAM-1 tyrosine phosphorylation was examined as described in the legend for Figure 3.

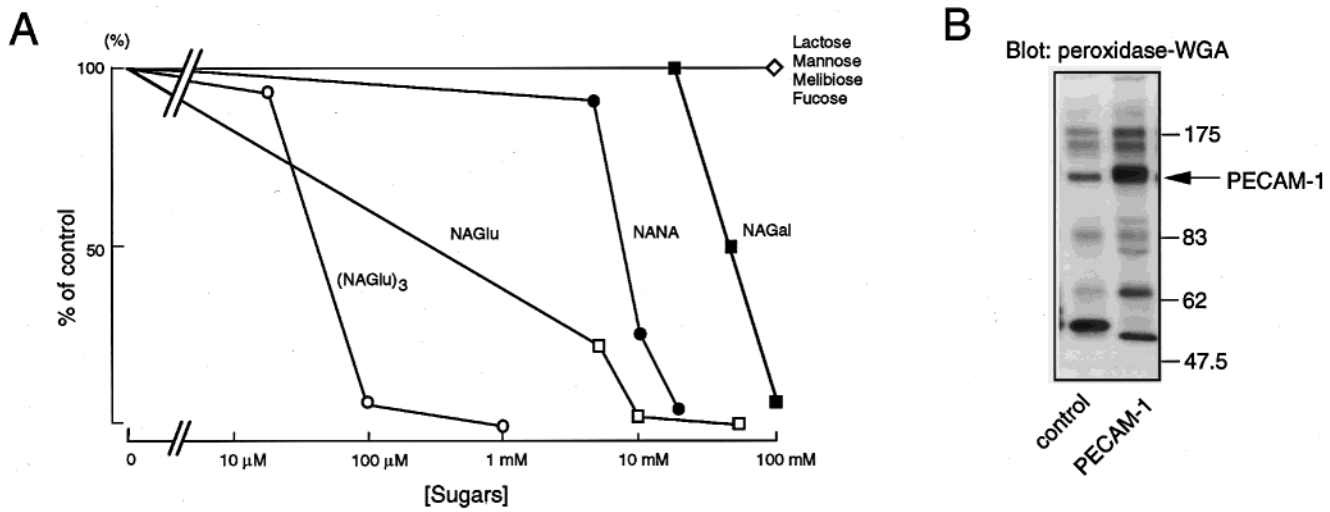

FIGURE 5: Association of WGA with platelet PECAM-1. In panel A, platelets were preincubated with various concentrations of sugars for 5 min, and then stimulated with 20 μg/mL of WGA for 5 min. Platelet aggregation was monitored by light transmission. The results were expressed as a percentage of the control without preincubation. The data are representative of three independent experiments. In panel B, platelet lysates were immunoprecipitated with control IgG or anti-PECAM-1 MoAb. The immunoprecipitates were resolved on an 8% SDS−PAGE, and then probed with 0.1 μg/mL of peroxidase-labeled WGA.

rylation, which is independent of αIIbβ3-mediated platelet aggregation or cell−cell contact.

Interaction of WGA with PECAM-1 on Platelets. To elucidate the mechanism by which WGA specifically and strongly phosphorylates PECAM-1, we examined the platelet membrane molecules with which WGA interacts. We first checked the effects of various sugars on WGA-induced platelet activation. WGA-induced platelet aggregation was strongly inhibited by $(NAGlu)_3$ or NAGlu, and weakly by sialic acid or *N*-acetyl galactosamine (Figure 5A), consistent with an established WGA selectivity toward sugar moieties (*17*). Gangliosides (sialic acid-containing glycosphingolipids) and their modified catabolites are highly expressed in the outer leaflet of plasma membrane and mediate a variety of biological processes (*38*, *39*). Since WGA reportedly interacts with GM3 (*40*), which is highly expressed on platelets (*41*), there was a possibility of WGA interacting with this ganglioside on platelets. However, pretreatment with GM3 (or GM1) did not affect WGA-induced platelet aggregation (data not shown). Furthermore, anti-GM3 MoAb did not inhibit WGA-elicited platelet activation (data not shown), indicating that GM3 is not a target for WGA.

We next examined the possibility of WGA interacting with the PECAM-1 molecule itself, which is highly *N*-glycosylated (*42*). When platelet PECAM-1 was immunoprecipitated using a specific antibody, resolved on an SDS−PAGE, and then probed with peroxidase-labeled WGA, PECAM-1 interaction with WGA was clearly detected (Figure 5B).

Since physical interaction of WGA with platelet PECAM-1 was confirmed, we examined the effect of anti-PECAM-1 antibody on WGA-induced platelet activation. Anti-PECAM-1 MoAb inhibited platelet aggregation induced by WGA (Figure 6D), but not by thrombin (data not shown). Furthermore, in contrast to the anti-PECAM-1 antibody, control IgG, anti-Fcg receptor IIA MoAb, and anti-GPIb MoAb failed to affect WGA-induced platelet aggregation (Figure 6). These findings further confirmed that WGA activates platelets through interaction with PECAM-1.

Recently, the collagen receptor GPVI was cloned and found to be coupled to the FcR γ-chain, which contains a consensus sequence known as the immune-receptor tyrosine-based activation motif (*34*, *43*). It is now established that tyrosine phosphorylation of this motif upon GPVI stimulation is a crucial step in platelet activation induced by collagen; platelets from FcR γ-chain-deficient mice fail to respond to collagen (*34*, *43*). Since GPVI, as well as PECAM-1, is a member of the Ig superfamily of proteins and since platelet activation induced by WGA is similar to that by collagen in some respects, we checked GPVI involvement in WGA-induced platelet activation, with the use of FcR γ-chain-deficient mice. Collagen activated platelets obtained from control mice but not from the knockout mice (Figure 7A), as reported previously (*34*), but WGA-induced platelet activation was observed for both (Figure 7B). These data indicate that FcR γ-chain and hence GPVI are not involved in signal transduction pathways elicited by WGA.

Involvement of Src Family Tyrosine Kinases in WGA-Induced Platelet Activation. We next examined the intracellular signaling events resulting from WGA interaction with PECAM-1. For this purpose, we first checked the association of kinase activity with several membrane receptors after a WGA challenge. As expected from PECAM-1 tyrosine phosphorylation by, and PECAM-1 interaction with, WGA, stimulation-dependent ^{32}Pi incorporation into enolase (added as an exogenous substrate), the 130K protein (confirmed as PECAM-1 with immunoblotting), and a 60K protein was observed specifically in immunoprecipitates obtained with the anti-PECAM-1 MoAb (Figure 8A). The phosphorylation levels reached their peak 15 s after activation and then rapidly decreased within 1 min (Figure 8A). Since Src family tyrosine kinase(s) reportedly phosphorylate PECAM-1 in several systems (*37*, *44*), we tested the possibility that the 60K tyrosine-phosphorylated protein may have been one of the Src family tyrosine kinases. Immunoblotting studies of PECAM-1 immunoprecipitates revealed that PECAM-1 constitutively associates with Src, Fyn, and Lyn (Figure 8B), as described previously (*37*). Furthermore, the PECAM-1-associated kinase activity was abolished by the specific Src family tyrosine kinase inhibitor PP2 (*45*) (data not shown). These data suggest that the activation of Src family tyrosine

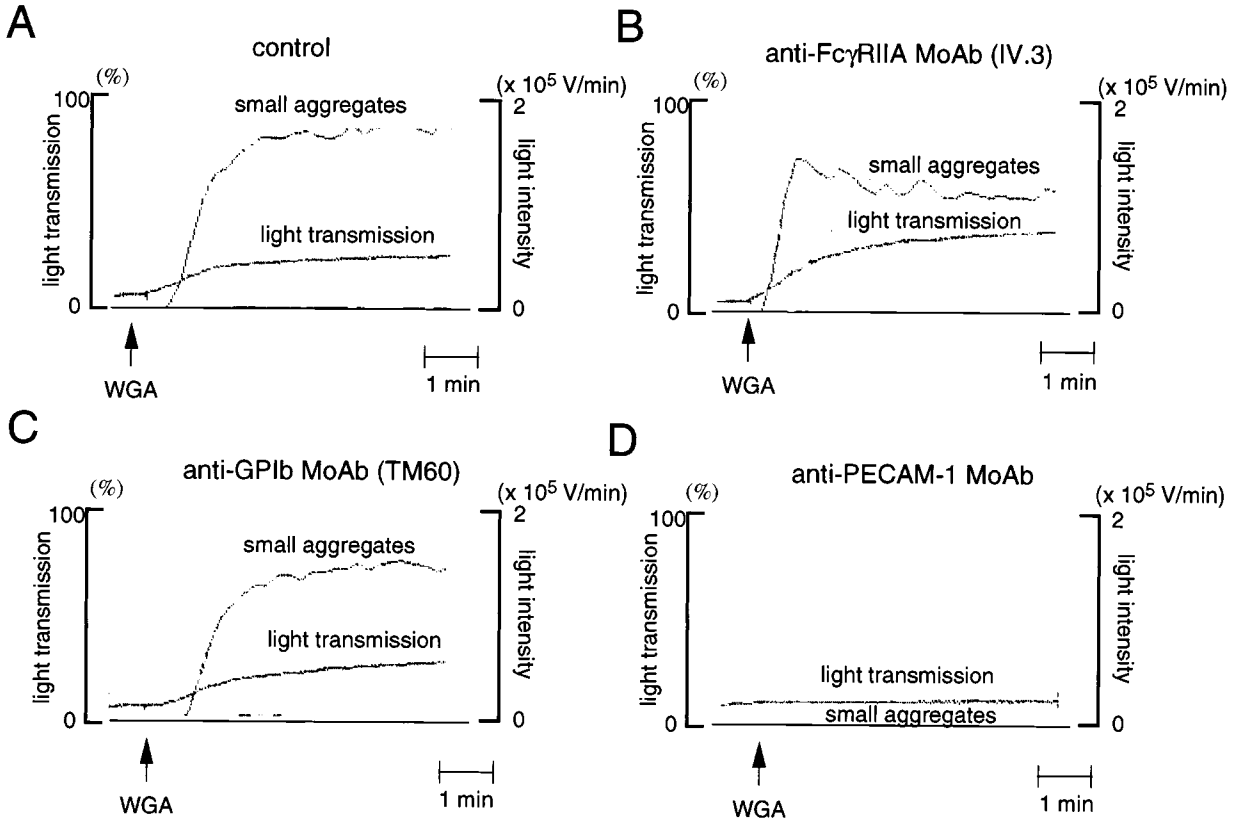

FIGURE 6: Effects of various antibodies on WGA-induced platelet aggregation. Platelets pretreated with 80 μg/mL of control IgG, anti-Fcγ receptor IIA MoAb (IV.3), anti-GP-Ib MoAb (TM60), or anti-PECAM-1 MoAb were challenged with 10 μg/mL of WGA. Platelet aggregation was monitored as described in the Experimental Procedures.

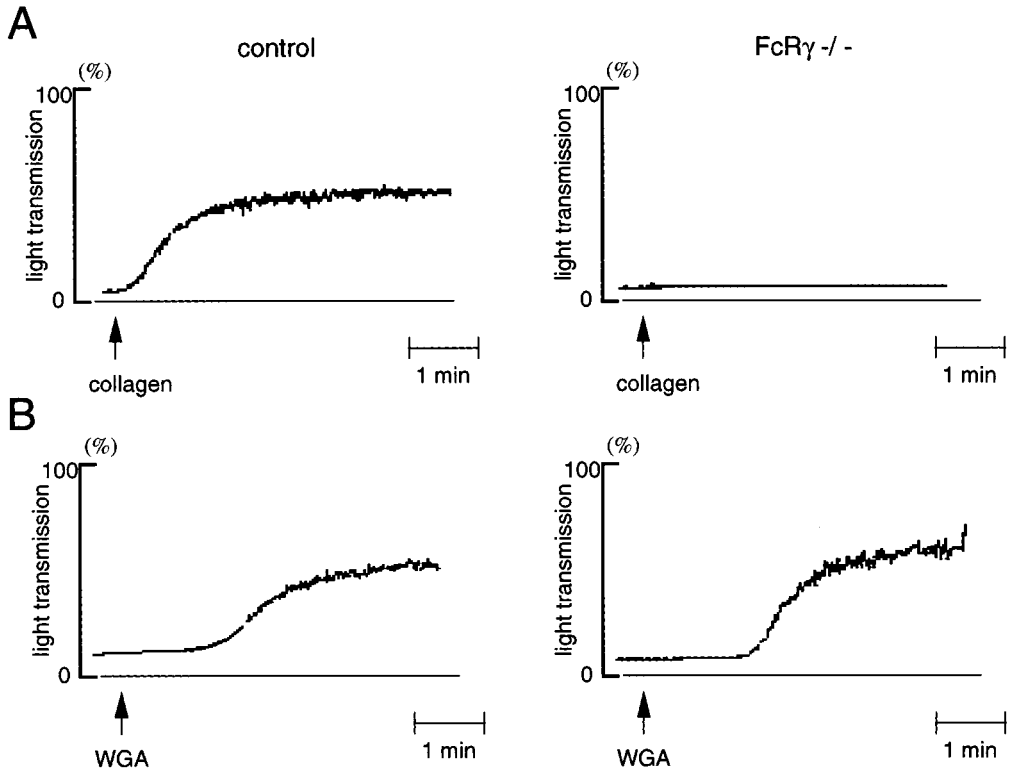

FIGURE 7: Effects of collagen or WGA on the aggregation response of platelets obtained from FcR γ-chain-deficient mice or control mice. Platelets prepared from control mice (control) or FcR γ-chain-deficient mice (FcRγ $-/-$) were stimulated with 50 μg/mL of collagen (A) or WGA (B), and platelet aggregation was monitored turbidometrically.

kinase(s) associated with PECAM-1 is the initial step in WGA-induced platelet activation.

The importance of Src family tyrosine kinase(s) in WGA-induced platelet activation was further confirmed by examin-

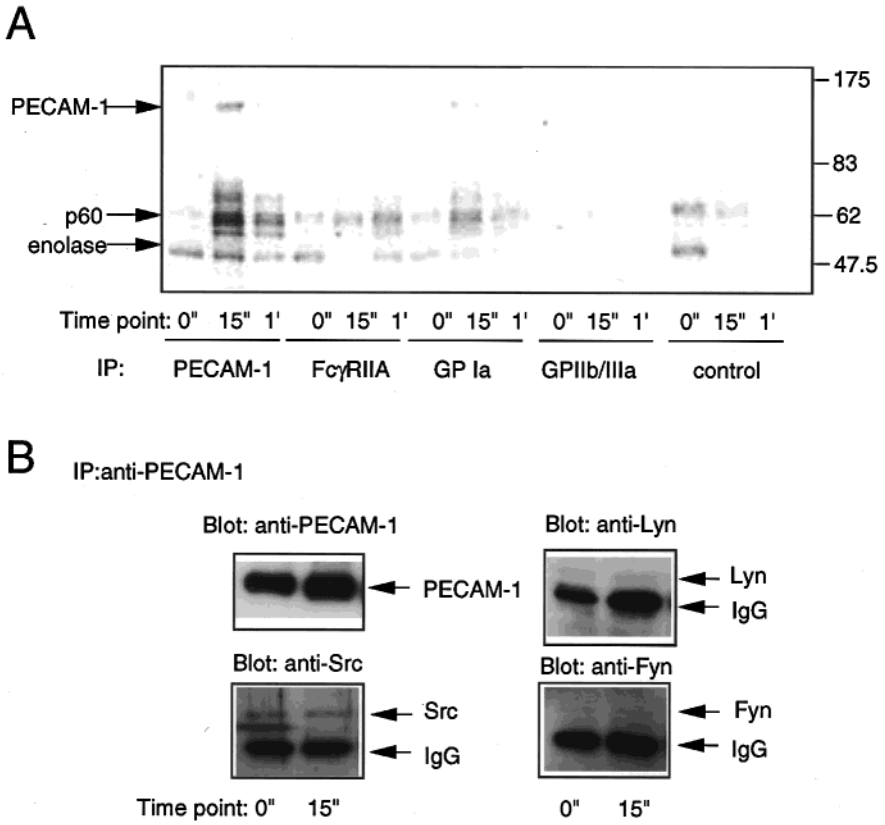

FIGURE 8: In vitro kinase assays associated with various platelet membrane proteins, and detection of Src family kinases in anti-PECAM-1 immunoprecipitates. In panel A, platelets were stimulated with 50 μg/mL of WGA for the indicated durations. The platelet lysates were immunoprecipitated with anti-PECAM-1 MoAb, anti-Fcγ receptor IIA MoAb (IV.3), anti-GPIa MoAb, anti-GPIIb/IIIa MoAb (NNKY-2-11), or control mouse IgG. An in vitro kinase assay was performed using acid-treated enolase as an exogenous substrate. In panel B, platelets were stimulated with 50 μg/mL of WGA for the indicated durations. The platelet lysates were immunoprecipitated with anti-PECAM-1 MoAb and then immunoblotted with anti-PECAM-1 MoAb, anti-Src MoAb, anti-Lyn MoAb, or anti-Fyn MoAb.

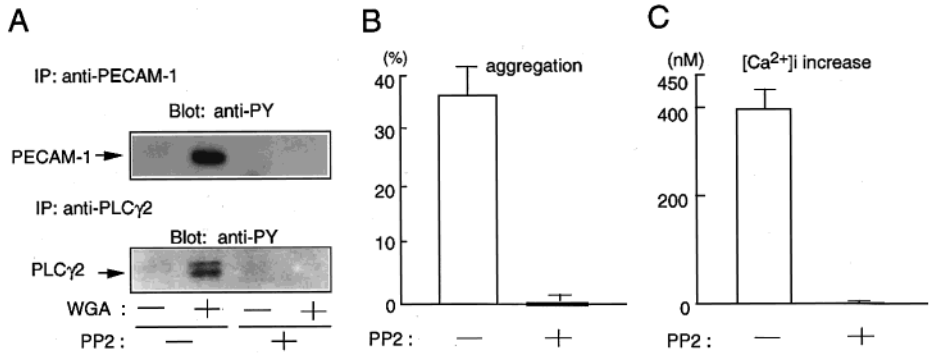

FIGURE 9: Effects of PP2 on tyrosine phosphorylation of PECAM-1 and PLCγ2, platelet aggregation, and Ca^{2+} mobilization induced by WGA. Platelets preincubated without (−) or with (+) 10 μM PP2 for 5 min were challenged with 10 μg/mL of WGA. In A, tyrosine phosphorylation of PECAM-1 (Upper panel) and PLCγ2 (Lower panel) was detected. In B, platelet aggregation was measured 2 min after a WGA challenge. In C, peak $[Ca^{2+}]_i$ increases after the WGA challenge were calculated. Columns and error bars represent the mean ± SD ($n = 3$).

ing the effects of PP2 on tyrosine phosphorylation of PECAM-1 and PLC-γ2, intracellular Ca^{2+} mobilization, and aggregation. PP2 almost completely inhibited tyrosine phosphorylation of PECAM-1 and PLCγ2, intracellular Ca^{2+} mobilization, and aggregation induced by WGA (Figure 9), while PP2 failed to affect Ca^{2+} mobilization and aggregation elicited by thrombin (data not shown). These data suggest that activation of Src family tyrosine kinases leading to tyrosine phosphorylation of PECAM-1 and PLCγ2 plays an important role in WGA-induced platelet activation.

WGA-Induced PECAM-1 Tyrosine Phosphorylation and Cytoskeletal Reorganization in Endothelial Cells. We finally examined whether WGA-induced activation via PECAM-1 was limited to platelets. When HUVECs were challenged with WGA, tyrosine phosphorylation of PECAM-1, reaching a maximal level 1 min after stimulation, was clearly observed (Figure 10A). We then examined the cytoskeletal reorganization induced by WGA. As can be seen in Figure 10B, actin was slightly concentrated in a peripheral band along cell−cell contacts in intact endothelial monolayers. WGA failed to induce stress fiber formation, cell contraction, or intercellular gaps (Figure 10B). WGA stimulated actin assembly at the cell periphery in cell−cell contact in endothelial monolayers (Figure 10B).

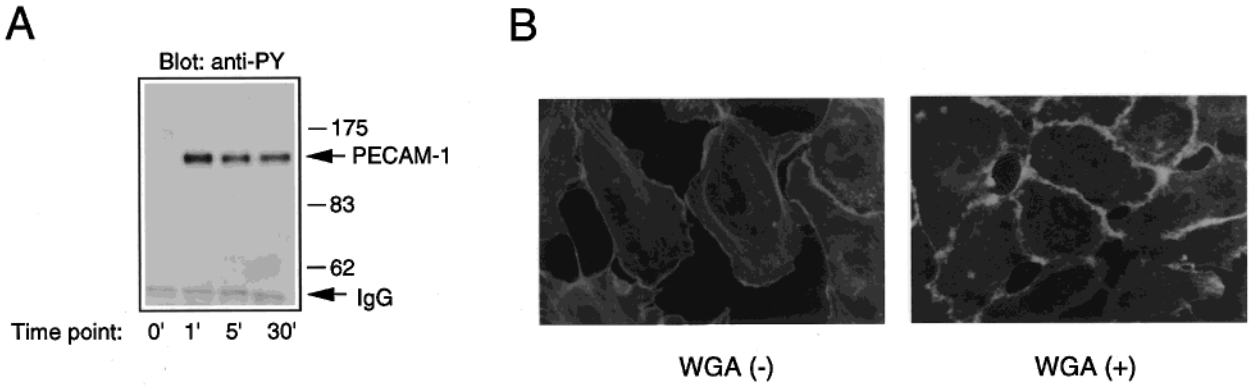

FIGURE 10: WGA-induced PECAM-1 tyrosine phosphorylation and cytoskeletal reorganization in vascular endothelial cells. In panel A, serum-starved HUVECs were challenged with 50 μg/mL of WGA for various durations. The cell lysates were immunoprecipitated with anti-PECAM-1 MoAb, resolved on an 8% SDS−PAGE, and then immunoblotted with anti-phosphotyrosine (PY) MoAb. In panel B, serum-starved HUVECs were challenged without (left panel) or with (right panel) 50 μg/mL of WGA for 30 min, fixed, and incubated with TRITC-phalloidin. Actin staining and immunofluorescent staining were performed as described in the Experimental Procedures.

DISCUSSION

WGA, a tetravalent plant lectin composed of two identical subunits, binds to the surface of platelets with high specificity for NAGlu and has been shown to elicit platelet reactions such as aggregation and release (*21*). We and others previously showed that protein-tyrosine phosphorylation plays a key role in eliciting PLC activation (leading to intracellular Ca^{2+} mobilization) and the resultant platelet functional responses induced by WGA (*19*, *20*, *22*, *23*), suggesting that WGA-induced platelet activation pathways resemble those induced by collagen. The collagen receptor GPVI is coupled to the FcR γ-chain, which contains a consensus sequence known as the immune-receptor tyrosine-based activation motif (*34*, *43*). It is now established that tyrosine phosphorylation of this motif upon GPVI stimulation is a crucial step in the activation of PLCγ2, through mediation of tyrosine kinases including Syk and Src family kinases (such as Fyn and Lyn) (*34*). As was the case with collagen, WGA induced protein-tyrosine phosphorylation of Syk and PLCγ2, and PLCγ2 phosphorylation, intracellular Ca^{2+} mobilization, and aggregation induced by WGA were inhibited by the Src family tyrosine kinase PP2. However, the platelet surface molecule(s) interacting with and transducing the WGA signal remained to be clarified until this study. In this study, we found that WGA interacts with and tyrosine-phosphorylates PECAM-1; WGA-mediated platelet activation was specifically inhibited by anti-PECAM-1 antibody. The involvement of GPVI in WGA-induced platelet activation was not likely since WGA activated platelets obtained from FcR γ-chain-deficient mice. These results of ours indicate that PECAM-1 is an important and functional receptor for WGA. However, it should be kept in mind that involvement of other cell surface molecule(s) cannot be ruled out.

Recent studies have revealed that the Ig superfamily member PECAM-1 is closely involved in signal transduction pathways (*1*, *2*). PECAM-1 tyrosine phosphorylation is a potential mode of regulation of its signal transduction, as integrin engagement and murine embryonic vascular development both modulate PECAM-1 phosphotyrosine levels (*3*). Engagement of PECAM-1 has been shown to affect the phosphorylation states of its cytoplasmic domain, creating a docking site for one or more signaling molecules (*2*, *4*, *15*). Since PECAM-1 does not possess a kinase domain in its cytoplasmic domain, it is important to identify the kinase(s) responsible for the observed tyrosine phosphorylation of PECAM-1. Many studies have shown that Src family tyrosine kinase(s) phosphorylates the cytoplasmic domain of PECAM-1 (*37*, *44*). In this study, Src family tyrosine kinases, and kinase activity which increased upon WGA stimulation, were detected in PECAM-1 immunoprecipitates. Furthermore, the Src family kinase inhibitor PP2 inhibited WGA-induced platelet aggregation, Ca^{2+} mobilization, and PLCγ2 tyrosine phosphorylation. Accordingly, PECAM-1-associated Src tyrosine kinases seem to play an important role in WGA-triggered downstream platelet activation signals leading to PLCγ2 activation. It should be noted that the collagen receptor GPVI is also a member of the Ig superfamily of proteins, with two extracellular Ig domains (*43*). It is likely that signals originating from cluster formation of Ig superfamily adhesion molecules and resulting in PLCγ2 activation, through mediation of Src family tyrosine kinases, may be an important group of signal transduction pathways in platelets, in addition to the interaction of soluble agonists with G protein-coupled heptahelical cell surface receptors.

Our present study is not the first to report platelet PECAM-1 tyrosine phosphorylation; this has been already reported for platelets stimulated with physiological agonists, including collagen and thrombin (*15*, *37*). However, the mode of PECAM-1 tyrosine phosphorylation induced by WGA is distinct from that by the receptor-mediated agonist. WGA-induced PECAM-1 phosphorylation is an early and rapid event which occurs before the onset of, and is independent of, integrin αIIbβ3-mediated platelet aggregation. In contrast, PECAM-1 phosphorylation observed upon agonist stimulation is a slower response and is dependent on platelet aggregation. Recently, it has been reported that platelet cell−cell contact, rather than aggregation, is important for PECAM-1 phosphorylation via agonist stimulation (*37*). However, WGA-induced PECAM-1 tyrosine phosphorylation proceeded even without stirring of platelets and hence cell−cell contact. Presumably, WGA clustering of PECAM-1 may transduce very strong and efficient signals leading to the tyrosine phosphorylation of this adhesion molecule itself, while PECAM-1 phosphorylation by soluble agonists is more of a secondary response.

WGA-induced PECAM-1 phosphorylation was reproduced in HUVECs. The cell−cell junction is the site where actin-based cytoskeletal structures are anchored to the plasma membrane and, therefore, is a potential site where mechanical stress generated by external forces is focused. PECAM-1 is localized to the cell−cell borders of vascular endothelial cells where it mediates the extravasation of monocytes and neutrophils during the inflammatory response (*4*) and is tyrosine-phosphorylated by mechanical stimulus, including fluid flow and hyper- and hypoosmotic shocks (*46*). Although mechanisms for fluid flow sensing and subsequent signaling in endothelial cells have not yet been well characterized (*47*), the flow-induced change in PECAM-1 tyrosine phosphorylation has been suggested to be a normal physiological process occurring in endothelial cells exposed to flow (*46*). When HUVECs were stimulated with WGA, PECAM-1 was markedly tyrosine-phosphorylated and the actin band at cell−cell contacts was thickened. The stimulation of endothelial cells with WGA seems to be a unique model for studying signaling pathways induced by shear stress.

As described above, we observed WGA induction of PECAM-1 phosphorylation and cellular responses in platelets and vascular endothelial cells, in which PECAM-1 was expressed. It has been reported that PECAM-1 is expressed not only in these cells, but also in neutrophils, monocytes, and lymphocytes (*1*, *2*). Basically, these PECAM-1-expressing cells are known to respond to a WGA challenge. WGA induces NADPH-oxidase activity in neutrophils (*48*), monocyte-mediated tumor cell killing (*49*), Syk activation in porcine splenocytes (*50*), and IL2 production and IL2 receptor expression in lymphocytes (*51*). It is possible that PECAM-1 is involved in these processes, and WGA may prove to be a useful tool for elucidating PECAM-1-mediated signaling and functional responses, with wide implications not limited to platelets.

PECAM-1 is postulated to contain functional immune-receptor tyrosine-based inhibitory motifs within its cytoplasmic domain; co-ligation of PECAM-1 with the T-cell antigen receptor results in tyrosine phosphorylation of PECAM-1, recruitment of Src homology 2 domain-containing protein tyrosine phosphatase-2, and attenuation of T-cell antigen receptor-mediated cellular signaling (*52*). Consistently with this finding in lymphocytes, it has very recently been shown that platelet activation via the GPVI/FcR γ-chain complex, which transduces collagen-elicited platelet activation through an immune-receptor tyrosine-based activation motif, is specifically exaggerated in PECAM-1-deficient murine platelets (*53*). Together with our present study, it is likely that PECAM-1 is deeply involved in platelet signal transduction pathways.

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