Collagen, Convulxin, and Thrombin Stimulate Aggregation-

independent Tyrosine Phosphorylation of CD31 in Platelets

EVIDENCE FOR THE INVOLVEMENT OF Src FAMILY KINASES*

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Platelet endothelial cell adhesion molecule-1 (CD31) is a 130-kDa glycoprotein receptor present on the surface of platelets, neutrophils, monocytes, certain T-lymphocytes, and vascular endothelial cells. CD31 is involved in adhesion and signal transduction and is implicated in the regulation of a number of cellular processes. These include transendothelial migration of leukocytes, integrin regulation, and T-cell function, although its function in platelets remains unclear. In this study, we demonstrate the ability of the platelet agonists collagen, convulxin, and thrombin to induce tyrosine phosphorylation of CD31. Furthermore, we show that this event is independent of platelet aggregation and secretion and is accompanied by an increase in surface expression of CD31. A kinase capable of phosphorylating CD31 was detected in CD31 immunoprecipitates, and its activity was increased following activation of platelets. CD31 tyrosine phosphorylation was reduced or abolished by the Src family kinase inhibitor PP2, suggesting a role for these enzymes. In accordance with this, each of the Src family members expressed in platelets, namely Fyn, Lyn, Src, Yes, and Hck, was shown to co-immunoprecipitate with CD31. The involvement of Src family kinases in this process was confirmed through the study of mouse platelets deficient in Fyn.

Platelet endothelial cell adhesion molecule-1 (CD31) is a membrane-spanning glycoprotein of 130 kDa that is expressed on the surface of platelets, endothelial cells, neutrophils, monocytes, and some T-lymphocyte subsets (1–4). The functions that have been identified for CD31 are diverse and include transendothelial migration of leukocytes (5, 6), angiogenesis (3, 7) vascularogenesis (8), integrin regulation (9, 10), and T-cell receptor function (11). The cloning of CD31 was as a consequence of its expression on platelets (4); however, a clear role for CD31 in platelets remains obscure. CD31 was classified to the family of cellular adhesion molecules on the basis of structural and amino acid sequence. Homophilic ligand binding interactions are believed to underlie the functions of CD31, although heterophilic interactions with integrin αβ3, CD38, and unidentified proteins have been suggested (12, 13).

In recent years, considerable attention has been directed to the ability of CD31 to participate in signal transduction. Numerous studies have shown that CD31 becomes tyrosine-phosphorylated in response to a variety of conditions that include CD31 cross-linking (14), activation of the high affinity receptor for immunoglobulin E (FcεRI) (15), platelet aggregation (16), shear (17), and oxidative stress (18). The sites of CD31 tyrosine phosphorylation have been mapped to two residues that fall within a conserved signaling motif known as an immunoreceptor tyrosine-based inhibitory motif (ITIM). ITIMs are found in an increasing list of receptors that regulate immune function and are defined by the consensus sequence (L/I/V/S)X(L/I/V) (5-30)-amino acid residue sequence (19). The phosphorylated ITIM provides a docking site for signaling proteins that bind via SH2 domains. The protein-tyrosine phosphatases SHP-2 and SHP-1 have been shown to associate with tyrosine-phosphorylated CD31 (16, 20); and for SHP-2, this interaction has been shown in vitro to stimulate its activation (14). As a consequence, ITIM-containing receptors may counter the effects of tyrosine kinases and thereby negatively regulate signaling pathways, although SHP-2 has been shown to positively regulate growth factor receptor signaling. It has been suggested recently that CD31 would be more correctly classified as a member of the Ig ITIM family of proteins (21).

In contrast to the effects of ITIM-containing receptors are the functions of a number of receptors that possess a conserved signaling motif termed the immunoreceptor tyrosine-based activation motif (ITAM). The consensus sequence for this motif is YXXLX(α-ω)YXXL (22) and is found on the cytoplasmic tail of several immune receptors, including T- and B-cell antigen receptors and a number of immunoglobulin receptors (23, 24). It is believed that ITAM- and ITIM-bearing receptors may in some contexts act antagonistically when expressed on the same cell. Indeed, a number of closely related receptors, such as FcγRIa (ITAM) and FcγRIb (ITIM), are thought to function in this way (25).

We and others have recently identified the collagen receptor that underlies platelet activation. Collagens are principal platelet agonists at sites of vascular damage and are therefore central to the process of hemostasis. This collagen receptor

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1 The abbreviations used are: FcεRI, high affinity receptor for immunoglobulin E; FcγRI, Fc receptor; ITAM, immunoreceptor tyrosine-based inhibitory motif; ITIM, immunoreceptor tyrosine-based activation motif; GPVI, glycoprotein VI; PP, protein phosphatase; Cvx, convulxin; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; 5-HT, 5-hydroxytryptamine.
comprises the recently cloned glycoprotein VI (GPVI) (26–28) and the Fc receptor (FcR) γ-chain (29). The FcR γ-chain contains an ITAM within its cytoplasmic domain that is tyrosine-phosphorylated upon collagen stimulation. The phosphorylated ITAM is responsible for recruiting signaling molecules such as Syk and phosphatidylinositol 3-kinase to the receptors; and thus, multiple signaling pathways are activated, leading to platelet secretion and aggregation (29–31).

The role of the collagen receptor GPVI in CD31 signaling in platelets is particularly interesting since this receptor signals through an ITAM. In this study, we have investigated the role of this collagen receptor in the stimulation of CD31 tyrosine phosphorylation. We demonstrate that activation of this receptor and also platelet thrombin receptors results in tyrosine phosphorylation of CD31 and that this is not dependent on integrin function or platelet aggregation. We show that platelet activation by GPVI and thrombin receptors leads to an increase in the platelet surface expression of CD31 and present data suggesting that Src family kinases are responsible for CD31 tyrosine phosphorylation in platelets.

EXPERIMENTAL PROCEDURES

Materials—Horn-Chemie collagen (collagen fibers from equine tendons) was purchased from Nycomed (Munich, Germany). Convolxin was purified from the venom of the rattlesnake (Crotalus durissus terrificus) as described previously (32). Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology, Inc. (Buckinghamshire, UK). Anti-CD31 monoclonal antibody HCl/6 was from Sero-tec (Oxford, UK); anti-CD31 polyclonal (C-20 and M-20) and monoclonal (AB468F and control antibody AB600F) antibodies were from Autogen-Bioclear Ltd. (Wiltshire, UK). Anti-Fyn, Anti-Lyn, anti-Src, anti-Yes, and anti-Hck polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-Fyn (clone 25) and anti-Lyn (clone 42) monoclonal antibodies were from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence detection system was purchased from Amersham Pharmacia Biotech (Buckinghamshire). The Src family kinase inhibitor PP2 and negative control PP3 were purchased from Amersham Pharmacia Biotech. The Src family kinase inhibitor PP2 and negative control PP3 were purchased from Amersham Pharmacia Biotech. The Src family kinase inhibitor PP2 and negative control PP3 were purchased from Amersham Pharmacia Biotech. The Src family kinase inhibitor PP2 and negative control PP3 were purchased from Amersham Pharmacia Biotech.

Preparation and Stimulation of Platelets—Human platelets from drug-free volunteers were prepared on the day of the experiment by differential centrifugation as described previously (31) and suspended in modified Tyrode's/Hepes buffer, and indomethacin, PP2, and PP3 were added such that the final solvent concentration was 0.2% (v/v) and replaced with assay buffer (105 mM NaCl, 20 mM Hepes, 5 mM glucose, and 1 mM MgCl2, pH 7.3) to a density of 8 × 10^10 cells/ml. Stimulation of platelets (450 μl) with collagen, convolxin (Cvx), and thrombin (dissolved in 50 μl) was performed at 37 °C in an aggregometer with continuous stirring (1200 rpm). Mouse platelets were prepared as described previously (30) and suspended in modified Tyrode's/Hepes buffer to a density of 1.3 × 10^10 cells/ml. 90 μl of suspension was used for each assay with collagen, Cvx, or thrombin, added in a volume of 10 μl. For protein precipitation experiments, platelets were resuspended in buffer containing 1 mM EGTA to prevent aggregation. In certain experiments, platelets were preincubated prior to stimulation with the tetrapeptide RGD(5) (16) or the fibrinogen γ-peptide DVIHHLGQRAG (125 μM) (34). Where required, inhibitors and other reagents were added such that the final solvent concentration was 0.2% (v/v) and incubated at 37 °C for 5 min before stimulation. Apyrase was prepared in modified Tyrode's/Hepes buffer, and indomethacin, PP2, and PP3 were dissolved in MeSO.

Immunoprecipitation Studies—Platelet stimulation was terminated by the addition of an equal volume of ice-cold lysis buffer (2% (v/v) Nonidet P-40 or 2% (v/v) Brij 96, 20 mM Tris, 300 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotenin, and 1 μg/ml pepstatin A, pH 7.3). Detergent-insoluble debris was removed, and the lysates were precleared by mixing with protein A-Sepharose for 1 h at 4 °C (20 μl of a 50% (v/v) suspension of protein A-Sepharose in Tris-buffered saline/Tween (TBS/T; 20 mM Tris, 137 mM NaCl, and 0.1% (v/v) Tween 20, pH 7.6). Protein A-Sepharose was removed from the lysates before the addition of relevant antibodies (1 or 2.5 μg of anti-CD31 or 1 μg of anti-Lyn, anti-Fyn, anti-Src, anti-Yes, or anti-Hck). Following rotation at 4 °C for 1 h, 0.5 μl of secondary antiserum was added where required (CD31 (human platelets), Lyn, and Fyn: rabbit anti-mouse IgG; and CD31 (mouse platelets) and Src: sheep anti-goat IgG) and mixed for a further 25 min. Serum-free suspension was added to each sample, and mixing was continued for 1 h before washing the Sepharose pellet in lysis buffer, followed by a second wash in TBS/T and the addition of Laemmli sample treatment buffer. Proteins were separated by SDS-PAGE using 10% gels and transferred to polyvinylidene difluoride membranes by semidy Western blotting.

Immunoblotting—Following Western blotting, polyvinylidene difluoride membranes were blocked in incubation in 10% (w/v) bovine serum albumin dissolved in TBS/T. Primary and secondary antibodies were diluted in TBS/T containing 2% (w/v) bovine serum albumin and incubated with polyvinylidene difluoride membranes for 1 h at room temperature. Blots were washed for 2 h in TBS/T following each incubation with antibodies for 1 h at room temperature and then developed using the enhanced chemiluminescence detection system. Primary antibodies were used at a concentration of 1 μg/ml (anti-phosphotyrosine, anti-CD31, anti-Lyn, anti-Fyn, anti-Src, anti-Yes, and anti-Hck). Horseradish peroxidase-conjugated secondary antibodies were diluted 1:10,000.

Flow Cytometry—Human platelets were stimulated at a density of 8 × 10^6 cells/ml with Cvx (62.5 or 125 ng/ml) or thrombin (0.1 or 1 unit/ml) in the presence of EGTA (1 mM), indomethacin (10 μM), and apyrase (2 units/ml). Stimulation was terminated by dilution to 10^8 cells/ml by the addition of ice-cold buffer (modified Tyrode's/Hepes buffer, pH 7.3, containing 1% (w/v) bovine serum albumin, 1 mM EGTA, and 0.04% (w/v) sodium azide. Platelet suspensions were then incubated at room temperature for 1 h with a fluorescein isothiocyanate-conjugated anti-CD31 monoclonal antibody (AB468F) diluted to 1:200. Nonspecific antibody binding was determined using a fluorescein isothiocyanate-conjugated isotype-matched IgG (AB600F). Data were collected using a FACScan flow cytometer (Becton Dickinson) and analyzed using CELLQuest software (Becton Dickinson).

RESULTS

Collagen, Convolxin, and Thrombin Stimulate CD31 Tyrosine Phosphorylation in Platelets—At sites of vascular damage, platelets may be activated in response to a number of factors, among which the extracellular matrix protein collagen and the enzyme thrombin are considered to be the most thrombogenic. Platelet activation is mediated through a tyrosine kinase-dependent pathway via the receptor GPVI, whereas thrombin stimulates an alternative signaling pathway via activation of G protein-coupled thrombin receptors.

Since the physiological significance of CD31 in platelet function has not been determined, we sought to characterize the effect of collagen- and thrombin receptor-mediated platelet activation on the tyrosine phosphorylation state of CD31. In addition to use of collagen and thrombin, platelets were also...
stimulated with Cvx, a protein purified from the venom of the rattlesnake (C. durissus terrificus) that is a selective GPVI agonist (31, 32).

Platelets were stimulated with collagen (10–100 μg/ml), Cvx (62.5–500 ng/ml), or thrombin (0.01–0.5 units/ml) for 90 s, and CD31 was immunoprecipitated from cell lysates. Proteins were separated by SDS-PAGE and immunoblotted to detect tyrosine phosphorylation. Each of the platelet agonists induced robust tyrosine phosphorylation of CD31 in a dose-dependent manner (Fig. 1A). With collagen, tyrosine phosphorylation was detectable at a concentration of 10 μg/ml and reached maximal phosphorylation at ~100 μg/ml. Cvx is a very potent GPVI agonist (32) and stimulated maximal tyrosine phosphorylation at 62.5 ng/ml. The level of CD31 tyrosine phosphorylation upon thrombin stimulation peaked at 0.1 unit/ml; however, at higher concentrations, additional tyrosine-phosphorylated proteins were co-immunoprecipitated. These unidentified proteins were also detected upon stimulation of platelets with supramaximal concentrations of collagen and convulxin (data not shown). Src family kinases have been implicated in the phosphorylation of CD31. Therefore, the possibility that the smaller tyrosine-phosphorylated protein that co-immunoprecipitated with CD31 (Fig. 1A) was a Src family kinase was investigated. The protein did not comigrate precisely with any of five Src family kinase members that are expressed in platelets. We report below that such kinases do associate with CD31 in platelets; however, this is not dependent on platelet activation. The larger tyrosine-phosphorylated co-immunoprecipitated protein has an apparent molecular mass of 68 kDa (determined using QuantityOne® software, Bio-Rad) and is likely to be SHP-2, whose association with tyrosine-phosphorylated CD31 is well documented.

The kinetics of CD31 tyrosine phosphorylation stimulated with these agonists are examined and are shown in Fig. 1B. Collagen (100 μg/ml)-stimulated tyrosine phosphorylation was detectable at 20 s of stimulation, and the level peaked at 90 s. This kinetic profile is similar to that previously reported for the tyrosine phosphorylation of the FcR γ-chain upon stimulation of platelets with collagen (29) and is consistent with this being an early signaling event. Cvx-stimulated tyrosine phosphorylation was rapid and reached maximal levels after 20 s of stimulation. Stimulation with thrombin also resulted in rapid tyrosine phosphorylation of CD31 that was detectable at 20 s; however, levels continued to rise over 300 s.

CD31 Tyrosine Phosphorylation Is Independent of Aggregation and Secretion—The stimulation of CD31 tyrosine phosphorylation in platelets following incubation with thrombin-related activatory peptide has been reported to be dependent on aggregation and integrin function. The results discussed above were therefore surprising since the stimulations were performed in the presence of EGTA (1 mM), which prevented platelet aggregation by chelating extracellular calcium that is required for stabilization and function of the fibrinogen receptor integrin αIIbβ3. To confirm this observation, platelets were stimulated with Cvx (62.5 ng/ml) or thrombin (0.1 unit/ml) under alternative conditions that prevent fibrinogen binding to integrin αIIbβ3, and thereby block aggregation (Fig. 2A). Tyrosine phosphorylation was induced by both agonists in the presence of EGTA, the tetrapeptide RGDS (0.5 mM), or the fibrinogen γ-chain peptide (125 μM). The treatments alone did not affect the basal levels of CD31 tyrosine phosphorylation (data not shown), and the differences in the levels of CD31 tyrosine phosphorylation in resting platelets are a consequence of variability between donors and platelet preparations. Aggregation assays were performed on the samples and confirmed complete inhibition of aggregation (data not shown).

Platelet activation leads to degranulation and the release or
secretion of various factors that further enhance platelet activation. These include thromboxane A2 formed from liberated arachidonic acid via the cyclooxygenase pathway and ADP, which is secreted from dense granules. To determine whether collagen-, Cvx-, or thrombin-stimulated CD31 tyrosine phosphorylation was dependent on the release of thromboxane A2, platelets were stimulated in the presence or absence of indomethacin (10 μM), an inhibitor of cyclooxygenase. Fig. 2B shows that the level of CD31 tyrosine phosphorylation was not altered in the presence of indomethacin. Furthermore, the inclusion of apyrase (2 units/ml), which catalyzes the conversion of ADP to AMP, had no effect on the level of CD31 tyrosine phosphorylation stimulated by these agonists (Fig. 2B). Slightly different levels of CD31 tyrosine phosphorylation in unstimulated cells in the two experiments shown in Fig. 2 are due to donor and platelet preparation variability.

Platelet degranulation following stimulation may be assessed through measurement of 5-HT secretion. Fig. 2C (panel i) shows the results of 5-HT secretion assays performed on cells stimulated in the absence or presence of EGTA (1 mM), indomethacin (10 μM), and apyrase (2 units/ml). High levels of 5-HT secretion were stimulated with Cvx (62.5 ng/ml) and thrombin (0.1 unit/ml), and this was almost abolished in the presence of EGTA, indomethacin, and apyrase (Cvx, reduced from 61.8 ± 6.5 to 5.6 ± 4.7%; and thrombin, reduced from 7.9 ± 1.7 to 6.2 ± 3.0%; and thrombin, reduced from 6.9 ± 2.8 to 5.6 ± 4.7% (mean ± S.E., n = 3)). However, Cvx- and thrombin-stimulated tyrosine phosphorylation of CD31 was maintained under these conditions (Fig. 2C, panel ii). These data support the notion that tyrosine phosphorylation of CD31 following stimulation of the GPVI collagen receptor or the thrombin receptor is an early signaling event that is not dependent on the activity of factors released by the platelets. All subsequent experiments were performed in the presence of EGTA (1 mM), indomethacin (10 μM), and apyrase (2 units/ml).

Platelet-Platelet Contact May Contribute to CD31 Tyrosine Phosphorylation—CD31 has been reported to participate in both homophilic and heterophilic binding, and CD31 dimerization or clustering has been shown to stimulate its tyrosine phosphorylation. However, the binding characteristics of this protein on platelets have not been studied. It was not clear...
from our experiments whether CD31 tyrosine phosphorylation was a direct consequence of intracellular signaling following stimulation of the collagen and thrombin receptors or whether CD31-ligand binding may contribute to the process. The stirring of platelets increases cell-cell collisions and increases the likelihood of intercellular ligand binding of CD31, which may lead to increased tyrosine phosphorylation of the protein. In accordance with this, we observed that stirring platelets induced low-level and time-dependent tyrosine phosphorylation of CD31. This effect is shown in CD31 immunoprecipitates from stirred platelets in Fig. 3A. The level of tyrosine phosphorylation was considerably lower than when stimulated with collagen, Cvx, or thrombin and continued to rise over a period of 5 min.

An increase in CD31 tyrosine phosphorylation upon stimulation with collagen, Cvx, and thrombin may be a direct consequence of activated intracellular signaling mechanisms and/or CD31-ligand binding. If CD31-ligand binding is important for this process, one may expect to observe an increased capacity for such binding in platelets stimulated with Cvx or thrombin. To examine this, the level of cell-surface expression of CD31 in resting platelets and platelets stimulated with Cvx or thrombin was examined by flow cytometry using a fluorescein isothiocyanate-conjugated anti-CD31 antibody. The results shown in Fig. 3B demonstrate that the cell-surface expression of CD31 was increased following stimulation of platelets with Cvx or thrombin in a dose-dependent manner. The mean fluorescence levels for Cvx-stimulated platelets (125 ng/ml) were increased by ~30% above resting levels; and for thrombin, the increase was ~190%. These results are representative of four separate experiments. Comparison of Fig. 3B with the CD31 tyrosine phosphorylation dose responses shown in Fig. 1A indicates that surface expression was increased at high concentrations where further increases in tyrosine phosphorylation were not observed. This indicates that increased surface expression of CD31 and the increased ligand binding that this would facilitate may contribute to CD31 tyrosine phosphorylation, but additional contributions by direct GPVI and thrombin receptor signaling are also likely to be involved. Due to sample processing for analysis by flow cytometry, the stimulation time in these experiments was 5 min. Maximal tyrosine phosphorylation of CD31 was maintained at this time point for each of the agonists (Fig. 1B and data not shown).

Cvx- and Thrombin-stimulated Tyrosine Phosphorylation: Evidence for Src Family Kinase Involvement—Several studies have implicated Src family kinases as the enzymes responsible for tyrosine phosphorylation of CD31; and in vitro, Src itself can phosphorylate CD31 (35, 36). Reconstitution studies have partially addressed which kinases can phosphorylate CD31 and have implicated Src, Lyn, and the Csk kinase (not a Src family kinase), although an exhaustive investigation has not been made. The identity of the kinase(s) that phosphorylate CD31 at normal cellular levels in vivo is, however, not clear; and this question has not been addressed in platelets. Receptors such as FcεRI (37), FcγRI (38), the T-cell antigen receptor (39), the B-cell antigen receptor (40), and the platelet-derived growth factor receptor (41) that are activated through phosphorylation by Src family kinases have been shown to physically associate with the appropriate kinase. We therefore conducted in vitro kinase assays on CD31 immunoprecipitates obtained from resting and stimulated platelets. Such experiments revealed that a kinase was co-immunoprecipitated with CD31 and was able to phosphorylate CD31 in vitro (Fig. 4A). Furthermore, the ability of this enzyme to phosphorylate CD31 was increased following stimulation of platelets with convulxin or thrombin (Fig. 4A, upper panel). Quantification of increased 32P incorporation into CD31 is shown in Fig. 4B. Blots were probed for tyrosine phosphorylation to reveal a similar profile to 32P incorporation in the kinase assays (Fig. 4A, middle panel). The identity of the protein on the PhosphorImager radiograph was confirmed by reprobing the blot for CD31.

We therefore examined the effect of the selective Src family kinase inhibitor PP2 (42) on Cvx- and thrombin-stimulated tyrosine phosphorylation of CD31 in platelets. 10 μM PP2 reduced basal levels of CD31 phosphorylation in comparison with cells stimulated in the presence of the negative control molecule for the inhibitor, PP3 (Fig. 5A). The same concentration of PP2 reduced Cvx (62.5 ng/ml)-stimulated tyrosine phosphorylation to approximately basal levels, and phosphorylation in response to thrombin (0.05 units/ml) was also reduced dramatically. This effect was shown to be dose-dependent (data not shown). In addition, stirring-induced phosphorylation of CD31 was completely abolished in the presence of 10 μM PP2 (data not shown).

Since a kinase co-immunoprecipitated with CD31 from platelets, which was able to phosphorylate CD31 in vitro and whose activity was increased upon stimulation of platelets with Cvx or thrombin, the possibility that the associated enzyme may be a Src family kinase was explored. Platelets have been shown to express five of the Src family kinase members: Fyn, Lyn, Src, Hck, and Yes. Each of the kinases was immunoprecipitated from platelet lysates under resting conditions or following stimulation with Cvx or thrombin. Proteins were separated by SDS-PAGE and immunoblotted to detect the presence of CD31.
FIG. 4. CD31 is associated with a kinase. A, platelets incubated with EGTA (1 mM), indomethacin (10 μM), and apyrase (2 units/ml) were stirred or stimulated with Cvx (62.5 ng/ml) or thrombin (Throm.; 0.1 unit/ml) for 90 s. CD31 was immunoprecipitated from cell lysates, and an in vitro kinase assay was performed on the precipitate. Samples were resolved by SDS-PAGE and Western-blotted onto polyvinylidene difluoride membranes. 32P incorporation into proteins was determined using a PhosphorImager (upper panel). Blots were immunoblotted for phosphorylated tyrosine residues (middle panel), and the identity of 32P-labeled CD31 was determined by stripping and reprobing for CD31 (lower panel). B, the relative increase in 32P incorporation into CD31 in the in vitro kinase assay (A, upper panel) was quantified using ImageQuant software. I.P., immunoprecipitates.

FIG. 5. Evidence for the involvement of Src family kinases. A, platelets incubated with EGTA (1 mM), indomethacin (10 μM), and apyrase (2 units/ml) were stirred or stimulated with Cvx (62.5 ng/ml) or thrombin (0.1 unit/ml) for 90 s in the presence of the selective Src family kinase inhibitor PP2 (10 μM) or the negative control PP3 (10 μM). CD31 was immunoprecipitated from platelet lysates and separated by SDS-PAGE. Levels of tyrosine phosphorylation were assessed by immunoblotting. Blots were reprobed for CD31 to ensure equal loading. B, platelets incubated with EGTA (1 mM), indomethacin (10 μM), and apyrase (2 units/ml) were only stirred or stimulated with Cvx (62.5 ng/ml) or thrombin (Thr; 0.1 unit/ml) for 90 s. The Src family kinases Fyn, Lyn, Src, Yes, and Hck were immunoprecipitated from platelet lysates. Proteins were separated by SDS-PAGE, and Western blots were probed for the presence of CD31. Following stripping of blots, levels of CD31 tyrosine phosphorylation and Src family kinases were determined by further immunoblotting. I.P., immunoprecipitates.
in the immunoprecipitates. CD31 co-immunoprecipitated with each of the kinases: Fyn, Lyn, Src, Hck, and Yes (Fig. 5B). CD31 and Src family kinase association was present in resting cells, and the level of association was unchanged upon stimulation with Cvx and thrombin. Immunoblots were reprobed to ascertain the levels of tyrosine phosphorylation of the co-immunoprecipitated CD31. CD31 was found to be tyrosine-phosphorylated in Fyn, Lyn, Src, Yes, and Hck immunoprecipitates (Fig. 5B), but tyrosine phosphorylation of CD31 was not detectably increased upon stimulation of platelets. Blots were further reprobed to confirm the presence and equivalent levels of the Src family kinases.

Given the lines of evidence presented to support a role for several of the Src family kinases in agonist-stimulated tyrosine phosphorylation of CD31 in platelets, experiments were conducted to investigate tyrosine phosphorylation of this protein in mouse platelets that do not express the kinase Fyn. Experiments were performed to confirm that similar levels of CD31 tyrosine phosphorylation are obtained from mouse platelets compared with the levels detected in human platelets upon stirring and by stimulation with Cvx and thrombin (data not shown). Stirring induced similar low levels of CD31 tyrosine phosphorylation in Fyn 

A critical difference between our experiments and those of Jackson et al. (16) lies behind the definition of non-aggregating conditions. In the above report, negligible increases in tyrosine phosphorylation were detected under non-aggregating conditions, but these experiments were conducted by treating plate-

FIG. 6. CD31 tyrosine phosphorylation is reduced in Fyn-deficient mouse platelets. Platelets were incubated with EGTA (1 mM), indomethacin (10 μM), and apyrase (2 units/ml), and CD31 was immunoprecipitated from lysates of control (+/+), Fyn-deficient (-/-), and Lyn-deficient (-/-) mouse platelets under stirring conditions or following stimulation with Cvx (125 ng/ml, 300 s) or thrombin (10 units/ml, 300 s). Proteins were separated by SDS-PAGE and immunoblotted for tyrosine-phosphorylated residues. Immunoprecipitation and equal loading were verified by reprobing for CD31. Results are representative of three experiments. I.P., immunoprecipitates.
Aggregation-independent Phosphorylation of CD31 in Platelets

Platelets under static conditions. Aggregation was induced by agonists and stirring and was prevented under stirring conditions through the addition of the tetrapeptide RGDW. It is possible that their observations may be explained by the fact that stimulation without stirring limits cell-cell contact, and this may in itself reduce CD31 tyrosine phosphorylation levels. As outlined above, CD31 is recognized for its propensity for homophilic ligand binding interactions, which may occur in an intercellular manner and give rise to activation of CD31. This is supported by our observation that stimulating platelets stimulates low-level time-dependent tyrosine phosphorylation of CD31 (Fig. 3A). Furthermore, we report here that stimulation of platelets with Cvx or thrombin leads to a substantial increase in the surface expression of CD31. This may lie behind the mechanism that leads to increased tyrosine phosphorylation in agonist-stimulated platelets. We have examined the membrane localization of CD31 by immunoblotting of external and internal platelet membrane preparations (prepared by high-voltage free-flow electrophoresis and kindly provided by Dr. K. Authi (Thrombosis Research Institute, London)). CD31 was detected on internal as well as external platelet membranes, which would be expected given our observation that surface expression may be up-regulated upon stimulation. This is in agreement with Cramer et al. (46), who showed the presence of CD31 in α-granules using immunogold electron microscopy. It is interesting to note that a greater increase in surface expression is obtained with thrombin in comparison with Cvx. This is consistent with higher levels of CD31 tyrosine phosphorylation upon thrombin stimulation (Fig. 1B) at the concentrations of agonists that were used in flow cytometry experiments. In addition, the association of CD31 with platelet cytoskeleton following stimulation with thrombin has been reported (47). This interaction may be important in the mechanism for increasing surface expression of this molecule.

In accordance with the observations of Jackson et al. (16), we also observed higher levels of CD31 tyrosine phosphorylation when platelets were allowed to aggregate (e.g., in the absence of EGTA). We suggest that this is more likely to be a consequence of greater platelet-platelet interactions in the aggregate fostering elevated CD31-ligand binding than a direct consequence of integrin αIIbβ3 engagement. However, the possibility that fibrinogen binding and other receptor interactions may contribute cannot be excluded. The respective contributions of CD31-ligand binding and signal transduction as a direct consequence of GPVI and thrombin receptor activation remain to be determined, although the rapid kinetics strongly suggest that direct signaling in addition to CD31-ligand binding is likely to be involved.

In this study, we have begun to address the identity of the kinase(s) that is responsible for tyrosine phosphorylation of CD31 following stimulation of GPVI and the thrombin receptor in platelets. The Src family of tyrosine kinases (more specifically, Src, Lyn, and Lck) has been widely implicated in the phosphorylation of CD31; and more recently, Csk and the related kinase Chk have been added to this list. Other proteins such as Syk, which is activated upon binding to the activated collagen receptor, have been shown to be unable to phosphorylate CD31 (36). Most of this work has been performed in vitro and with recombinant proteins or through overexpression reconstitution studies, where elevated kinase levels may promote phosphorylation events that may not occur in vivo. It is important, however, to note the kinases that are capable of phosphorylating CD31 on tyrosine.

We have demonstrated that a kinase is associated with CD31 in platelets and that it is able to phosphorylate CD31 in vitro. Furthermore, the ability of this enzyme to phosphorylate CD31 is increased upon stimulation with Cvx or thrombin. We therefore sought to identify which kinase(s) may be present in CD31 immunoprecipitates and are responsible for phosphorylating CD31 in platelets. A selective Src family inhibitor (PP2) was used to determine whether Src family kinases are likely to be involved in the phosphorylation of CD31 in platelets (42). This compound was able to inhibit or to substantially reduce the level of CD31 tyrosine phosphorylation upon stimulation with Cvx or thrombin in dose-dependent manner. The activation of the GPVI collagen receptor has been shown to involve the kinases Fyn and Lyn (48, 49); and therefore, the effect on CD31 phosphorylation upon Cvx stimulation may be a consequence of inhibition of GPVI activation. However, stirring-induced CD31 phosphorylation was abolished using PP2, and thrombin-stimulated platelet activation has not been reported to be dependent on Src family kinases. This strongly implicates Src family kinase involvement in agonist- and stirring-mediated CD31 tyrosine phosphorylation.

An investigation was conducted to determine if any of the five Src family kinases that are expressed in platelets, namely Fyn, Lyn, Src, Hck, and Yes, are able to associate with CD31 in resting and stimulated platelets. Through co-immunoprecipitation studies, we have discovered that each of the enzymes is able to physically associate with CD31, although whether this association is direct or indirect is presently not known. Interactions between the Src family kinases and CD31 are stable in the weak detergent Brij 96, but are reduced or not detected when immunoprecipitation is performed from platelets solubilized in stronger detergents such as Nonidet P-40. CD31 present in Src family kinase immunoprecipitates was also shown to be tyrosine-phosphorylated, although an increase in tyrosine phosphorylation or an increase in protein association upon stimulation was not observed. Taken together, these results indicate that this interaction is not dependent on tyrosine phosphorylation and is unlikely to be mediated by kinase SH2 domains. Lu et al. (35) have shown in vitro that CD31, when phosphorylated on Tyr(683) and Tyr(686), is able to support binding to the SH2 domain of Src expressed as a glutathione S-transferase fusion protein; however, the relevance of this observation in vivo is uncertain. We have also detected Fyn, Lyn, and Src to be present in CD31 immunoprecipitates (data not shown). In such experiments where isolation of considerably greater levels of CD31 is possible (in comparison with co-association in Src family kinase immunoprecipitates), the levels of CD31 tyrosine phosphorylation are increased upon stimulation with Cvx or thrombin. It is therefore likely that increases in CD31 tyrosine phosphorylation were not observed in Src family kinase immunoprecipitates due to insufficient assay sensitivity.

To further investigate a role for Src family kinases in this process, we studied the phosphorylation of CD31 in mouse platelets lacking Fyn. Staining-induced phosphorylation was detectable in Fyn+/− and Fyn−/− platelets, and was indistinguishable. Cvx- and thrombin-induced CD31 tyrosine phosphorylation was reduced in Fyn−/− platelets. The reduction was variable between experiments, probably due to the presence of the remaining four Src family kinases in these cells. The Src family kinases share a great deal of structural and functional homology, and it is possible that there is functional redundancy in their abilities to phosphorylate CD31 in platelets. Co-isolation of Fyn with the FeR γ-chain in platelets has implicated this kinase in the activation of the GPVI collagen receptor. This has been recently confirmed using Fyn-deficient platelets, in which GPVI-stimulated signaling is diminished in comparison with platelets.

M. Cicmil and J. M. Gibbins, unpublished results.
control platelets. Therefore, reduced CD31 tyrosine phosphorylation in Fyn-deficient platelets stimulated with Cvvx is consistent with reduced collagen receptor activation in these cells. We conclude that Src family kinases are associated with CD31 in platelets and play a role in the tyrosine phosphorylation of this molecule upon stimulation of the ITAM-bearing collagen receptor complex or the thrombin receptor. Diminished thrombin-stimulated CD31 tyrosine phosphorylation in Fyn-deficient platelets indicates the involvement of this enzyme and validates our observations made using the Src family kinase inhibitor PP2 on thrombin and stirring-induced phosphorylation. Although we have demonstrated the involvement of Fyn in tyrosine phosphorylation of CD31 in mouse platelets, a multiple knockout approach may be necessary to unravel which enzymes are essential for CD31 phosphorylation in platelets upon stimulation with given agonists.

It remains to be established whether signal transduction pathways downstream of the collagen and thrombin receptors participate directly in the tyrosine phosphorylation of CD31. Work is presently underway in this laboratory to address this question. Directly likely that additional receptors and signaling pathways that are involved in platelet activation, such as integrin αIIbβ3, may influence the tyrosine phosphorylation of CD31. We have, however, established that this is not dependent on integrin function.

The intriguing question still remains as to the function of CD31 in platelets. It is presently unclear whether phosphorylation of CD31 and stimulation of the signaling from this receptor enhance or reduce platelet activation. Cross-linking of platelet CD31 has been reported to increase platelet adhesion, although it is tempting to speculate that CD31 may antagonize the actions of the collagen receptor due to the opposing nature of their signaling motifs. This may be beneficial upon contact of platelets with healthy endothelium surrounding an area of tissue damage, where homophilic interactions of platelet and endothelial cell CD31 may make platelets refractory to activation and may stimulate the release of prostacelyn from the endothelium. Further work is required to address this complex question.

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