THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 271, No. 43, Issue of October 25, pp. 26668–26676, 1996
Printed in U.S.A.

The $\alpha_2\beta_1$ Integrin Is a Necessary Co-receptor for Collagen-induced Activation of Syk and the Subsequent Phosphorylation of Phospholipase Cγ2 in Platelets*

(Received for publication, January 25, 1996, and in revised form, July 31, 1996)

Patricia J. Keely‡ and Leslie V. Parise

From the Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599

Although there are multiple potential collagen-binding proteins on platelets, the contribution of each to collagen-induced signaling events and platelet activation is unclear. We investigated which early platelet signaling events, if any, could be attributed specifically to the binding of collagen to one of its receptors, the $\alpha_2\beta_1$ integrin. Treatment of platelets with collagen induced a rapid activation of the non-receptor tyrosine kinase, Syk, as measured by an increase in phosphorylation and kinase activity. Collagen also induced the rapid phosphorylation of phospholipase Cγ2 (PLCγ2). The phosphorylation of both Syk and PLCγ2, as well as platelet aggregation, was blocked by an anti-$\alpha_2\beta_1$ integrin monoclonal antibody (P1E6), demonstrating that collagen binding to $\alpha_2\beta_1$ is necessary for signaling. Cross-linking of the $\alpha_2\beta_1$ integrin with stimulatory monoclonal antibody against either the β_1 or α_2 subunit stimulated the phosphorylation of both Syk and PLCγ2. However, antibody stimulation was dependent on co-stimulation of the FcγII receptor (CD32) since specific F(ab′)$_2$ fragments did not induce Syk and PLCγ2 phosphorylation. Thus, these results suggest that occupancy of $\alpha_2\beta_1$ by collagen is necessary, but that a co-receptor, in addition to $\alpha_2\beta_1$, is required for these collagen-induced signaling events. Moreover, the P1E6 antibody did not inhibit all collagen-induced tyrosine phosphorylation events, demonstrating that collagen also induces phosphorylation events that are independent of the $\alpha_2\beta_1$ integrin. In addition to Syk and PLCγ2, we identified the FcγII receptor (FcγRII) as being rapidly phosphorylated in response to collagen stimulation, even in the absence of antibodies. Finally, to determine if Syk activation precedes and directly contributes to the phosphorylation of PLCγ2, platelets were preincubated with the Syk-selective kinase inhibitor, piceatannol. A concentration of piceatannol that inhibited the phosphorylation and kinase activity of Syk, but had no effect on Src kinase activity, blocked the collagen-induced phosphorylation of PLCγ2 and also inhibited collagen-induced platelet aggregation. Our results begin to delineate a signaling pathway whereby occupancy of the $\alpha_2\beta_1$ integrin is required, but not sufficient, for collagen-induced activation of Syk and the subsequent phosphorylation of PLCγ2. These events are necessary for platelet activation and aggregation in response to collagen.

Platelet adhesion to collagen at sites of vascular injury initiates a cascade of signaling events leading to platelet activation and aggregation. Several collagen-binding proteins exist on the platelet surface that may mediate platelet activation by collagen, including the $\alpha_2\beta_1$ integrin (1, 2), glycoprotein (GP)[1] VI (3), GPIV (also known as GPIIIa or CD36 (4, 5)), and a 65-kDa protein (6, 7). Although multiple potential collagen receptors exist on platelets, the relative contribution of each in signaling collagen-induced platelet activation is unknown.

The $\alpha_2\beta_1$ integrin is necessary for the adhesion of platelets to collagen since patient platelets lacking this integrin do not adhere to or aggregate in response to soluble collagen fibers (8, 9). Additionally, antibodies against the $\alpha_2\beta_1$ integrin block platelet aggregation in response to soluble collagen (10) and adhesion to immobilized collagen (2, 11, 12). A more direct role for the $\alpha_2\beta_1$ integrin in early collagen-induced platelet signaling events, other than mediating the initial adhesion to collagen, has been assumed (13), but has not yet been clearly established. Platelet adhesion to immobilized collagen induces the phosphorylation of unknown 40-, 101-, and 105-kDa proteins and of pp125^{FAK} (14–16). However, the role of $\alpha_2\beta_1$ in these events is difficult to establish since anti-$\alpha_2\beta_1$ integrin antibodies almost totally inhibit adhesion to immobilized collagen (12, 14). Therefore, it is not clear whether blocking the $\alpha_2\beta_1$ integrin disrupts specific signaling events or simply the initial adhesion of platelets to collagen.

A direct role for the $\alpha_2\beta_1$ integrin in mediating signal transduction in platelets would be consistent with emerging data linking integrins to various signaling events (for review, see Ref. 17). For example, the phosphorylation of numerous proteins during platelet aggregation has been shown to be dependent on the $\alpha_{IIb}\beta_3$ integrin (18–20), which becomes functionally active following platelet activation. Recently, Lin *et al.* (21) have found that engagement of β_1 integrins on monocytes induces Syk activation and NF-κB-dependent gene expression. Additionally, integrins have been shown to mediate the phosphorylation of numerous proteins (22–27), the activation of mitogen-activated protein kinases (28), the stimulation of metalloproteinase gene expression (29), and the activation of the Na^+/H^+ antiporter (30). The $\alpha_2\beta_1$ integrin has been implicated in signaling events in cells other than platelets since it mediates the activation of Ras in T-cells (31), the morphological organization of mammary cells in three-dimensional collagen gels (32), and collagenase expression in osteosarcoma cells (33). However, the mechanism by which the $\alpha_2\beta_1$ integrin or any other integrin transduces signals in cells is still poorly understood.

Although the $\alpha_2\beta_1$ integrin is likely to play a role in collagen-

* This work was supported by National Research Service Award Fellowship F32-HL09399-01 (to P. J. K.) and National Institutes of Health Grant 1-P01-HL45100 (to L. V. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, CB 7365, 1106 FLOB, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-962-1058; Fax: 919-966-5640; E-mail: pkeely@med.unc.edu.

[1] The abbreviations used are: GP, glycoprotein; PLC, phospholipase C; FcγRII, FcγII receptor; RIPA, radioimmune precipitation assay; mAb, monoclonal antibody.

induced platelet activation, its role is confused by data suggesting that other collagen-binding proteins also contribute to platelet signaling. For example, it is clear that GPVI plays an important role in collagen-induced platelet activation since patient platelets deficient in GPVI are defective in collagen-induced aggregation and exhibit partially decreased cation-independent adhesion to collagen (3, 34, 35). GPVI directly contributes to signaling in the platelet, as shown by the recent finding that cross-linking of GPVI with antibodies causes the Fc-independent activation and phosphorylation of the non-receptor tyrosine kinase, Syk (36). A role for the p65 collagen-binding protein, which has a similar molecular mass compared with GPVI, is also suggested since an anti-p65 antibody inhibits its collagen-induced platelet aggregation (7). A role for GPIV/CD36 is less clear since platelets from patients lacking GPIV (the Nak^a negative phenotype) aggregate and adhere normally to collagen (37–40). In spite of this finding, GPIV may play some role since it is physically associated with the Src family kinases, Fyn, Yes, and Lyn (20). The presence of other collagen receptors on platelets may explain the finding that triple-helical collagen-like synthetic peptides are able to stimulate platelet aggregation in an $\alpha_2\beta_1$-independent manner (41).

Collagen stimulation of platelets leads to the phosphorylation of numerous proteins (Refs. 4, 13, 18, and 42; for review, see Ref. 43). Collagen induces the tyrosine phosphorylation and activation of phospholipase Cγ2 (44, 45), which cleaves phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate. These second messengers, in turn, cause the activation of protein kinase C and the release of intracellular calcium, which are necessary for the subsequent events leading to granule secretion, activation of the $\alpha_{IIb}\beta_3$ integrin, aggregation, and actin polymerization (46–49). The 72-kDa non-receptor tyrosine kinase, Syk, a member of the Zap70 kinase family, is also tyrosine-phosphorylated and activated in platelets following stimulation by collagen (50). Like collagen, stimulation of platelets through either the FcγII receptor or CD9 also causes activation of both PLCγ2 and Syk (44, 51–55). Additionally, a number of other agonists (including thrombin (56) and the thromboxane A_2 mimetic, U46619 (57)) also stimulate Syk phosphorylation. The activation of platelets by thrombin causes the relocation of Syk to the cytoskeletal fraction (58, 59) and its association with phosphatidylinositol 3-kinase (60), suggesting that Syk has multiple roles in platelet signaling. Currently, the collagen-induced signaling pathways in platelets leading to the activation of either PLCγ2 or Syk have not been fully defined.

Because multiple collagen-binding proteins exist on platelets, but the relative contribution of each to collagen-induced platelet activation has not yet been determined, the purpose of this study was to determine which intracellular collagen-induced signaling events, if any, could be directly linked to the function of the $\alpha_2\beta_1$ integrin. Additionally, we sought to determine whether these signaling events contribute to collagen-induced platelet aggregation. We find that collagen stimulation of both Syk and PLCγ2 is dependent on the $\alpha_2\beta_1$ integrin. However, antibody cross-linking of $\alpha_2\beta_1$ is not sufficient to induce the phosphorylation of Syk and PLCγ2 unless the FcγII receptor is also engaged, suggesting that $\alpha_2\beta_1$ functions in a co-stimulatory role with other receptors. Moreover, we find that blocking the $\alpha_2\beta_1$ integrin does not inhibit all collagen-induced phosphorylation events, which further suggests that multiple collagen receptors are simultaneously activated during platelet binding to collagen. We also identify one additional phosphorylation event induced by collagen, that of FcγRII, even in the absence of added antibody. Finally, our data suggest that Syk kinase activity is upstream of PLCγ2; these signaling events had not previously been ordered on the same pathway in platelets. Moreover, inhibition of Syk activity suppresses collagen-induced platelet aggregation, suggesting an important role for Syk in the activation of platelets.

EXPERIMENTAL PROCEDURES

Materials—Piceatannol was a generous gift of Dr. Robert Geahlen (Purdue University, West Lafayette, IN). The anti-$\alpha_2\beta_1$ integrin antibody JBS2 was a generous gift of Dr. John Wilkins (RDU Research Laboratory, Winnipeg, Manitoba, Canada). The anti-$\alpha_2\beta_1$ integrin antibody P1H5 was a generous gift of Dr. William Carter (Fred Hutchinson Cancer Research Center, Seattle, WA), and P1E6 was purchased from Life Technologies, Inc. The anti-Src antibody 327 was a generous gift of Dr. Patricia Maness (University of North Carolina, Chapel Hill, NC). The polyclonal anti-Syk antibody was a generous gift of Dr. Andrew Chan (Washington University, St. Louis, MO) and was also purchased, along with PLCγ2, from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphotyrosine antibody PY-20 and the recombinant RC-20 were purchased from Transduction Labs (Lexington, KY). Secondary $F(ab')_2$ anti-mouse IgG and horseradish peroxidase-conjugated anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Hybridomas for the anti-β_1 integrin antibody (TS2/16), anti-FcγRII antibody (IV.3), and isotype-matched control IgGs were obtained from American Type Culture Collection, and antibodies were prepared in the monoclonal core facility of the Department of Pharmacology (University of North Carolina, Chapel Hill). Protein G-Sepharose was purchased from Pharmacia (Uppsala). Tubulin was a generous gift of Dr. Michael Caplow (University of North Carolina, Chapel Hill). Collagen fibers were formed from bovine skin collagen (type I; Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) by neutralizing the collagen solution in 10 mM Na_2HPO_4 to give a final concentration of 2 mg/ml. Prostaglandin E_1 and U46619 were obtained from Cayman Chemical Co., Inc. (Ann Arbor, MI). Deoxycholic acid was from Calbiochem. All other chemicals were from Sigma unless otherwise noted. Gel electrophoresis and transfers were performed using a Novex Xcell II system.

Platelet Preparation, Stimulation, and Aggregation—Blood was obtained from healthy consenting human donors using acid-citrate/dextrose as an anticoagulant, and platelets were prepared by centrifugation as described (61). Briefly, platelet-rich plasma was separated from whole blood at 800 rpm in a Beckman GP tabletop centrifuge for 25 min, treated with prostaglandin E_1 (50 ng/ml), and centrifuged at 1800 rpm for 20 min to pellet the platelets. Platelets were washed once in citrate/dextrose/NaCl (pH 7.0) and resuspended in buffer A (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 50 mM HEPES (pH 7.3), 2 mM $MgCl_2$, and 1 mM $CaCl_2$). The platelet concentration was determined using a Thrombocounter (Coulter Corp., Hialeah, FL). Platelets were diluted in buffer A to 10^9/ml and, where indicated, treated with 200 μg/ml bovine skin collagen fibers (Collaborative Research).

Platelet aggregation studies were performed to determine platelet function for every platelet preparation. Platelets were resuspended in buffer A at a concentration of 2×10^8/ml, incubated at 37 °C under stirring conditions in an aggregometer (Chrono-Log Corp., Havertown, PA), and activated by the addition of collagen (200 μg/ml) or anti-integrin or control antibodies in the presence or absence of added fibrinogen. Incubation of isolated platelets in an aggregometer with added fibrinogen in the absence of agonist demonstrated that the platelets were not pre-activated.

Immunoprecipitation and Immunoblotting—Platelets were lysed by the addition of an equal volume of ice-cold 2 × RIPA buffer (50 mM HEPES (pH 7.3), 150 mM NaCl, 2% Nonidet P-40, 1% deoxycholate, 0.2% SDS, 2 mM sodium vanadate, 100 mM NaF, 20 mM sodium pyrophosphate, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 5 mM Pefabloc (Boehringer Mannheim), 5 mM benzamidine HCl, and 2 mM EDTA) at 4 °C for 30 min, centrifuged at 15,000 × *g* for 15 min, and precleared for 1 h by the addition of protein G-Sepharose (GammaBind, Pharmacia). Cleared lysates were incubated overnight with the polyclonal anti-Syk or anti-PLCγ2 antibody or with the monoclonal antibody IV.3 to immunoprecipitate FcγRII plus protein G-Sepharose at 4 °C and then washed three times with 1 × RIPA lysis buffer. Immunoprecipitated proteins were eluted in 1 × Laemmli sample buffer, reduced, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. To determine phosphotyrosine content, membranes were incubated with the anti-phosphotyrosine antibody (PY-20 or RC-20) conjugated to horseradish peroxidase and visualized using the ECL reagent (Amersham Corp.). To reprobe membranes for PLCγ2 or Syk, membranes were incubated overnight with NaN_3 in order to

inhibit horseradish peroxidase activity, washed, and reprobed with the anti-PLCγ2 or anti-Syk antibody, followed by horseradish peroxidase-conjugated anti-rabbit IgG.

Treatment with Antibodies, Peptides, and Inhibitors—For antibody inhibition studies, platelets were incubated with the anti-α_2 integrin antibody, P1E6, at a dilution of 1:1000 or isotype-matched IgG or without antibody for another 3 min, followed by stimulation with or without collagen for 2.5 min. For antibody stimulation experiments, platelets were preincubated with rabbit IgG for 3 min in order to block Fc receptors and incubated with anti-β_1 integrin TS2/16 or IgG for 3 min, and where indicated, antibodies were cross-linked with anti-mouse F(ab′)$_2$ for 5 min. It was later determined that treatment with rabbit IgG was not sufficient to completely block the Fc receptors (see below). F(ab′)$_2$ fragments of TS2/16 were prepared by digesting the antibody with immobilized ficin and removing Fc fragments with a protein A column (Pierce). Gel electrophoresis confirmed that no contaminating intact IgG remained. Platelets were then lysed by the addition of 2 × RIPA buffer as described above. For peptide inhibition studies, the peptide GPAGKDGEAGA, representing residues 430–440 from the α_1 chain of collagen I (62), was synthesized at the University of North Carolina core protein synthesis facility (UNCCH-NIEHS). The peptide was diluted in buffer A, and platelets were preincubated with the indicated concentration of peptide for 5 min, stimulated with collagen, and then lysed in 2 × RIPA buffer.

For FcγRII blocking experiments, platelets were preincubated for 5 min with 20 μg/ml IV.3 antibody and then incubated with anti-integrin antibodies that were biotinylated using the ECL protein biotinylation module (Amersham Corp.) according to the manufacturer's instructions. Biotinylated antibodies were dialyzed against phosphate-buffered saline prior to use. Biotinylated antibodies were cross-linked with 10 μg/ml avidin (Pierce) for 5 min prior to lysing the platelets in 2 × RIPA buffer.

Kinase Assay—Precleared lysates, prepared as described above, were immunoprecipitated with the anti-Syk or anti-Src antibody plus protein G-sepharose for 2 h and then washed three times with 1 × RIPA buffer without detergent. Immunoprecipitates were split into two samples: one for phosphotyrosine determination and one for the kinase assay, which was resuspended in 20 μl of kinase buffer (25 mM HEPES (pH 7.5), 10 mM $MnCl_2$, and 10 mM *p*-nitrophenyl phosphate) plus 1 μM ATP, 5 μCi of [γ-^{32}P]ATP (Amersham Corp.), and 10 μg of tubulin and incubated at 24 °C for 2 min. The assay was terminated by adding 3 × Laemmli sample buffer and boiling the samples for 3 min. Samples were electrophoresed, and the gels were stained with Coomassie Brilliant Blue, dried, and subjected to autoradiography.

RESULTS

Kinetics of the Collagen-induced Phosphorylation of PLCγ2 and Syk—Treatment of platelets with collagen (200 μg/ml) under non-stirring conditions caused the phosphorylation of Syk on tyrosine within 1 min (Fig. 1). This collagen-induced phosphorylation persisted for 20 min. Other investigators have also noted similar kinetics of Syk phosphorylation following collagen stimulation (48). Stimulation of platelets by thrombin (Fig. 1) or the thromboxane A_2 mimetic, U46619 (data not shown), also induced the tyrosine phosphorylation of Syk between 30 s and 2.5 min. Collagen-induced tyrosine phosphorylation of PLCγ2 lagged behind that of Syk since obvious phosphorylation did not occur until 2.5 min, with only a faint phosphorylated PLCγ2 band visible at 1 min (Fig. 1). Stimulation of Syk and PLCγ2 phosphorylation was independent of platelet aggregation since it occurred in the absence of stirring or when aggregation was blocked by the addition of a synthetic RGDW-containing peptide, which blocks fibrinogen binding to the $\alpha_{IIb}\beta_3$ integrin (data not shown). We also observed the phosphorylation of PLCγ2 after treatment with thrombin (Fig. 1) or U46619 (data not shown) for 2.5 min. This result is in contrast to the results of Daniel *et al.* (45) or Blake *et al.* (44), who did not find PLCγ2 phosphorylation following thrombin treatment, but is consistent with the results of Tate and Rittenhouse (63).

Tyrosine Phosphorylation of PLCγ2 and Syk Is Dependent on the $\alpha_2\beta_1$ Integrin—To determine the role of the $\alpha_2\beta_1$ integrin in collagen-induced platelet signaling events, platelets were pre-

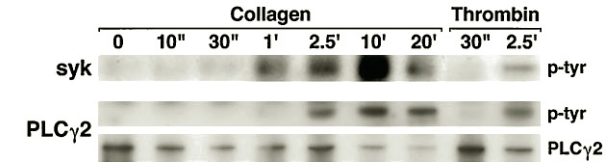

FIG. 1. **Kinetics of collagen-induced Syk and PLCγ2 tyrosine phosphorylation.** Platelets (10^9) were stimulated with collagen (200 μg/ml) or thrombin (0.4 unit/ml) for the indicated length of time and then lysed by the addition of 2 × RIPA buffer. Precleared lysates were immunoprecipitated with anti-Syk or anti-PLCγ2 antiserum as indicated, subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotted for phosphotyrosine (*p-tyr*) with horseradish peroxidase-conjugated RC-20. Blots were reprobed for Syk (not shown) or PLCγ2 to control for the amount of protein in each lane. Similar kinetics were determined in three separate experiments.

treated with an anti-$\alpha_2\beta_1$ integrin mAb (P1E6) that blocks $\alpha_2\beta_1$-mediated binding of platelets to collagen (11). As shown in Fig. 2*A*, P1E6 treatment dramatically decreased the phosphorylation of both Syk and PLCγ2. In control experiments, an isotype-matched IgG did not inhibit the phosphorylation of Syk or PLCγ2 (Fig. 2*A*). Inhibition of Syk and PLCγ2 phosphorylation was confirmed using a different function-blocking anti-α_2 integrin antibody (P1H5) (data not shown). These results suggest that collagen interaction with $\alpha_2\beta_1$ is necessary for the phosphorylation of PLCγ2 and Syk.

We also examined the effect of the anti-$\alpha_2\beta_1$ integrin mAb on the total phosphotyrosine content of collagen-treated platelets. Antibody blocking of the $\alpha_2\beta_1$ integrin caused the loss of several phosphotyrosine bands, including a band just larger than 100 kDa. However, not all signaling events were affected since the collagen-induced tyrosine phosphorylation of several other unidentified proteins, including at least three major bands at about 46, 50, and 65 kDa, was not inhibited by the presence of the anti-$\alpha_2\beta_1$ integrin mAb (Fig. 2*B*), suggesting that collagen interacts with the platelet surface even when the $\alpha_2\beta_1$ integrin has been blocked.

In aggregation assays, the anti-$\alpha_2\beta_1$ integrin mAb (P1E6) inhibited collagen-induced platelet aggregation, while control IgG had no effect (Fig. 2*C*). This inhibition was specific since P1E6 did not inhibit platelet aggregation stimulated by thrombin (data not shown).

As an additional approach to block the $\alpha_2\beta_1$ integrin, we used a synthetic peptide from the α1(I) collagen chain, GPAGKDGEAGA (KDGEA), a putative ligand of $\alpha_2\beta_1$ that blocks the cation-dependent adhesion of platelets and breast cells to collagen (62). Pretreatment of platelets with a 3 mM concentration of the KDGEA peptide resulted in the partial inhibition, and pretreatment with 10 mM resulted in the total inhibition of both Syk and PLCγ2 tyrosine phosphorylation (data not shown). The concentration of the KDGEA peptide that was necessary for inhibition is in close agreement with the concentration necessary to inhibit platelet adhesion to collagen, which was partial at 3 mM and total at 6 mM (62). As noted for treatment with the anti-$\alpha_2\beta_1$ integrin antibody, pretreatment of platelets with KDGEA did not inhibit all tyrosine phosphorylation events (data not shown). Thus, inhibition of Syk and PLCγ2 phosphorylation with both an $\alpha_2\beta_1$-specific antibody and peptide suggests that occupancy of the $\alpha_2\beta_1$ integrin is required for collagen-induced Syk and PLCγ2 phosphorylation.

Antibody Cross-linking of the $\alpha_2\beta_1$ Integrin Results in the Phosphorylation of Syk and PLCγ2 in an Fc Receptor-dependent Manner—To determine whether cross-linking of $\alpha_2\beta_1$ is sufficient to induce tyrosine phosphorylation of Syk and PLCγ2, platelets were pretreated with either a stimulatory anti-β_1 integrin mAb (TS2/16) or an isotype-matched control IgG, followed by cross-linking of the primary antibody with a

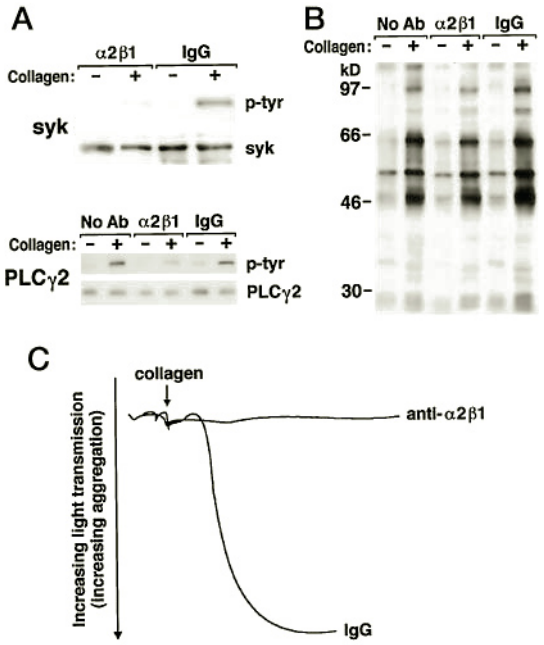

FIG. 2. **An anti-$\alpha_2\beta_1$ integrin-blocking antibody inhibits Syk and PLCγ2 phosphorylation.** Platelets were pretreated with the anti-α_2 integrin mAb, P1E6, or isotype-matched control mouse IgG or with no antibody, followed by treatment with collagen as indicated. Following lysis in 2 × RIPA buffer, precleared lysates were immunoprecipitated with antiserum against Syk or PLCγ2 as indicated (*A*) or anti-phosphotyrosine (PY-20) (*B*). Protein blots in both *A* and *B* were probed for phosphotyrosine (*p-tyr*) with horseradish peroxidase-conjugated RC-20. Blots in *A* were reprobed for Syk or PLCγ2 as noted. In *C*, platelets were pretreated with the anti-α_2 integrin mAb (P1E6) or control IgG for 3 min and then stimulated with collagen (200 μg/ml) under stirring conditions in an aggregometer. The experiment shown is representative of four similar experiments.

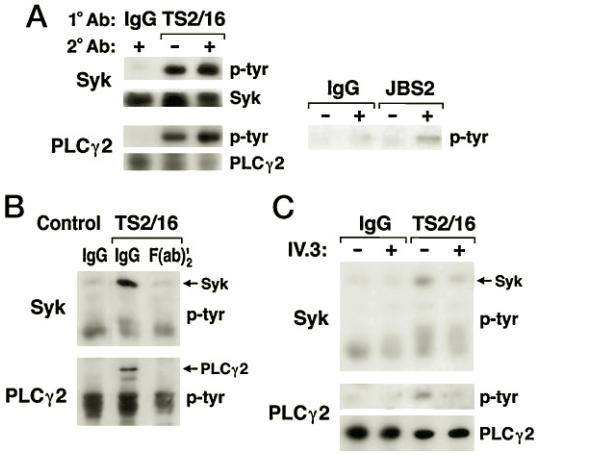

FIG. 3. **Cross-linking the $\alpha_2\beta_1$ integrin with antibody induces Syk and PLCγ2 phosphorylation in an FcγRII-dependent manner.** *A*, platelets were incubated with a stimulatory anti-β_1 integrin mAb (TS2/16; *left panel*), a stimulatory anti-α_2 subunit mAb (JBS2; *right panel*), or an isotype-matched control mouse IgG as indicated. Following primary antibody treatment, platelets were incubated with or without a secondary anti-mouse F(ab′)$_2$ antibody in order to further cross-link receptors as indicated. Precleared lysates from stimulated platelets were immunoprecipitated with the anti-Syk or anti-PLCγ2 antibody as indicated and probed for phosphotyrosine (*p-tyr*) using horseradish peroxidase-conjugated RC-20. Syk immunoprecipitation following cross-linking with JBS2 was not performed. The TS2/16 experiment is one of three such experiments giving similar results. *B*, F(ab′)$_2$ fragments fail to induce phosphorylation of Syk and PLCγ2. Platelets were incubated with intact IgG, an F(ab′)$_2$ fragment of TS2/16, or an isotype-matched control mouse IgG as indicated, followed by incubation with a secondary antibody. Precleared lysates from stimulated platelets were immunoprecipitated with the anti-Syk or anti-PLCγ2 antibody as indicated and probed for phosphotyrosine. Shown is one of three similar experiments. *C*, preblocking FcγRII blocks TS2/16 antibody stimulation. To block Fc receptors, platelets were preincubated with or without 20 μg/ml IV.3 anti-FcγRII mAb for 5 min as indicated. Platelets were then incubated with 20 μg/ml biotinylated TS2/16 or control IgG for 3 min, followed by incubation for 5 min with 10 μg/ml avidin in order to cross-link the biotinylated antibodies. Syk or PLCγ2 was immunoprecipitated as described for *A* and *B*, and blots were probed for phosphotyrosine. Shown is one of two such experiments.

secondary anti-mouse F(ab′)$_2$ antibody to cluster the integrin receptors. Treatment of platelets in suspension with the TS2/16 antibody, but not control IgG, caused the phosphorylation of both Syk and PLCγ2 (Fig. 3*A*, *left panel*). Interestingly, cross-linking TS2/16 with the secondary antibody was not necessary since TS2/16 alone also induced the phosphorylation of Syk and PLCγ2. However, there was an enhancement following cross-linking with the secondary antibody (compare lanes ± secondary). These results are consistent with the results of Lin *et al.* (21), who found that TS2/16 alone, in the absence of secondary antibody, could stimulate Syk phosphorylation in a monocytic cell line.

To determine whether cross-linking of the α_2 subunit stimulates signaling in platelets, we used JBS2, an anti-α_2 integrin mAb that stimulates collagen binding and Ras activation in T-cells (31). Treatment of platelets in suspension with JBS2 stimulated the tyrosine phosphorylation of PLCγ2 (Fig. 3*A*, *right panel*). In contrast to the findings with TS2/16, it was necessary to cross-link the JBS2 antibody in order to induce PLCγ2 phosphorylation. This difference may reflect a difference in the way that the TS2/16 and JBS2 antibodies bind to and stimulate the α_2 or β_1 integrin subunits. It is possible that the TS2/16 antibody cross-links two integrin heterodimers even in the absence of the secondary antibody. We also found that the level of JBS2-induced phosphorylation was less than that noted when TS2/16 was used. This difference may be due to the very strong stimulatory activity of the TS2/16 antibody compared with the JBS2 antibody. Furthermore, since platelets have $\alpha_5\beta_1$ and $\alpha_6\beta_1$, stimulation of these β_1 integrins may also contribute to the stronger signal noted with TS2/16. Our results with JBS2 demonstrate that specifically cross-linking the $\alpha_2\beta_1$ integrin with intact mAb induces signaling in platelets.

Although control IgG did not result in the phosphorylation of Syk or PLCγ2, we investigated whether Fc receptors contributed to signaling following cross-linking with anti-integrin antibodies. F(ab′)$_2$ fragments of the TS2/16 antibody failed to stimulate the phosphorylation of either Syk or PLCγ2 (Fig. 3*B*), demonstrating that the Fc portion of the TS2/16 antibody contributes to its activity. To check the activity of the F(ab′)$_2$ fragments, we took advantage of the fact that the TS2/16 antibody enhances binding of the integrin to collagen and thus enhances platelet aggregation in response to suboptimal collagen concentrations. We found that the F(ab′)$_2$ fragments of TS2/16 retained this ability (data not shown), indicating that the failure of F(ab′)$_2$ fragments to stimulate platelets is not due to a loss of their activity.

To determine more directly whether FcγRII, which is the only Fc receptor on platelets, contributes to anti-integrin antibody signaling, FcγRII was blocked with 20 μg/ml IV.3 antibody prior to stimulation with intact antibodies. To avoid using a secondary antibody to cluster anti-integrin antibodies, which would stimulate the platelets by also cross-linking the IV.3 antibody, the anti-integrin and control IgG antibodies were biotinylated and cross-linked using avidin. Pretreatment of platelets with IV.3 blocked stimulation of Syk and PLCγ2 phosphorylation by biotinylated TS2/16 (Fig. 3*C*), once again suggesting a contribution of FcγRII. Similar results were obtained

with biotinylated JBS2 (data not shown). Anti-integrin antibody stimulation led not only to the phosphorylation of Syk and PLCγ2, but also to the tyrosine phosphorylation of FcγRII itself (data not shown). This phosphorylation of FcγRII was also blocked by the IV.3 antibody. It is unlikely that anti-integrin antibody signaling is due solely to a nonspecific effect of antibody binding to FcγRII since control IgG antibodies consistently failed to activate Syk or PLCγ2 phosphorylation. These results suggest that antibody clustering of the $\alpha_2\beta_1$ integrin alone is insufficient to induce Syk and PLCγ2 phosphorylation and that FcγRII co-stimulates platelets treated with anti-integrin antibodies. Cross-linking the $\alpha_2\beta_1$ integrin on the platelet surface was not sufficient to induce aggregation since treatment of platelets with intact TS2/16 or JBS2 antibodies with or without the secondary antibody did not cause the aggregation of platelets (data not shown).

Collagen Causes an $\alpha_2\beta_1$-independent Phosphorylation of FcγRII—Because FcγRII was necessary for anti-$\alpha_2\beta_1$ integrin antibody-stimulated phosphorylation of Syk and PLCγ2, we determined whether FcγRII was also a target for tyrosine phosphorylation following collagen stimulation. Collagen treatment of platelets induced the phosphorylation of FcγRII within 1 min (Fig. 4*A*). This phosphorylation proceeded for at least 5 min following collagen stimulation. Unlike the collagen-induced phosphorylation of Syk and PLCγ2, the phosphorylation of FcγRII was not blocked by pretreatment of platelets with the anti-α_2 integrin antibody (P1E6) (Fig. 4*B*). In fact, the experiment shown in Fig. 4*B* (*third lane*)) suggests that P1E6 may cause a slight stimulation of FcγRII phosphorylation even in the absence of collagen. Even with this background level of phosphorylation, there is still a further stimulation of FcγRII phosphorylation upon collagen addition (Fig. 4*B*, compare the *third* and *fourth lanes*), indicating that P1E6 does not inhibit collagen stimulation of FcγRII phosphorylation. Thus, although antibody stimulation of β_1 integrins can induce FcγRII phosphorylation, collagen stimulation of FcγRII phosphorylation is $\alpha_2\beta_1$ integrin-independent.

Collagen stimulation of FcγRII phosphorylation was not inhibited by pretreating the platelets with the blocking IV.3 anti-FcγRII antibody (Fig. 4*C*). This implies that collagen probably does not bind directly to the FcγII receptor. Additionally, this result suggests that collagen stimulation of FcγRII phosphorylation does not occur by inducing platelet release of immune complexes that subsequently bind the FcγII receptor, but more likely by an internal signaling pathway. Pretreatment of platelets with IV.3 also did not inhibit the collagen-induced phosphorylation of Syk or PLCγ2 (Fig. 4*D*) or collagen-induced platelet aggregation (data not shown), suggesting that if FcγRII plays a role in collagen stimulation of platelets, this role is not mediated by its ability to bind Fc ligands. Thus, stimulation of Syk and PLCγ2 phosphorylation by collagen differs from stimulation by anti-$\alpha_2\beta_1$ integrin antibodies, which was blocked by preincubation with the IV.3 antibody. This difference suggests that stimulation by collagen does not share the same requirement for FcγRII binding as stimulation by anti-integrin antibodies. In the case of antibody stimulation, FcγRII may substitute for a co-receptor that is normally stimulated by collagen.

Inhibition of Syk Kinase Activity Also Inhibits the Phosphorylation of PLCγ2—To determine whether Syk is on the same pathway and upstream of PLCγ2, platelets were treated with the Syk-selective kinase inhibitor, piceatannol (64). Platelets were pretreated for 10 min with various concentrations of piceatannol or Me_2SO, followed by treatment with collagen for 2.5 min. Syk phosphorylation was dramatically reduced following treatment with 10 μg/ml piceatannol and completely eliminated following treatment with 30 μg/ml piceatannol (Fig. 5*A*). The tyrosine phosphorylation of PLCγ2 was closely correlated with that of Syk since the phosphorylation of PLCγ2 was also decreased following treatment with 10 μg/ml piceatannol and completely eliminated following treatment with 30 μg/ml piceatannol (Fig. 5*A*). This finding is consistent with a model placing Syk activity upstream of PLCγ2. Piceatannol was less effective in inhibiting the collagen-induced phosphorylation of FcγRII since treatment with 10 μg/ml piceatannol did not inhibit, and 30 μg/ml piceatannol only partially inhibited FcγRII phosphorylation (Fig. 5*A*, *bottom panel*). This is consistent with results suggesting that FcγRII is phosphorylated by Src in platelets (65). Pretreatment of platelets with piceatannol also inhibited the tyrosine phosphorylation of several other (but not all) proteins (Fig. 5*B*), implying that Syk may be involved in several phosphorylation events in stimulated platelets.

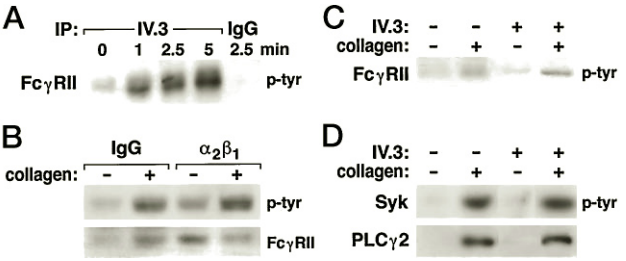

FIG. 4. **Collagen induces the $\alpha_2\beta_1$-independent phosphorylation of FcγRII.** *A*, platelets were incubated with 200 μg/ml collagen and, at the indicated times, lysed by the addition of 2 × RIPA buffer. Precleared lysates were immunoprecipitated with the IV.3 anti-FcγRII mAb or control IgG, subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotted for phosphotyrosine with horseradish peroxidase-conjugated RC-20. Similar kinetics were observed on two separate occasions. *B*, the anti-α_2 integrin mAb (P1E6) does not inhibit collagen-induced FcγRII phosphorylation. Platelets were pretreated with P1E6 or isotype-matched control mouse IgG as indicated, followed by treatment with collagen for 2.5 min. Lysates were immunoprecipitated with the IV.3 mAb and probed for phosphotyrosine as described for *A*. The blot was reprobed for FcγRII with the IV.3 antibody as indicated. The experiment shown is representative of three separate experiments. *C* and *D*, pretreatment with the IV.3 antibody does not block the collagen-induced phosphorylation of FcγRII, Syk, or PLCγ2. Platelets were pretreated with or without the IV.3 antibody in order to block FcγRII and then incubated with collagen for 2.5 min as indicated. Lysates were immunoprecipitated with additional IV.3 antibody (*C*) or with anti-Syk or anti-PLCγ2 antiserum (*D*) and probed for phosphotyrosine (*p-tyr*). The experiments shown are representative of two similar experiments.

Syk is known to have autophosphorylating activity, and its level of tyrosine phosphorylation correlates with its kinase activity in platelets (58). To verify that piceatannol inhibited Syk kinase activity as well as the phosphotyrosine content of Syk, immune complex kinase assays were performed. Pretreatment of platelets with piceatannol inhibited Syk kinase activity as measured by autophosphorylation and by the phosphorylation of an exogenous substrate, tubulin (Fig. 5*C*). Treatment of platelets with 30 μg/ml piceatannol inhibited Syk kinase activity by 72% (Table I). In contrast, Src kinase activity was not significantly affected by pretreatment of platelets with piceatannol (Fig. 5*C* and Table I). Treatment of platelets with 10 μg/ml piceatannol did not significantly inhibit Syk kinase activity (Fig. 5*C* and Table I), even though this concentration partially inhibited the tyrosine phosphorylation of Syk (Fig. 5*A*). The reason for this difference is not clear, but may be due to the removal of piceatannol in washing steps before the kinase assay; kinase activity may therefore appear to be less sensitive to inhibition by piceatannol than in the direct phosphotyrosine blots shown in Fig. 5*A*. Our results are consistent with those of Oliver *et al.* (64), who found that pretreatment of mast cells with 50 μg/ml piceatannol inhibited Syk kinase

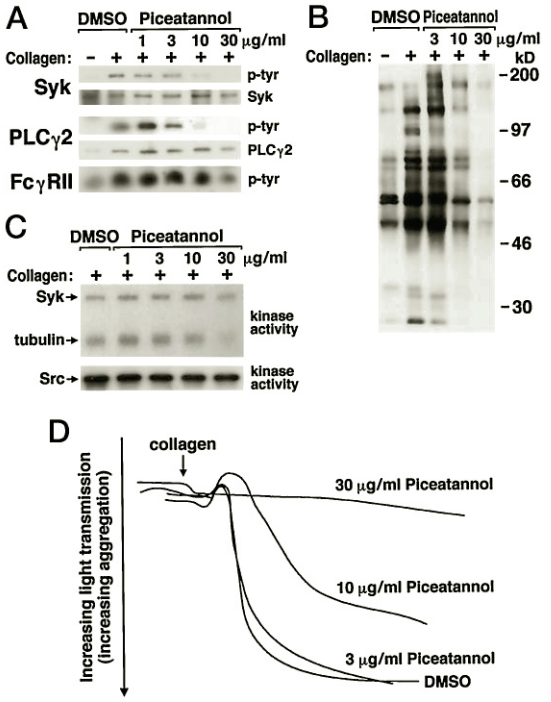

FIG. 5. **Piceatannol inhibits both Syk and PLCγ2 phosphorylation.** *A* and *B*, platelets were pretreated for 10 min with increasing concentrations of piceatannol or Me_2SO (*DMSO*) as a control. Platelets were then stimulated with collagen for 2.5 min and lysed by the addition of 2 × RIPA buffer. Precleared lysates were immunoprecipitated with antibodies against Syk, PLCγ2, or FcγRII (*A*) or with PY-20 to determine total phosphotyrosine (*B*) and immunoblotted for phosphotyrosine with horseradish peroxidase-conjugated RC-20. Blots in *A* were reprobed for Syk or PLCγ2 as indicated. Note that 10 μg/ml piceatannol partially inhibits, and 30 μg/ml piceatannol totally inhibits both Syk and PLCγ2 phosphorylation (*A*) as well as the phosphorylation of numerous other proteins (*B*). 10 μg/ml piceatannol does not inhibit, and 30 μg/ml piceatannol only partially inhibits FcγRII phosphorylation (*A*). These experiments are representative of four such experiments, with consistent sensitivity of phosphorylation to the concentrations shown. *C*, immune complex kinase assay: platelets were pretreated with piceatannol or Me_2SO, stimulated with collagen for 1 min, and lysed with 2 × RIPA buffer. Precleared lysates were immunoprecipitated for 2 h with antiserum against Syk or mAb 327 against Src, washed three times in buffer without detergent, and resuspended in kinase buffer. Phosphorylated proteins were subjected to SDS-polyacrylamide gel electrophoresis, and the resulting gels were stained, dried, and subjected to autoradiography. A quantification of kinase activity is found in Table I. *D*, platelets were pretreated for 10 min with piceatannol or Me_2SO as indicated and then stimulated with collagen under stirring conditions in an aggregometer. This experiment is representative of six similar experiments from different donors, all of which had similar sensitivity to inhibition by piceatannol.

activity, but not the Src family kinase Lyn. Thus, the concentrations of piceatannol used here seem to be relatively specific for Syk and do not affect Src family kinases, which suggests that the inhibition of PLCγ2 phosphorylation by piceatannol is due to an effect on Syk activity. However, the possibility still exists that piceatannol also affects other kinases in platelets as well.

The effects of piceatannol on platelet aggregation were also studied. Collagen-induced platelet aggregation was partially inhibited by pretreatment of platelets with 10 μg/ml piceatannol and totally inhibited by pretreatment with 30 μg/ml piceatannol (Fig. 5*D*). Collagen-induced platelet shape change was also inhibited by 30 μg/ml piceatannol. Piceatannol also inhibited platelet aggregation in response to thrombin and U46619. As with collagen-induced aggregation, 10 μg/ml piceatannol caused a partial inhibition, and 30 μg/ml piceatannol caused a total inhibition of thrombin-induced aggregation (Fig. 6). Unlike collagen stimulation, platelets pretreated with 30 μg/ml piceatannol still exhibited shape change in response to thrombin or U46619 (Fig. 6). This suggests that the mechanisms leading to collagen-induced platelet shape change are different from those stimulated by G-protein-coupled receptors. The inhibition of platelet aggregation by piceatannol suggests an important role for Syk in platelet activation by a number of agonists.

TABLE I
Syk and Src kinase activities following piceatannol treatment of platelets

[Piceatannol]	Kinase activity[a]	
	Syk[b]	Src[c]
	% of control	
1 μg/ml	101	103
3 μg/ml	82	90
10 μg/ml	76	84
30 μg/ml	28	81

[a] A quantification of kinase activity was determined by excising the phosphorylated bands from the dried gel shown in Fig. 5*C* and counting them in a scintillation counter. Percent of control is the ratio of the dpm in the sample treated with piceatannol to the dpm in the Me_2SO control band.

[b] Syk kinase activity was determined by excising the tubulin band from the dried gel shown in Fig. 5*C*.

[c] Src kinase activity was determined by excising the Src band from the dried gel shown in Fig. 5*C*.

DISCUSSION

We have shown that occupancy of the $\alpha_2\beta_1$ integrin is necessary, but not sufficient, for the collagen-induced tyrosine phosphorylation of Syk and PLCγ2 in platelets. Blocking of the $\alpha_2\beta_1$ integrin using either inhibitory antibodies or a collagen-derived peptide that is a putative $\alpha_2\beta_1$ ligand inhibited the phosphorylation of both Syk and PLCγ2. Additionally, cross-linking of the β_1 or α_2 subunits with stimulatory antibodies in solution co-stimulated, along with FcγRII, the phosphorylation of both Syk and PLCγ2. These early platelet signaling events had not previously been linked directly to occupancy of the $\alpha_2\beta_1$ integrin. Furthermore, we find evidence for a possible role for the FcγII receptor in collagen stimulation since collagen induced the phosphorylation of FcγRII, a finding that, to our knowledge, had not previously been reported. By using the Syk-selective inhibitor, piceatannol, we are able to place Syk activation upstream of PLCγ2 in platelets. Our results begin to define a pathway whereby collagen binding to the $\alpha_2\beta_1$ integrin contributes to the activation of Syk and the subsequent phosphorylation of numerous proteins including PLCγ2, leading to platelet activation and aggregation.

Although Syk activation has been assumed by some investigators to occur upstream of PLCγ2, this order of events in platelets was not previously established. Several pieces of evidence support the positioning of Syk activation upstream of PLCγ2. First, inhibition of Syk by the Syk-selective kinase inhibitor, piceatannol, inhibited not only Syk kinase activity and tyrosine phosphorylation, but also PLCγ2 phosphorylation. In our hands, in agreement with Oliver *et al.* (64), concentrations of piceatannol that inhibited Syk activity had no significant effect on Src family kinase activity, suggesting that piceatannol specifically inhibits Syk. However, it remains possible that other kinases are also affected by piceatannol. Second, kinetic studies demonstrated that Syk phosphorylation occurred before PLCγ2 phosphorylation. This agrees with the observation that, in B-cells, a knockout of the *syk* gene results in cells that no longer phosphorylate PLCγ2 in response to B-cell receptor cross-linking (66). It remains to be determined whether Syk directly phosphorylates PLCγ2 or whether there are other kinases in between Syk and PLCγ2.

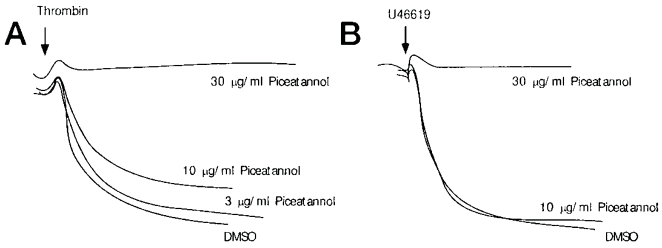

FIG. 6. **Piceatannol inhibits platelet aggregation induced by thrombin and U46619.** Platelets were pretreated for 10 min with piceatannol or Me_2SO (*DMSO*) as indicated and then stimulated with 0.4 unit/ml thrombin (*A*) or 1 μM thromboxane A_2 mimetic, U46619 (*B*), under stirring conditions in an aggregometer. Shown is one of two such experiments.

Our data demonstrate that both the $\alpha_2\beta_1$ integrin and the subsequent activation of Syk and PLCγ2 are necessary for collagen-induced platelet aggregation since either anti-$\alpha_2\beta_1$ integrin antibodies or piceatannol inhibited aggregation. Other investigators have also noted that collagen-induced platelet aggregation is inhibited by some anti-$\alpha_2\beta_1$ integrin antibodies (10) or in platelets missing the $\alpha_2\beta_1$ integrin (2, 8). Although the $\alpha_2\beta_1$ integrin/Syk pathway was required for collagen-induced platelet aggregation, it was not sufficient since intact antibody cross-linking of the $\alpha_2\beta_1$ integrin and subsequent Syk activation did not induce platelet aggregation. This suggests that multiple signaling events, most likely mediated through additional collagen-binding proteins, are necessary to cause full platelet activation. Antibody cross-linking of one putative collagen-binding protein, GPVI, induces the Fc-independent activation of Syk and platelet aggregation (36). It is of interest that both GPVI and the $\alpha_2\beta_1$ integrin seem to be necessary to elicit the full range of platelet responses to collagen since patients deficient in either receptor have platelets that are defective in collagen-induced aggregation (3, 8, 9). The potential necessity for both receptors to elicit the full range of platelet responses to collagen suggests that there may be other signals independent of the Syk/PLCγ2 pathway that contribute to platelet responses to collagen, some of which are linked to the $\alpha_2\beta_1$ integrin and some to GPVI, such that eliminating either receptor might eliminate a portion of the collagen-induced signaling pathway in platelets. Evidence that stimulation of platelets by collagen occurs through two different sites, both of which are necessary for platelet activation by collagen, has also been suggested by Santoro *et al.* (67). It remains to be determined just how the $\alpha_2\beta_1$ integrin and other collagen receptors, including GPVI, cooperate in producing the total platelet response to collagen.

Our observation that some collagen-induced phosphorylation events occur even when the $\alpha_2\beta_1$ integrin is blocked by antibodies or peptides suggests, first, that blocking the $\alpha_2\beta_1$ integrin blocks specific signaling events rather than totally inhibiting all interactions of platelets with soluble collagen. Second, this observation suggests that collagen binding to other receptors occurs independent of, and in addition to, collagen binding to the $\alpha_2\beta_1$ integrin. This finding differs from the observation that platelets deficient in the $\alpha_2\beta_1$ integrin do not adhere to collagen (8, 9) and that anti-$\alpha_2\beta_1$ integrin mAbs almost completely block adhesion to immobilized collagen (11). It is possible that soluble collagen simultaneously binds the $\alpha_2\beta_1$ integrin and other collagen receptors on platelets, but that the $\alpha_2\beta_1$ integrin is required for stable adhesion to immobilized collagen. The other collagen-binding proteins on platelets may be too weak or interact too transiently to mediate stable adhesion to collagen in the absence of the $\alpha_2\beta_1$ integrin, but are probably necessary to elicit the full range of platelet responses to collagen.

The notion that the $\alpha_2\beta_1$ integrin works in conjunction with other receptors is consistent with FcγRII and the $\alpha_2\beta_1$ integrin acting together in antibody cross-linking experiments. Anti-integrin antibodies did not induce phosphorylation of Syk and PLCγ2 when the Fc portion of the antibody was removed or when FcγRII was blocked with the IV.3 antibody. However, antibody-induced tyrosine phosphorylation was not solely due to FcγRII since only the anti-β_1 integrin antibody, and not control IgG, caused the phosphorylation of Syk and PLCγ2. It is likely that the anti-integrin antibody caused a clustering of the integrin with FcγRII. This heterotypic clustering differs from homotypic clustering of FcγRII alone since anti-integrin antibodies did not induce the platelet aggregation that is observed upon cross-linking with the anti-FcγRII antibody (65). Functional cooperation between integrins and the FcγII receptor has also been described for β_2 integrins in neutrophils (68, 69).

Our results are not the first in which co-stimulation of FcγRII contributes to antibody stimulation of platelets. Most notably, antibodies against the tetraspanin CD9 strongly stimulate platelet aggregation, but only in conjunction with FcγRII (51, 70). It is thus of interest that CD9 has recently been co-immunoprecipitated with β_1 integrins from various cells (71, 72) and modulates β_1 integrin function in B-cells (73). An interaction between CD9 and the $\alpha_2\beta_1$ integrin was specifically not found by Berditchevski *et al.* (72), but remains to be tested in platelets.

Unexpectedly, collagen stimulation of platelets, in the absence of antibody, led to the $\alpha_2\beta_1$-independent phosphorylation of FcγRII. Previously, the tyrosine phosphorylation of an unidentified 40-kDa protein following collagen stimulation had been reported (15, 16). Our results suggest that this 40-kDa protein might be FcγRII. Interestingly, this phosphorylation was not blocked by an anti-$\alpha_2\beta_1$ integrin antibody, suggesting that the phosphorylation of FcγRII might occur by collagen binding through a different platelet receptor. This receptor might be GPVI since anti-GPVI antibodies stimulate the phosphorylation of a 40-kDa protein (34). Additionally, collagen-induced FcγRII phosphorylation was also not blocked by the IV.3 anti-FcγRII antibody, implying that it did not occur as a result of binding to an Fc ligand and may, instead, be due to internal signaling events following collagen stimulation. Collagen is not known to bind FcγRII directly. Although collagen binding directly to FcγRII is unlikely, if it does occur, it would have to bind at a site not blocked by IV.3. Our finding differs from that of Yanaga *et al.* (55), who observed no phosphorylation of FcγRII following collagen stimulation. However, their experiments were performed in the presence of EGTA, which would affect the divalent cation-dependent functioning of the $\alpha_2\beta_1$ integrin and possibly other collagen receptors and alter the overall response of the platelets to collagen.

In spite of these observations, the role of FcγRII in collagen signaling, if any, is unclear. Blocking FcγRII with the IV.3 antibody had no effect on either collagen-induced FcγRII, Syk, and PLCγ2 phosphorylation or subsequent platelet aggregation. One possibility is that FcγRII plays no role in collagen-induced platelet activation, but substitutes for the role of a co-receptor or co-stimulator in the case of anti-$\alpha_2\beta_1$ integrin antibody stimulation. In the more physiologic case where $\alpha_2\beta_1$ is bound by collagen, this co-receptor may instead be one of the other collagen-binding proteins such as GPVI. Alternatively, it is possible that the presence of phosphorylated FcγRII, in the absence of Fc ligand binding, is sufficient to contribute to collagen-induced platelet activation. FcγRII contains a tyrosine-based activation motif that, when tyrosine-phosphorylated, binds and activates Syk in platelets (55, 74), consistent with the role of tyrosine-based activation motifs in T- and B-cell

receptor signaling (75–78). Integrin α and β subunits lack such a motif, although the β_1 subunit does have two tyrosine residues with similar spacing as those found in tyrosine-based activation motifs. An attractive model is that FcγRII becomes phosphorylated as a result of collagen activation and then serves as a docking molecule to localize Syk to receptor complexes that might include the $\alpha_2\beta_1$ integrin and perhaps other collagen receptors. Because FcγRII phosphorylation was less sensitive to inhibition by piceatannol than was Syk or PLCγ2 phosphorylation, it is likely that FcγRII is phosphorylated upstream of Syk by another kinase. This kinase may be Src since the phosphorylation of tyrosine-based activation motifs is thought to occur through the action of Src family kinases (79, 80), which are not inhibited by the concentrations of piceatannol used in our assays (64). Furthermore, Src is implicated as the kinase that phosphorylates FcγRII following cross-linking of FcγRII in platelets (65). The determination of whether FcγRII contributes to collagen activation, or how it does so, awaits further study.

While other platelet agonists, like collagen, stimulate downstream signaling events that include protein kinase C activation and calcium second messenger signaling, there is evidence that part of the signaling pathway stimulated by either collagen or the FcγII receptor diverges from that stimulated by G-protein-coupled agonists such as thrombin or thromboxane A_2. Both collagen- and FcγRII-induced platelet activation are insensitive to elevated cAMP (15, 48). In contrast, cAMP is a potent inhibitor of platelet activation induced by thrombin, the thromboxane mimetic (U46619), or ADP (48). Additionally, stimulation by collagen or via the FcγII receptor is similarly sensitive to inhibition by phenylarsine oxide, unlike thrombin stimulation of platelets (16, 54). Finally, stimulation of Syk phosphorylation by collagen and via FcγRII is insensitive to the combined inhibition of protein kinase C and intracellular calcium chelation, also unlike stimulation by thrombin (55).

Some investigators have proposed that the difference between collagen *versus* G-protein-coupled receptors could be explained by the fact that collagen (and FcγRII) activates the PLCγ2 isoform, whereas thrombin activates PLCβ. However, conflicting reports exist as to whether thrombin also stimulates PLCγ2 (44, 45, 63, 65). Our finding that thrombin and U46619 also stimulated PLCγ2 phosphorylation is consistent with the findings of Tate and Rittenhouse (63), but is in contrast to the findings of Blake *et al.* (44) and Daniel *et al.* (45). This may relate to the fact that these investigators examined PLCγ2 phosphorylation only up to 2 min following stimulation by thrombin or U46619, while in this study, we noted PLCγ2 phosphorylation beginning at 2.5 min.

Aggregation studies following treatment of platelets with piceatannol suggest the general importance of Syk activity to platelet aggregation. Piceatannol inhibited platelet aggregation not only in response to collagen, but also in response to thrombin and U46619. Interestingly, piceatannol completely inhibited platelet shape change, as determined by aggregation traces, in response to collagen, but not in response to thrombin or U46619. These results imply a role for Syk in the initial collagen-induced shape change, a role that may not be shared by G-protein-coupled receptors. A role for Syk upstream of platelet aggregation is further supported by the findings that Syk activation is rapid (50, 58, 60) and occurs even in the absence of platelet aggregation and $\alpha_{IIb}\beta_3$ integrin engagement (58). Thus, we found that pretreatment of platelets with the RGDW peptide, which blocks fibrinogen binding to the $\alpha_{IIb}\beta_3$ integrin, did not inhibit collagen-induced Syk activation. In spite of our finding that Syk is important to platelet aggregation, recent studies of Syk null mice demonstrate no obvious platelet defects (81, 82). Further studies will be needed to fully determine the role of Syk in platelet aggregation. Additionally, although Syk activation might be required for platelet aggregation, activation of Syk is not sufficient to cause platelet aggregation since cross-linking the $\alpha_2\beta_1$ integrin by intact antibodies caused phosphorylation of Syk, but not platelet aggregation.

A role for Syk in integrin-mediated signaling was also found by Lin *et al.* (21) in studies of the $\alpha_5\beta_1$ integrin in the monocytic cell line THP-1. Thus, Syk activation may be downstream of a number of integrin receptors in hematopoietic cells. In support of this, about half of the Syk that becomes activated in aggregating platelets occurs following engagement of the $\alpha_{IIb}\beta_3$ integrin during platelet aggregation (58), although Syk is activated prior to $\alpha_{IIb}\beta_3$ engagement as well. It will be interesting to determine whether this represents different subpopulations or localization of Syk in the platelet.

Activation of focal adhesion kinase differs from Syk since its activation in stimulated platelets occurs only after platelet aggregation and requires $\alpha_{IIb}\beta_3$ integrin engagement (19, 83–85). Focal adhesion kinase phosphorylation is also known to be downstream of the $\alpha_2\beta_1$ integrin in the case of platelet adhesion to immobilized collagen (14). Haimovich *et al.* (14) also found a role for the $\alpha_2\beta_1$ integrin in the phosphorylation of two other unidentified 100- and 105-kDa proteins. In our studies, treatment of platelets with soluble collagen also caused the phosphorylation of a similar sized protein (100–110 kDa) that was inhibited by blocking the $\alpha_2\beta_1$ integrin with antibody. Additionally, treatment of platelets with piceatannol also caused the loss of a 100–110-kDa phosphorylated band. The identity of this protein and whether it is the same as that noted previously (14) are not known.

In summary, we have elucidated some steps of $\alpha_2\beta_1$ integrin signaling in platelets by linking specific events to the receptor. It will be of future interest to determine the proximal signaling events that lead to Syk activation and how these steps are regulated by the $\alpha_2\beta_1$ integrin. Additionally, it will be of interest to identify the signaling events unrelated to Syk that may also have a role in collagen-induced aggregation. Finally, it will be important to determine how other collagen-binding proteins contribute to the overall response of platelets to collagen.

Acknowledgments—We thank Dr. Robert Geahlen for generously providing piceatannol, Dr. John Wilkins for providing the JBS2 mAb, Dr. William Carter for providing P1H5 supernatant, Dr. Andrew Chan for contributing polyclonal anti-Syk antibody, Dr. Patricia Maness for providing the anti-Src antibody, and Dr. Michael Caplow for supplying tubulin. We also thank Dr. Carlos Rosales for helpful discussion and supplying the TS2/16 mAb, Dr. Christel Boudignon-Proudhon for helpful suggestions and review of the manuscript, David Shock and Dr. Ulhas Naik for useful suggestions, and Dr. Karen Webb (Department of Pharmacology monoclonal core facility, University of North Carolina, Chapel Hill) for producing control antibodies.

Note Added in Proof—The recent finding by Gibbins *et al.* that collagen induces the phosphorylation of the Fc receptor γ-chain in platelets suggests that the Fc receptor γ-chain, in addition to or instead of FcγRII, may play a role as a docking molecule for Syk following collagen stimulation of platelets. Interestingly, although they do not make the point, their results are like ours in that they also demonstrate a tyrosine-phosphorylated band of an appropriate molecular weight to be FcγRII following collagen stimulation.

REFERENCES

1. Santoro, S. A. (1986) *Cell* **46,** 913–920
2. Kunicki, T. J., Nugent, D. J., Staats, S. J., Orchekowski, R. P., Wayner, E. A. & Carter, W. G. (1988) *J. Biol. Chem.* **263,** 4516–4519
3. Moroi, M., Jung, S. M., Okuma, M. & Shinmyozu, K. (1989) *J. Clin. Invest.* **84,** 1440–1445
4. Tandon, N. N., Kralisz, U. & Jamieson, G. A. (1989) *J. Biol. Chem.* **264,** 7576–7583
5. Asch, A. S., Liu, I., Briccetti, F. M., Barnwell, J. W., Kwakye-Berko, F., Dokun, A., Goldberger, J. & Pernambuco, M. (1993) *Science* **262,** 1436–1440
6. Chiang, T. M. & Kang, A. H. (1982) *J. Biol. Chem.* **257,** 7581–7586

7. Chiang, T. M., Jin, A. & Kang, A. H. (1987) *J. Immunol.* **139,** 887–892
8. Nieuwenhuis, H. K., Akkerman, J. W. N., Houdijk, W. P. M. & Sixma, J. J. (1985) *Nature* **318,** 470–472
9. Kehrel, B., Balleisen, L., Kokott, R., Mesters, R., Stenzinger, W., Clemetson, K. J. & van de Loo, J. (1988) *Blood* **71,** 1074–1078
10. Coller, B. S., Beer, J. H., Scudder, L. E. & Steinberg, M. H. (1989) *Blood* **74,** 182–192
11. Staatz, W. D., Rajpara, S. M., Wayner, E. A., Carter, W. G. & Santoro, S. A. (1989) *J. Cell Biol.* **108,** 1917–1924
12. Saelman, E. U. M., Nieuwenhuis, H. K., Hese, K. M., de Groot, P. G., Heijnen, H. F. G., Sage, E. H., Williams, S., McKeown, L., Gralnick, H. R., and Sixma, J. J. (1994) *Blood* **83,** 1244–1250
13. Polanowska-Grabowska, R., Geanacopoulos, M. & Gear, A. R. L. (1993) *Biochem. J.* **296,** 543–547
14. Haimovich, B., Lipfert, L., Brugge, J. S. & Shattil, S. J. (1993) *J. Biol. Chem.* **268,** 15868–15877
15. Smith, J. B., Dangelmaier, C. & Daniel, J. L. (1993) *Biochem. Biophys. Res. Commun.* **191,** 695–700
16. Greenwalt, D. E. & Tandon, N. N. (1994) *Br. J. Haematol.* **88,** 830–838
17. Rosales, C., O'Brien, V., Kornberg, L. & Juliano, R. (1995) *Biochim. Biophys. Acta* **1242,** 77–98
18. Golden, A., Brugge, J. S. & Shattil, S. J. (1990) *J. Cell Biol.* **111,** 3117–3127
19. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T. & Brugge, J. S. (1992) *J. Cell Biol.* **119,** 905–912
20. Huang, M.-M., Bolen, J. B., Barnwell, J. W., Shattil, S. J. & Brugge, J. S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7844–7848
21. Lin, T. H., Rosales, C., Mondal, K., Bolen, J. B., Haskill, S. & Juliano, R. L. (1995) *J. Biol. Chem.* **270,** 16189–16197
22. Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C. & Juliano, R. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 8392–8396
23. Lin, T. H., Yurochko, A., Kornberg, L., Morris, J., Walker, J. J., Haskill, S. & Juliano, R. L. (1994) *J. Cell Biol.* **126,** 1585–1593
24. Nojima, Y., Morino, N., Mimua, T., Hamasaki, K., Furuya, H., Sakai, R., Sato, T., Tachibana, K., Morimoto, C., Yazaki, Y., and Hirai, H. (1995) *J. Biol. Chem.* **270,** 15398–15402
25. Jewell, K., Kapron-Bras, C., Jeevaratnam, P. & Dedhar, S. (1995) *J. Cell Sci.* **108,** 1165–1174
26. Petch, L. A., Bockholt, S. M., Bouton, A., Parsons, J. T. & Burridge, K. (1995) *J. Cell Sci.* **108,** 1371–1379
27. Vuori, K. & Ruoslahti, E. (1995) *J. Biol. Chem.* **270,** 22259–22262
28. Chen, Q., Kinch, M. S., Lin, T. H., Burridge, K. & Juliano, R. L. (1994) *J. Biol. Chem.* **269,** 26602–26605
29. Werb, Z., Tremble, P. M., Behrendtsen, O., Crowley, E. & Damsky, C. H. (1989) *J. Cell Biol.* **109,** 877–889
30. Schwartz, M. A., Lechene, C. & Ingber, D. E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7849–7853
31. Kapron-Bras, C., Fitz-Gibbon, L., Jeevaratnam, P., Wilkins, J. & Dedhar, S. (1993) *J. Biol. Chem.* **268,** 20701–20704
32. Keely, P. J., Fong, A. M., Zutter, M. M. & Santoro, S. A. (1995) *J. Cell Sci.* **108,** 595–607
33. Riikonen, T., Westermarck, J., Koivisto, L., Broberg, A., Kahari, V.-M. & Heino, J. (1995) *J. Biol. Chem.* **270,** 13548–13552
34. Sugiyama, T., Okuma, M., Ushikubi, F., Sensaki, S., Kanaji, K. & Uch, H. (1987) *Blood* **69,** 1712–1720
35. Arai, M., Yamamoto, N., Moroi, M., Akamatsu, N., Fukutake, K. & Tanoue, K. (1995) *Br. J. Haematol.* **89,** 124–130
36. Ichinohe, T., Takayama, H., Ezumi, Y., Yanagi, S., Yamamura, H. & Okuma, M. (1995) *J. Biol. Chem.* **270,** 28029–28036
37. Yamamoto, N., Ikeda, H., Tandon, N. N., Herman, J., Tomiyama, Y., Mitani, T., Sekiguchi, S., Lipsky, R., Kralisz, U., and Jamieson, G. A. (1990) *Blood* **76,** 1698–1703
38. Kehrel, B., Kronenberg, A., Rauterberg, J., Niesing-Bresch, D., Niehues, U., Kardoeus, J., Schwippert, B., Tschope, D., van de Loo, J., and Clemetson, K. J. (1993) *Blood* **82,** 3364–3370
39. Saelman, E. U. M., Kehrel, B., Hese, K. M., de Groot, P. G., Sixma, J. J. & Nieuwenhuis, H. K. (1994b) *Blood* **83,** 3240–3244
40. McKeown, L., Vail, M., Williams, S., Kramer, W., Hansmann, K. & Gralnick, H. (1994) *Blood* **83,** 2866–2871
41. Morton, L. F., Hargreaves, P. G., Farndale, R. W., Young, R. D. & Barnes, M. J. (1995) *Biochem. J.* **306,** 337–344
42. Nakamura, S. & Yamamura, H. (1989) *J. Biol. Chem.* **264,** 7089–7091
43. Shattil, S. J., Ginsberg, M. H. & Brugge, J. S. (1994) *Curr. Opin. Cell Biol.* **6,** 695–704
44. Blake, R. A., Schieven, G. L. & Watson, S. P. (1994) *FEBS Lett.* **353,** 212–216
45. Daniel, J. L., Dangelmaier, C. & Smith, J. B. (1994) *Biochem. J.* **302,** 617–622
46. Karniguian, A., Grelac, F., Levy-Toledano, S., Legrand, Y. J. & Rendu, F. (1990) *Biochem. J.* **268,** 325–331
47. Smith, B., Selak, M. A., Dangelmaier, C. & Daniel, J. L. (1992) *Biochem. J.* **288,** 925–929
48. Smith, J. B., Dangelmaier, C., Selak, M. A., Ashby, B. & Daniel, J. (1992) *Biochem. J.* **283,** 889–892
49. Sorisky, A., King, W. G. & Rittenhouse, S. E. (1992) *Biochem. J.* **286,** 581–584
50. Fujii, C., Yanagi, S., Sada, K., Nagai, K., Taniguchi, T. & Yamamura, H. (1994) *Eur. J. Biochem.* **226,** 243–248
51. Worthington, R. E., Carroll, R. C. & Boucheix, C. (1990) *Br. J. Haematol.* **74,** 216–222
52. Chacko, G. W., Duchemin, A.-M., Coggeshall, K. M., Osborne, J. M., Brandt, J. T. & Anderson, C. L. (1994) *J. Biol. Chem.* **269,** 32435–32440
53. Ozaki, Y., Satoh, K., Kuroda, K., Qi, R., Yatomi, Y., Yanagi, S., Sada, K., Yamamura, H., Yanabu, M., Nomura, S., and Kume, S. (1995) *J. Biol. Chem.* **270,** 15119–15124
54. Yanaga, F., Asselin, J., Schieven, G. L. & Watson, S. P. (1995) *FEBS Lett.* **368,** 377–380
55. Yanaga, F., Poole, A., Asselin, J., Blake, R., Schieven, G. L., Clark, E. A., Law, C.-L. & Watson, S. P. (1995) *Biochem. J.* **311,** 471–478
56. Taniguchi, T., Kitagawa, H., Yasue, S., Yanagi, S., Sakai, K., Asahi, M., Ohta, S., Takeuchi, F., Nakamura, S., and Yamamura, H. (1993) *J. Biol. Chem.* **268,** 2277–2279
57. Maeda, H., Taniguchi, T., Inazu, T., Yang, C., Nakagawara, G. & Yamamura, H. (1993) *Biochem. Biophys. Res. Commun.* **197,** 62–67
58. Clark, E. A., Shattil, S. J., Ginsberg, M. H., Bolen, J. & Brugge, J. S. (1994) *J. Biol. Chem.* **269,** 28859–28864
59. Tohyama, Y., Yanagi, S., Sada, K. & Yamamura, H. (1994) *J. Biol. Chem.* **269,** 32796–32799
60. Yanagi, S., Sada, K., Tohyama, Y., Tsubokawa, M., Nagai, K., Yonezawa, K. & Yamamura, H. (1994) *Eur. J. Biochem.* **224,** 329–333
61. Hillery, C. A., Smyth, S. S. & Parise, L. V. (1991) *J. Biol. Chem.* **266,** 14663–14669
62. Staatz, W. D., Fok, K. F., Zutter, M. M., Adams, S. P., Rodriguez, B. A. & Santoro, S. A. (1991) *J. Biol. Chem.* **266,** 7363–7367
63. Tate, B. F. & Rittenhouse, S. E. (1993) *Biochim. Biophys. Acta* **1178,** 281–285
64. Oliver, J. M., Burg, D. L., Wilson, B. S., McLaughlin, J. L. & Geahlen, R. L. (1994) *J. Biol. Chem.* **269,** 29697–29703
65. Huang, M.-M., Indik, Z., Brass, L. F., Hoxie, J. A., Schreiber, A. D. & Brugge, J. S. (1992) *J. Biol. Chem.* **267,** 5467–5473
66. Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H. & Kurosaki, T. (1994) *EMBO J.* **13,** 1341–1349
67. Santoro, S. A., Walsh, J. J., Staatz, W. D. & Baranski, K. J. (1991) *Cell Regul.* **2,** 905–913
68. Graham, I. L., Lefkowith, J. B., Anderson, D. C. & Brown, E. J. (1993) *J. Cell Biol.* **120,** 1509–1517
69. Katz, D. A., Haimovich, B. & Greco, R. S. (1995) *Surgery* (*St. Louis*) **118,** 154–161
70. Kuroda, K., Ozaki, Y., Qi, R., Asazuma, N., Yatomi, Y., Satoh, K., Nomura, S., Suzuki, M. & Kume, S. (1995) *J. Immunol.* **155,** 4427–4436
71. Rubinstein, E., Le Naour, F., Billard, M., Prenant, M. & Boucheix, C. (1994) *Eur. J. Immunol.* **24,** 3005–3013
72. Berditchevski, F., Zutter, M. M. & Hemler, M. E. (1996) *Mol. Biol. Cell* **7,** 193–207
73. Shaw, A. R. E., Domanska, A., Mak, A., Gilchrist, A., Dobler, K., Visser, L., Poppeman, S., Fliegel, L., Letarte, M., and Willett, B. J. (1995) *J. Biol. Chem.* **270,** 24092–24099
74. Chacko, G. W., Brandt, J. T., Coggeshall, K. M. & Anderson, C. L. (1996) *J. Biol. Chem.* **271,** 10775–10781
75. Kolanus, W., Romeo, C. & Seed, B. (1993) *Cell* **74,** 171–183
76. Samelson, L. T. & Klausner, R. D. (1992) *J. Biol. Chem.* **267,** 24913–24916
77. Geahlen, R. L. & Burg, D. L. (1994) *The Role of syk in Cell Signaling*, pp. 103–107, Plenum Press, New York
78. Weiss, A. & Littman, D. R. (1994) *Cell* **76,** 263–274
79. Iwashima, M., Irving, B. A., van Oers, N. S., Chan, A. C. & Weiss, A. (1994) *Science* **263,** 1136–1139
80. Ting, A. T., Dick, C. J., Schoon, R. A., Karnitz, L. M., Abraham, R. T. & Leibson, P. J. (1995) *J. Biol. Chem.* **270,** 16415–16421
81. Cheng, A. M., Rowley, B., Pao, W., Hayday, A., Bolen, J. B. & Pawson, T. (1995) *Nature* **378,** 303–306
82. Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L. & Tybulewicz, V. L. J. (1995) *Nature* **378,** 298–302
83. Ferrell, J. E., Jr. & Martin, G. S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 2234–2238
84. Huang, M.-M., Lipfert, L., Cunningham, M., Brugge, J. S., Ginsberg, M. H. & Shattil, S. J. (1993) *J. Cell Biol.* **122,** 473–483
85. Shattil, S. J., Haimovich, B., Cunningham, M., Lipfert, L., Parsons, J. T., Ginsberg, M. H. & Brugge, J. S. (1994) *J. Biol. Chem.* **269,** 14738–14745
86. Gibbins, J., Asselini, J., Farndale, R., Barnes, M., Law, C.-L., and Watson, S. P. (1996) **271,** 18095–18099