THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 44, Issue of October 31, pp. 27539–27542, 1997

Printed in U.S.A.

Communication

Syk and Fyn Are Required by Mouse Megakaryocytes for the Rise in Intracellular Calcium Induced by a Collagen-related Peptide*

(Received for publication, June 30, 1997, and in revised form, August 14, 1997)

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Stimulation of platelets by collagen leads to activation of a tyrosine kinase cascade resulting in secretion and aggregation. We have recently shown that this pathway involves rapid tyrosine phosphorylation of an Fc receptor γ chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM), enabling interaction with the tandem SH2 domains of the tyrosine kinase Syk. Activation of Syk lies upstream of tyrosine phosphorylation of phospholipase Cγ2. In the present study we sought to test directly the role of the ITAM/Syk interaction and the role of the Src-related kinases in collagen receptor signaling using mouse megakaryocytes. We demonstrate that the calcium-mobilizing action of a collagen-related peptide (CRP) is kinase-dependent, inhibited by the microinjection of the tandem SH2 domains of Syk and abolished in Syk-deficient mice. Furthermore, the CRP response is abolished by the Src family kinase inhibitor PP1 and inhibited in Fyn-deficient mice. In contrast, the calcium response to the G-protein-linked receptor agonist thrombin is not significantly altered under these conditions. These results provide direct evidence of the functional importance of Fyn and Syk in collagen receptor signaling and support the megakaryocyte as a model for the study of proteins involved in this pathway.

The extracellular matrix protein collagen has a fundamental role in hemostasis. Upon vascular damage, platelets adhere to subendothelial collagen leading to platelet degranulation, aggregation, and development of a hemostatic plug.

Collagen activates platelets through a tyrosine kinase-dependent pathway, which involves tyrosine phosphorylation of Syk and phospholipase Cγ2 (PLCγ2),[1] leading to formation of the Ca^{2+}-mobilizing second messenger inositol 1,4,5-trisphosphate and a rise in intracellular calcium ($[Ca^{2+}]_i$) (1–4). Syk is a 72-kDa non-receptor tyrosine kinase, which is assembled into signaling complexes via interaction between its tandem Src homology 2 (SH2) domains and an immunoreceptor tyrosine-based activation motif (ITAM) found in receptors of the immune system (5, 6). The ITAM has the amino acid sequence $YXX(L/I)X_{6-8}YXX(L/I)$ (7) and is phosphorylated on the conserved tyrosine residues by a member of the Src family of kinases upon receptor activation (8). We have recently shown that stimulation of platelets with collagen, or a collagen-related peptide (CRP), induces tyrosine phosphorylation of the Fc receptor γ chain (FcR-γ chain), which contains an ITAM, allowing formation of an association with Syk (9). We have also demonstrated, via use of genetically deficient mice, the absolute requirement for both Syk and the FcR-γ chain for collagen-induced phosphorylation of PLCγ2 and functional responses in mouse platelets (10).

Syk is also phosphorylated on tyrosine when platelets are stimulated with thrombin (11). This occurs in the absence of significant PLCγ2 phosphorylation (1). Thrombin elicits a rise in $[Ca^{2+}]_i$ via a G-protein link to PLCβ (12). However, since Syk-deficient platelets have normal functional responses to thrombin the significance of Syk phosphorylation in thrombin receptor signaling remains unclear (10).

The range of techniques that can be used to verify the role of Syk in collagen and thrombin receptor signaling in platelets is hampered by the absence of a nucleus and by their small size. It is possible to overcome these limitations through experiments on the platelet precursor cell, the megakaryocyte (13). Megakaryocytes are large (20–50 μm), terminally differentiated polyploid cells comprising 0.1% of bone marrow cells. They can be obtained in reasonable yield only through surgery, limiting experimentation on human tissue. In the present study we have therefore used mouse megakaryocytes in conjunction with single-cell video imaging to investigate the role of Src and Syk family kinases in collagen receptor signaling.

CRP has been used to activate the collagen receptor in these experiments to limit contributions from other receptors. CRP mimics platelet activation by collagen, inducing marked tyrosine phosphorylation of the FcR-γ chain, Syk, and PLCγ2 but is unable to bind to the integrin $\alpha_2\beta_1$ (9, 14, 15), which has been proposed as a co-receptor in collagen-induced activation of platelets (16). A number of other proteins have also been proposed as candidate collagen receptors including glycoprotein IV (GPIIIb, CD36) and uncharacterized 65- and 85–90-kDa glycoproteins (17–19). The use of CRP eliminates the potential involvement of $\alpha_2\beta_1$ and possibly other receptors enabling selective study of the signaling events believed to directly underlie platelet activation by collagen. There is strong evidence that glycoprotein VI (GPVI) is the receptor responsible for activation of platelets by collagen and CRP (19–22). Platelet activation by collagen is absent in donors deficient in GPVI (20), whereas activation of the glycoprotein stimulates phosphorylation of FcR-γ chain, Syk, and PLCγ2 (19, 21, 22).

We demonstrate the requirement of Syk for CRP-induced $[Ca^{2+}]_i$ rises via introduction of a protein comprising the tan-

* This work was supported by the Wellcome Trust, the British Heart Foundation, and the Medical Research Council. 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.

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[1] The abbreviations used are: PLC, phospholipase C; SH2, Src homology 2; CRP, collagen-related peptide; FcR-γ chain, Fc receptor γ chain; IP_3R, inositol 1,4,5-trisphosphate receptor.

dem SH2 domains of Syk and studies of Syk-deficient megakaryocytes. Neither treatment has a significant effect on the Ca^{2+}-mobilizing response of the G-protein receptor-coupled agonist, thrombin. We further demonstrate the importance of the Src family kinases, and in particular the kinase Fyn, for CRP-induced $[Ca^{2+}]_i$ rises. These data provide direct evidence for a functional role of Syk and Fyn in collagen receptor signaling. These studies support the megakaryocyte as a model of signaling pathways in platelets (23) and suggest that the megakaryocyte could be an important model system for the study of other proteins involved in signaling by collagen.

EXPERIMENTAL PROCEDURES

Materials—CRP (GCP*(GPP*)$_{10}$GCP*G (single-letter amino acid code, where P* is hydroxyproline), cross-linked as described (14)) was kindly donated by Dr. M. Barnes (Strangeways Research Laboratory, Cambridge, U.K.). PP1 was provided by Dr. J. Hanke (Pfizer, U.K.). Thrombin, apyrase, staurosporine, and poly-L-lysine (M_r 70,000) were from Sigma (Poole, Dorset, U.K.). Fura-2 and calibration standards were from Molecular Probes (Eugene, OR). All other agents were from previously reported sources (3).

Fusion Protein—A glutathione *S*-transferase fusion protein containing the tandem SH2 domains of Syk was constructed and expressed as described previously (3) using the pGEX vector (Pharmacia Biotech Inc.), which introduces a thrombin cleavage site facilitating the removal of glutathione *S*-transferase.

Protein-deficient Mice—The generation of BALB/c radiation chimeric mice reconstituted with Syk-deficient fetal liver has been described (10). Fyn-deficient mice on a C57BL/6 background were obtained from Dr. P. Soriano (Division of Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, WA) (24).

Preparation of Mouse Megakaryocytes—Mice (4–8 weeks) were killed by cervical dislocation, and the femoral bones were extracted. Control data were generated on male BALB/c mice. The bone marrow was gently washed through a 23-gauge needle into a Ca^{2+}/Mg^{2+}-free Hanks' solution containing 0.4% (w/v) bovine serum albumin and 0.2 units/ml apyrase. The cells were centrifuged at 200 × *g* for 10 min in the presence of prostacyclin (1 μg/ml) and resuspended for experimentation in a modified Hanks' solution (143 mM NaCl, 5.6 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, 0.2 mM $CaCl_2$, 0.4% bovine serum albumin, pH 7.2). Experiments were performed at room temperature (22 ± 2 °C).

Single-cell Studies—Bone marrow cells were plated onto coverslips coated in poly-L-lysine (M_r 70,000). Microinjection of proteins and Fura-2 (25) with glass capillaries was performed with an Eppendorf microinjector 5242 and manipulator 5170. Fura-2 was present in the microinjection needle at a concentration of 2.5 mM. The tandem SH2 domains of Syk were present in the microinjection needle at a concentration of 135 μg/ml and introduced to the cells at a calculated concentration of approximately 0.5 μM (26). Agonists were dissolved in the external solution and added to the well containing the cells by pipetting; the tyrosine kinase inhibitor staurosporine was given 180 s before CRP. Single-cell digital imaging of $[Ca^{2+}]_i$ was performed using Ionvision software (Improvision, Warwick, U.K.). Fluorescence video images were captured at excitation wavelengths of 340 and 380 nm with emission at 510 nm. Calculation of $[Ca^{2+}]_i$ from the 340:380 ratio was performed by the use of a previously established calibration curve using standard solutions of various free Ca^{2+} concentrations applying a viscosity correction factor (27). Analysis was performed using Ionvision software for the Macintosh. Results are shown as mean ± S.E. of data collected from a minimum of three mice. Statistical analysis was by Student's *t* test.

RESULTS

Isolated Megakaryocytes Respond to Platelet Agonists—Megakaryocytes were identified on the basis of their size and morphology. Cells possessing the characteristics of granular megakaryocytes (Stage III) or mature megakaryocytes (Stage IV) were used for experimentation. No discernible difference in response could be detected between the two stages.

Megakaryocytes injected with Fura-2 exhibited a uniform distribution of Ca^{2+} throughout the cytosol. There were no oscillations in resting $[Ca^{2+}]_i$, unlike those seen in rat megakaryocytes (23, 28). Fura-2-loaded megakaryocytes demonstrated a rise in $[Ca^{2+}]_i$ when treated with CRP (2.5 μg/ml) and thrombin (1 unit/ml) as shown in Fig. 1*A*. The steady

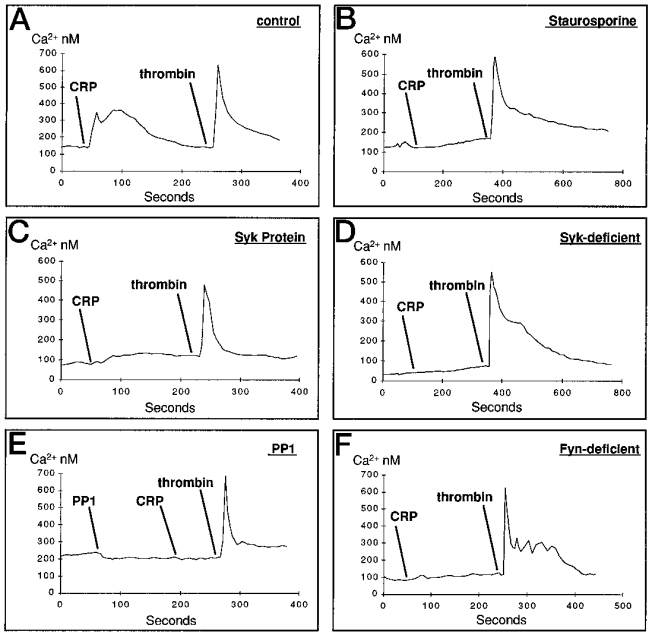

FIG. 1. **Single-cell calcium traces of mouse megakaryocytes microinjected with Fura-2.** Each *trace* is representative of between 8 and 18 cells from at least three animals. *A*, control response to CRP (2.5 μg/ml) and thrombin (1 unit/ml). These concentrations refer to all traces. *B*, the effect of staurosporine (3 μM) added 180 s prior to agonist addition. *C*, the effect of microinjecting Syk protein to a calculated concentration of 0.5 μM, 120 s prior to agonist addition. *D*, the effect of agonist addition in megakaryocytes lacking the Syk protein. *E*, the effect of the Src family kinase inhibitor PP1 (10 μM) applied at 1 min. *F*, the effect of agonist addition in megakaryocytes lacking the Fyn protein.

resting basal $[Ca^{2+}]_i$ was measured as 92 ± 13 nM (n = 18). CRP induced a rapid rise in $[Ca^{2+}]_i$, which peaked at a mean of 348 ± 31 nM (Table I) and returned to basal levels within 100 s. The G-protein receptor-coupled agonist, thrombin, caused a larger peak $[Ca^{2+}]_i$ rise to 456 ± 36 nM (Table I), which returned rapidly to basal within a period of 100 s. The response to thrombin was not significantly different whether given before or after exposure to CRP (not shown).

Effect of the Nonspecific Kinase Inhibitor Staurosporine—To investigate whether CRP signals through a kinase-dependent pathway we applied the nonspecific kinase inhibitor staurosporine (29) to the external solution bathing the megakaryocytes, at a concentration of 3 μM. Staurosporine had no significant effect on resting $[Ca^{2+}]_i$ but abolished the $[Ca^{2+}]_i$ rise induced by CRP (2.5 μg/ml) (Table I). The example experimental record in Fig. 1*B* clearly shows an abolition of the response to CRP. Staurosporine did not significantly alter the peak response to thrombin (1 unit/ml; mean peak value, 456 ± 71 nM (Table I)) but prolonged the period of increased $[Ca^{2+}]_i$, taking in excess of 400 s to return to basal levels.

Effect of Microinjected Syk Protein—The involvement of the non-receptor tyrosine kinase Syk in the collagen signaling pathway was investigated by co-microinjection of the tandem SH2 domains of Syk and Fura-2. It was hypothesized that the protein would compete with endogenous Syk for binding to the Fc receptor γ chain ITAM, preventing downstream phosphorylation events and a rise in $[Ca^{2+}]_i$.

Injection of the Syk protein did not significantly alter resting $[Ca^{2+}]_i$ (109 ± 17 nM). The peak $[Ca^{2+}]_i$ response induced by CRP was significantly reduced to a mean of 158 ± 20 nM ($p < 0.01$) (Fig. 1*C* and Table I). This value is significantly higher than basal ($p < 0.02$), possibly reflecting the fact that not all endogenous Syk is being competed out. The mean peak $[Ca^{2+}]_i$ value obtained after thrombin stimulation was 412 ± 26 nM,

TABLE I

Role of tyrosine kinases in the rise in $[Ca^{2+}]_i$ by CRP and thrombin

Combined data are shown from a series of Fura-2-microinjected cells showing comparison between control cells, cells treated with staurosporine (3 μM), cells injected with the tandem SH2 domains of Syk, cells from Syk-deficient mice, cells treated with PP1 (10 μM), and cells from Fyn-deficient mice. The table shows peak intracellular calcium values of cells at rest and upon stimulation with CRP (2.5 μg/ml) and thrombin (1 unit/ml). The values are shown as mean ± S.E. ($n \geq 8$, from at least three animals).

	Peak $[Ca^{2+}]_i$ value[a]			n
	Resting	2.5 μg/ml CRP	1 unit/ml thrombin	
		nM		
Control	92 ± 13	348 ± 31	456 ± 36	18
+ 3 μM staurosporine	118 ± 23	149 ± 28[b,c]	456 ± 71	8
+ Syk SH2 protein	109 ± 17	158 ± 20[b]	412 ± 26	11
Syk-deficient	64 ± 15	73 ± 15[b,c]	411 ± 30	9
+ 10 μM PP1	121 ± 16	133 ± 14[b,c]	516 ± 42	8
Fyn-deficient	109 ± 14	147 ± 19[b]	540 ± 49	13

[a] Mean ± S.E.

[b] Indicates significant inhibition of response compared with control cells ($p < 0.01$).

[c] Indicates no significant difference compared with resting levels.

which is not significantly different from non-injected control cells (Fig. 1*C* and Table I). Prolongation of the thrombin response was not observed.

Effect of Agonists on Syk-deficient Megakaryocytes—The apparent requirement of Syk for CRP-induced $[Ca^{2+}]_i$ rises was further investigated in megakaryocytes from radiation chimeric mice reconstituted with fetal liver deficient in Syk. Successful reconstitution was demonstrated by the absence of Syk in immunoblots of platelet lysates (not shown). Syk-deficient megakaryocytes showed no significant difference in resting $[Ca^{2+}]_i$ from controls (Fig. 1*D*, Table I). Treatment with CRP (2.5 μg/ml) failed to induce a significant rise in $[Ca^{2+}]_i$ above basal levels. The mean peak $[Ca^{2+}]_i$ value obtained by thrombin stimulation was not significantly different from the wild type control at 411 ± 30 nM but showed a slight prolongation of the period of raised $[Ca^{2+}]_i$. This demonstrates that Syk is essential for $[Ca^{2+}]_i$ increases induced by CRP.

Effect of the Src Family Kinase Inhibitor PP1—It is proposed that a member of the Src family of kinases phosphorylates the ITAM upon activation of antigen receptors (8). It can therefore be speculated that a member of this family is involved in phosphorylation of the FcR-γ chain following stimulation by CRP. This hypothesis was investigated using the pyrazolopyrimidine PP1, a reported Src family kinase inhibitor (30). This inhibitor is reported to be selective for the kinases Fyn and Lck over other members of the family of Src-like kinases and also over Zap-70, a member of the Syk family of kinases. Fura-2-injected megakaryocytes were treated with PP1 for 3 min prior to agonist addition. PP1 abolished the rise in $[Ca^{2+}]_i$ in response to CRP (Fig. 1*E*, Table I). In contrast, thrombin induced a rise in $[Ca^{2+}]_i$ to a mean peak value of 516 ± 42 nM, a value higher than that seen in control cells although this effect did not reach statistical significance. This indicates that one or more members of the Src family kinases are essential for the calcium-mobilizing action of CRP but that they are not required for thrombin signaling.

The Effect of Agonists on Fyn-deficient Megakaryocytes—Given that PP1 is reported to be selective for Fyn and Lck and that Lck is absent in platelets, studies were performed on mice genetically manipulated to lack the Fyn protein to see if CRP signaling was impaired. Fyn was not detected by immunoblot in the platelets from Fyn-deficient animals, and the absence of Fyn was confirmed with polymerase chain reaction (not shown). Fyn-deficient megakaryocytes injected with Fura-2 showed no significant difference in resting $[Ca^{2+}]_i$ in comparison with control cells. Treatment with CRP elicits a small rise in $[Ca^{2+}]_i$ from the resting value of 109 ± 14 nM to a peak of 147 ± 19 nM (Fig. 1*F*, Table I). This rise is significantly higher than resting ($p < 0.05$) but is dramatically reduced compared with control cells ($p < 0.01$). The peak $[Ca^{2+}]_i$ response to thrombin was greater than control at 540 ± 49 nM, but this did not reach statistical significance. In addition a short period of oscillations in $[Ca^{2+}]_i$ was observed after the initial thrombin-induced peak. These results suggest that Fyn plays a crucial role in signaling by CRP but little or no role in that of thrombin.

DISCUSSION

Various biochemical studies have provided evidence for the involvement of the non-receptor tyrosine kinase Syk in the signaling pathway elicited by collagen stimulation in platelets (1–4). The aim of this study was to verify this model through direct evaluation of the role of Syk and the Src family of kinases in signaling by collagen using single-cell imaging and microinjection techniques in megakaryocytes, the precursor cell for the platelet.

We have shown that mouse megakaryocytes exhibit a rise in $[Ca^{2+}]_i$ when treated with CRP and thrombin. Through the use of the nonspecific kinase inhibitor staurosporine we have shown that the Ca^{2+}-mobilizing action of CRP is dependent on kinase activity. In contrast the peak Ca^{2+} response to thrombin is unaffected. This is in agreement with data from platelets where thrombin is known to elicit a rise in $[Ca^{2+}]_i$ via a G-protein-dependent pathway acting through PLCβ (10). The prolonged period of increased $[Ca^{2+}]_i$ induced by thrombin in the presence of staurosporine could reflect an inhibition of kinase-dependent extrusion mechanisms, loss of an inhibitory action of protein kinase C at the level of PLCβ (31), or prolongation of Ca^{2+} influx mechanisms (32).

Microinjection of the tandem SH2 domains of Syk into mouse megakaryocytes significantly inhibited the Ca^{2+}-elevating action of CRP but had no significant effect on the action of thrombin. We propose that the injected protein is competing with endogenous Syk for binding to the ITAM in the FcR-γ chain, which is phosphorylated in CRP-stimulated platelets. The lack of a catalytic domain in the injected protein inhibits downstream phosphorylation of PLCγ2, preventing a rise in $[Ca^{2+}]_i$. Current evidence suggests that the receptor recognizing CRP is one component of the collagen receptor complex and is associated with the FcR-γ chain (9). Upon receptor activation endogenous Syk binds to the collagen receptor complex via interaction of its SH2 domains with the ITAM on the FcR-γ chain (6). Several groups have demonstrated the importance of the tandem SH2 domain region of Syk for optimal association with the tyrosine-phosphorylated ITAMs (33, 34). For example, introduction of Syk SH2 domains to RBL-2H3 cells by permeabilization of the membrane (35) or in T cells by overexpression (36) inhibits signaling via the high affinity IgE receptor (FcϵRI) and the T cell antigen receptor, respectively. Importantly, inhibition was seen at concentrations similar to those used in the present study. The equilibrium binding affinity for association of tandem Syk SH2 domains to the phosphorylated ITAM of the FcϵRI receptor has been shown to be 1.4 nM, demonstrating that this is a very high affinity interaction (37). The high binding affinity supports a specific action of the Syk tandem SH2 domain protein. Specificity in the action of Syk SH2 domains is also suggested by the fact that several other SH2 domains, including the tandem SH2 domains of PLCγ1, do not alter the response to CRP.[2] Specific inhibitory action of SH2

[2] B. Gross, S. K. Melford, and S. P. Watson, manuscript in preparation.

domains has also been demonstrated for stimuli in other cells (36, 38, 39). Moreover, the complete lack of a Ca^{2+} mobilization by CRP in megakaryocytes lacking Syk confirms an essential role for Syk in transducing the signal and is in agreement with the observation that platelets from Syk-deficient mice do not respond to collagen (10). These studies demonstrate that the action of Syk lies upstream of Ca^{2+} mobilization. The lack of inhibitory effect of both the injected Syk SH2 domains and absence of Syk on thrombin stimulation suggests that Syk does not perform an important role in the Ca^{2+}-mobilizing action of this G-protein-coupled receptor pathway. The physiological significance of phosphorylation of Syk caused by thrombin stimulation may occur downstream of PLCβ and Ca^{2+} mobilization or may have no involvement in the rise of $[Ca^{2+}]_i$. The role of Syk may be in ending the Ca^{2+} response, given the extended Ca^{2+} flux in Syk-deficient megakaryocytes stimulated with thrombin.

Inhibition of the Src family of kinases by the selective agent PP1 abolishes the Ca^{2+} rise induced by CRP. This suggests a crucial involvement of Src kinases in this receptor pathway, possibly through phosphorylation of the ITAM on the FcR-γ chain, analogous to current models of signaling by immune receptors. The large inhibition of CRP response seen in Fyn-deficient mice suggests that Fyn may act upstream of Ca^{2+} mobilization and may be the crucial kinase responsible for phosphorylation of the ITAM. It could also be possible that Fyn lies between Syk and PLCγ2. The small rise in $[Ca^{2+}]_i$ in Fyn-deficient mouse megakaryocytes in response to CRP presumably reflects redundancy among members of the Src family of kinases but could reflect a Syk-dependent, Fyn-independent signaling pathway, which is not via the FcR-γ chain. It has been proposed that Fyn can influence the function of the inositol 1,4,5-trisphosphate receptor (IP_3R) in T cells (40). In our studies it is unlikely that the inhibitory action of Fyn deficiency on CRP signaling is at the level of the IP_3R given that the Ca^{2+}-mobilizing action of thrombin, which also signals via the IP_3R, is not inhibited either in the presence of PP1 or in the Fyn-deficient mice.

Our studies highlight several comparisons with the role of tyrosine kinases in signaling via other immune receptors. Syk deficiency abolished Ca^{2+} mobilization mediated by the FcϵRI in mast cells (41) and by the B-cell antigen receptor in the chicken B-cell line, DT40 (42). In mast cells, it has been suggested that signaling via FcϵRI, which is associated with the FcR-γ chain, involves sequential activation of Lyn and then Syk (41, 43). In this cell type Lyn deficiency results in a reduced Ca^{2+} response to activation of FcϵRI (43).

These studies provide the first demonstration of the functional importance and relative action of Syk and Fyn in the mobilization of Ca^{2+} by the receptor for collagen in mice megakaryocytes. The data provide further evidence that the collagen receptor, presumably GPVI (see Introduction), signals in a manner similar to an immune receptor. The megakaryocyte could provide a model for the study of other proteins that participate in signaling by collagen and by immune receptors in general, *e.g.* SLP-76, PLCγ, or p38 (44).

Acknowledgments—We are grateful to Dr. M. Barnes for CRP, Dr. Che-Leung Law for the fusion construct of Syk, Dr. P. Soriano for Fyn-deficient mice, and Dr. J. Hanke for PP1. We appreciate the help of Dr. A. Poole in the early part of this study.

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Communications:

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J. Biol. Chem. 1997, 272:27539-27542.
doi: 10.1074/jbc.272.44.27539