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

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*g*2. In the present study

dem SH2 domains of Syk and studies of Syk-deficient megakaryocytes. Neither treatment has a significant effect on the Ca^{21}-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 $[Ca^{21}]_i$ 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

MaterialsÐCRP (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

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

Effect of Agonists on Syk-deficient MegakaryocytesÐ The apparent requirement of Syk for CRP-induced [Ca ^{21}]$_i$ 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^{21}]$_i$ from controls (Fig. 1 D, Table I). Treatment with CRP (2.5 *m*g/ml) failed to induce a significant rise in [Ca ^{21}]$_i$ above basal levels. The mean peak [Ca ^{21}]$_i$ value obtained by thrombin stimulation was not significantly different from the wild type control at 411 6 30 nM but showed a slight prolongation of the period of raised [Ca ^{21}]$_i$. This demonstrates that Syk is essential for [Ca ^{21}]$_i$ increases induced by CRP.

Effect of the Src Family Kinase Inhibitor PP1Ð 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- *g* 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 ^{21}]$_i$ in response to CRP (Fig. 1E, Table I). In contrast, thrombin induced a rise in [Ca ^{21}]$_i$ to a mean peak value of 516 6 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 MegakaryocytesÐ 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 ^{21}]$_i$ in comparison with control cells. Treatment with CRP elicits a small rise in [Ca^{21}]$_i$ from the resting value of 109 6 14 nM to a peak of 147 6 19 nM (Fig. 1F, 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^{21}]$_i$ response to thrombin was greater than control at 540 6 49 nM, but this did not reach statistical significance. In addition a short period of oscillations in [Ca ^{21}]$_i$ 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^{21}]$_i$ when treated with CRP and thrombin. Through the use of the nonspecific kinase inhibitor staurosporine we have shown that the Ca ^{21}

domains has also been demonstrated for stimuli in other cells (36, 38, 39). Moreover, the complete lack of a Ca^{21} 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^{21} 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^{21}-mobilizing action of this G-protein-coupled receptor pathway. The physiological significance of phosphorylation of Syk caused by thrombin stimulation may occur downstream of PLC *b* and Ca^{21} mobilization or may have no involvement in the rise of $[Ca^{21}]_i$. The role of Syk may be in ending the Ca^{21} response, given the extended Ca^{21} flux in Syk-deficient megakaryocytes stimulated with thrombin.

Inhibition of the Src family of kinases by the selective agent PP1 abolishes the Ca^{21} 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- *g* 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^{21} mobilization and may be the crucial kinase responsible for

Communications:

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