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Tyrosine Phosphorylation of SLP-76 Is Downstream of Syk following Stimulation of the Collagen Receptor in Platelets*

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Barbara S. Gross‡§, Jong Ran Lee¶, James L. Clements¶, Martin Turner, Victor L. J. Tybulewicz**, Paul R. Findell‡‡, Gary A. Koretzky¶, and Steve P. Watson‡§§**

*From the ‡Department of Pharmacology, Mansfield Road, Oxford University, Oxford OX1 3QT, United Kingdom, the ¶Department of Internal Medicine, Physiology and Biophysics and the Interdisciplinary Immunology Program, University of Iowa College of Medicine, Iowa City, Iowa 52242, the **National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom, and ‡‡Roche Biosciences, Palo Alto, California 94304*

Collagen-related peptide (CRP), a collagen homologue, induces platelet activation through a tyrosine kinase-dependent pathway, leading to sequential tyrosine phosphorylation of Fc receptor (FcR) γ-chain, Syk, and phospholipase C-γ_2. Here we report that CRP and the platelet low affinity immune receptor FcγRIIA stimulate tyrosine phosphorylation of the T cell adapter SLP-76, whereas the G protein-coupled receptor agonist thrombin induces only minor tyrosine phosphorylation. This suggests that SLP-76 has a specific role downstream of receptors that signal via an immunoreceptor tyrosine-based activation motif. Immunoprecipitation studies demonstrate association of SLP-76 with SLAP-130, Vav, Fyn, Lyn, and the FcR γ-chain in CRP-stimulated platelets. Several of these proteins, including SLP-76, undergo tyrosine phosphorylation in *in vitro* kinase assays performed on SLP-76 immunoprecipitates. Tyrosine phosphorylation of all of these proteins in the *in vitro* kinase assay was abrogated by the Src family kinase inhibitor PP1, suggesting that it is mediated by either Fyn or Lyn. The physiological significance of this is uncertain, however, since tyrosine phosphorylation of SLP-76 *in vivo* is not altered in either Fyn- or Lyn-deficient platelets. CRP stimulation of Syk-deficient platelets demonstrated that *in vivo* tyrosine phosphorylation of SLP-76 is downstream of Syk. The absence of Syk in the SLP-76 immunoprecipitates raises the possibility that another protein is responsible for bringing SLP-76 to Syk. Candidates for this include those proteins that co-immunoprecipitate with SLP-76, including the FcR γ-chain. Tyrosine phosphorylation of PLC-γ_2 and Ca^{2+} mobilization is markedly attenuated in SLP-76-deficient platelets following CRP stimulation, suggesting that the adapter plays a critical role in the regulation of the phospholipase. The increase in tyrosine phosphorylation of SLAP-130 in response to CRP is also inhibited in SLP-76-deficient platelets, placing it downstream of SLP-76. This work identifies SLP-76 as an important adapter molecule that is regulated by Syk and lies upstream of SLAP-130 and PLC-γ_2 in CRP-stimulated platelets.

SLP-76 (SH2[1] domain-containing leukocyte protein of 76 kDa) was identified by association with the SH3 domain of the Grb2 adapter protein in T cells and becomes tyrosine-phosphorylated upon T cell receptor (TCR) stimulation (1). SLP-76 has three potential tyrosine phosphorylation sites within its amino terminus region: Tyr^{113}, Tyr^{128}, and Tyr^{145}. Tyr^{113} and Tyr^{128} have a consensus binding site for the SH2 domain of Vav (DYESP) (2–5) and are heavily tyrosine-phosphorylated following TCR engagement, whereas Tyr^{145}, which falls in the sequence DYEPP, is phosphorylated to a lesser extent (6). SLP-76 also contains a central proline-rich region that mediates the association with Grb2 (7) and a carboxyl-terminal SH2 domain that binds to at least two tyrosine-phosphorylated proteins, SLAP-130 (SLP-76-associated phosphoprotein of 130 kDa) (8), a 62-kDa protein, and an uncharacterized serine/threonine kinase after TCR engagement (7). SLP-76 is believed to be an essential adapter protein in T cells. Overexpression of SLP-76 results in an enhancement of TCR-mediated induction of nuclear factor of activated T cell and interleukin-2 promoter activity (3, 5–7, 9, 10). More recently, lack of expression of SLP-76 in Jurkat cells demonstrated that SLP-76 is necessary for tyrosine phosphorylation of phospholipase C-γ_1 (PLC-γ_1) and activation of the Ras pathway (11). Moreover, SLP-76 is required for normal thymocyte development, since SLP-76 knockout mice lack peripheral T cells (12, 13).

The three tyrosine phosphorylation sites, the proline-rich region, and the SH2 domain of SLP-76 have all been shown to be important for the regulation of T cell interleukin 2 production (10). The inducible tyrosine phosphorylation of SLP-76 is mediated by ZAP-70 or Syk in COS cells (9) and rat basophilic leukemia cells (14), respectively. The mechanism by which SLP-76 is phosphorylated by ZAP-70 or Syk is not known.

We have previously reported the association of tyrosine-phosphorylated SLP-76 with the SH3 domain of Grb2 in platelets in response to stimulation of the low affinity IgG immunoreceptor FcγRIIA (15). Increasing evidence suggests that the collagen receptor underlying the major increase in tyrosine phosphorylation in platelets also signals like an immune receptor. The collagen receptor is believed to comprise a multimeric structure, containing the glycoprotein VI (GPVI), and the Fc receptor (FcR) γ-chain (16–19). Binding of collagen to GPVI induces tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif in the cytoplasmic tail of FcR γ-chain (18), leading to tyrosine phosphorylation of Syk and

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§ A Wellcome Prize Student. To whom correspondence should be addressed: Dept. of Pharmacology, Mansfield Rd., Oxford OX1 3QT, UK. Tel.: 44-1865-271590; Fax: 44-1865-271853; E-mail: barbara.gross@users.jesus.ox.ac.uk.

§§ A British Heart Foundation Senior Research Fellow.

[1] The abbreviations used are: SH2, Src homology 2; CRP, collagen-related peptide; GST, gluthatione *S*-transferase; FcR, Fc receptor; PLC-γ, phospholipase C-γ; TCR, T cell receptor; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; PVDF, polyvinylidene difluoride; GPVI, glycoprotein VI.

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PLC-γ_2 (20).

SLP-76 was recently reported to be a crucial adapter protein in collagen-stimulated platelets, since aggregation and tyrosine phosphorylation of PLC-γ_2 in response to collagen is abolished in SLP-76-deficient platelets.[2] In this study, we have investigated the mechanism of tyrosine phosphorylation of SLP-76 and the function of SLP-76 in platelets following stimulation by a collagen-related peptide (CRP), through the specific binding to GPVI (22). CRP is a synthetic, triple helical peptide composed of Gly-Pro-hydroxyproline repeats, cross-linked by cysteines at the N and C termini (23). CRP activates platelets through the GPVI but, in contrast to collagen, is unable to bind the integrin $\alpha_2\beta_1$ (23, 24). It is a more powerful agonist than collagen and exhibits less variation in response between individuals.

MATERIALS AND METHODS

Antibodies and Reagents—A CRP (GCP*(GPP)$_{10}$GCP*G; single amino acid code P* represents hydroxyproline) was cross-linked via cysteine residues as described previously (23); CRP was kindly donated by Dr. M. Barnes (Cambridge, UK). Collagen (native collagen fibrils from equine tendons) was from Nycomed (Munich, Germany). FcγRIIA-specific monoclonal antibody (mAb) was purchased from Madarex Inc (Annandale, NJ). Sheep F(ab′)$_2$ raised against mouse IgG (M-1522) and thrombin were purchased from Sigma (Poole, UK). Fura-2/AM was from Molecular Probes, Inc. (Eugene, OR). Anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology, Inc. (TCS Biologicals Ltd., Botolph Claydon, UK); polyclonal anti-Lyn antibody, Lyn(44), and polyclonal anti-Fyn antibody, Fyn(FYN3), were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-SLP-76 mAb and the GST-SLP-SH2 were described previously (1), and polyclonal anti-SLAP-130 rabbit antiserum was described previously (8). Polyclonal anti-FcR γ-chain rabbit antiserum was a gift from Dr. J. P. Kinet (Beth Israel Hospital, Boston, MA). PP1 (CP118,556) was kindly donated by Dr. J. Hanke (Pfizer Central Research, CT). The Syk-deficient mice and murine anti-Syk antiserum were described previously (25). The SLP-76 knock-out mice were previously described (12). The Lyn knock-out mice were a gift from Dr. A. Dunn (Ludwig Institute, Melbourne, Australia) (26). The Fyn knock-out mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Preparation and Stimulation of Platelets—Human platelets were isolated from blood taken on the day of the experiment as described previously (27). Mouse platelets were prepared as described previously (20). Stimulations were performed at 37 °C in the presence of 1 mM EGTA and 10 μM indomethacin with continuous stirring at 1,200 rpm. Platelets were stimulated with 3 μg/ml CRP, 30 μg/ml collagen, or 1 unit/ml thrombin for 90 s. Platelets were stimulated via FcγRIIA using mAb IV.3 (1 μg/ml) for 1 min and then the cross-linker F(ab′)$_2$ (30 μg/ml) for 90 s.

GST Precipitation, Immunoprecipitation, and Immunoblotting—Platelets were lysed with an equal volume of lysis buffer (2% Nonidet P-40, 300 mM NaCl, 20 mM Tris, 10 mM EDTA containing 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml pepstatin A, pH 7.3). Insoluble cell debris was removed by centrifugation. Cell lysates were precleared with glutathione-agarose or Protein A-Sepharose for GST precipitation and immunoprecipitation, respectively. For some experiments, antibodies were covalently linked to Protein A-Sepharose as described previously (28). For GST precipitation, lysates were incubated with 5 μg of fusion protein immobilized on agarose. Endogenous SLP-76 was immunoprecipitated using 4 μg of anti-SLP-76 mAb. The resulting protein complexes and immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblotting was carried out as described previously (24) with protein detection by enhanced chemiluminescence. Sequential immunoprecipitation was performed following kinase assay as described previously (29).

In Vitro Kinase Assay—Protein immunoprecipitations or precipitations were submitted to kinase assay as described (16). Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were treated with 1 M KOH for 1 h at 55 °C to cleave serine/threonine phosphorylation and then subjected to autoradiography and immunoblotting.

GST Fusion Proteins—Wild type SLP-76 (1) and the triple mutant SLP-76 YYY/FFF (5) were used as template for polymerase chain reaction using the following primers: SLP-TyrF (5′-ATT GGA TCC GGG GGT TGG TCG TCC TTT GAA-3′) and SLP-TyrR (5′-ATT CCC GGG GCT TCC TCG TCA TTG GAG GG-3′). The polymerase chain reaction products were expressed in pGEX-2T (Amersham Pharmacia Biotech, St. Albans, UK) as described previously (30).

Measurements of Cytosolic Ca^{2+} Levels—Mouse platelets (10^8 cells/ml) were loaded with 1 μM Fura-2/AM for 30 min at 37 °C in RPMI 1640 medium containing 1% fetal calf serum and resuspended at 2×10^8 cells/ml in Hepes-buffered salt solution (135 mM NaCl, 5 mM KCl, 10 mM Hepes, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$) containing 1% fetal calf serum. Stimulation and measurements were performed at 37 °C. Cytosolic Ca^{2+} levels were measured as described previously (31). The intracellular Ca^{2+} concentration was estimated using the equation described by Grynkiewicz *et al.* (32): $(R - R_{min})/(R_{max} - R) \times K$, where K represents the value $K_d \times$ Sf2/Sb2. The experimentally determined value of K used in this study is 3.6 μM.

RESULTS

SLP-76 Is Involved in Early Signaling Events Induced by CRP—We have previously reported that FcγRIIA cross-linking stimulates marked tyrosine phosphorylation of SLP-76 in human platelets (15), a result that is confirmed in the present study (Fig. 1*A*). Collagen was also observed to stimulate marked tyrosine phosphorylation of SLP-76, in agreement with the observation in mouse platelets,[2] whereas the G protein-coupled receptor agonist thrombin induced only a low level of tyrosine phosphorylation of SLP-76 (Fig. 1*A*). CRP, a synthetic peptide based on the triple-helical structure of collagen, also stimulated tyrosine phosphorylation of SLP-76 (Fig. 1*A*). Reprobing the membrane for SLP-76 to check loading revealed that the anti-SLP-76 antibody has a better affinity for the unphosphorylated form of SLP-76 (Fig. 1*A*).

Further studies were performed with CRP, since this is a powerful agonist for GPVI but is unable to bind to $\alpha_2\beta_1$ (23, 24). Tyrosine phosphorylation of SLP-76 by CRP occurred within 10 s and reached a maximum at 60 s, being sustained for up to 10 min (Fig. 1*B*). The time course showing tyrosine phosphorylation of total platelet protein indicated that a major band of 75 kDa displayed the same tyrosine phosphorylation pattern as SLP-76 (not shown). SLP-76 was identified as a component of this band. We have previously shown that Syk is also a component of this band. Pretreatment of platelets with the Ca^{2+} chelator bis(*O*-aminophenoxy)-*N*,*N*,*N*′,*N*′-tetraacetic acid and the protein kinase C antagonist Ro 31-8220 to inhibit the action of the second messengers produced by PLC-γ_2 indicated that tyrosine phosphorylation of SLP-76 is independent of PLC-γ_2 activation (Fig. 1*A*).

SLP-76 Is Associated with Lyn and a 130-kDa Phosphoprotein following Platelet Stimulation—SLP-76 co-immunoprecipitated with a tyrosine phosphoprotein of 130 kDa following stimulation by CRP, collagen, and thrombin (Fig. 1*A*). Cross-linking of FcγRIIA only induces a small increase in phosphorylation of this 130-kDa phosphoprotein. SLAP-130, which associates with the SH2 domain of SLP-76 following TCR engagement (8), and PLC-γ_2, the only PLC-γ isoform to be tyrosine-phosphorylated in CRP and collagen-stimulated platelets (24, 33), have a similar electrophoretic mobility to this tyrosine-phosphorylated protein of 130 kDa. However, subsequent immunoblotting with SLAP-130- or PLC-γ_2-specific antibodies failed to identify conclusively the 130-kDa co-precipitated protein, possibly because the level of protein was below that of the sensitivity of detection. The presence of SLAP-130 in the 130-kDa band was subsequently shown by sequential immunoprecipitations as described below.

Immunoprecipitation of SLP-76 using an antibody covalently linked to Protein A-Sepharose revealed the presence of a ty-

[2] Clements, J. L., Lee, J. Ran, Gross, B., Yang, B., Olson, J. D., Sandra, A., Watson, S., Lentz, S. R., and Koretzky, G. A., (1999) *J. Clin. Invest.* **103,** 19–25

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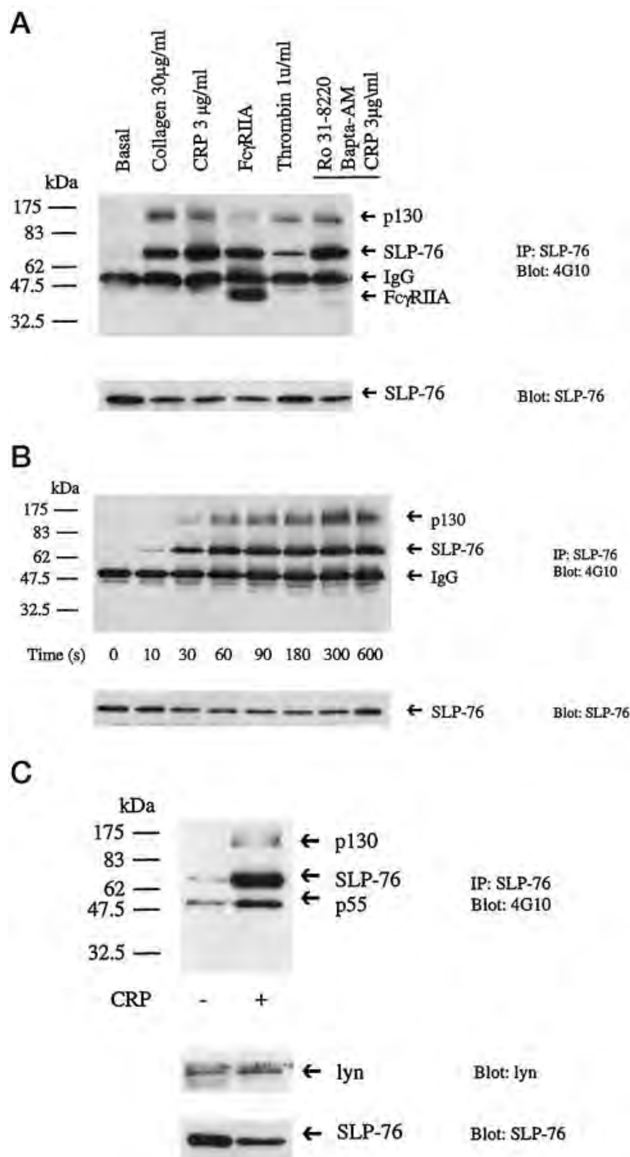

FIG. 1. **SLP-76 represents a major tyrosine-phosphorylated protein in stimulated platelets.** *A*, platelets were incubated in Tyrode-Hepes buffer and stimulated by the addition of 30 μg/ml collagen for 90 s, 3 μg/ml CRP for 90 s, anti-FcγRIIA mAb IV.3 (1 μg/ml) for 1 min followed by cross-linker F(ab$'$)$_2$ 30 μg/ml for 90 s and 1 unit/ml thrombin for 90 s. Platelets were preincubated with 10 μM Ro 31–8220 and 40 μM bis(*O*-aminophenoxy)-*N*,*N*,*N'*,*N'*-tetraacetic acid 5 min prior to CRP stimulation. Stimulation was stopped by the addition of an equal volume of Nonidet P-40 lysis buffer. SLP-76 was immunoprecipitated from precleared lysate using the anti-SLP-76 mAb. Immunoprecipitated proteins were separated by 10% SDS-PAGE, electroblotted onto PVDF membranes, and detected by immunoblotting using the anti-phosphotyrosine mAb 4G10. Membranes were stripped and reprobed for SLP-76 using the anti-SLP-76 mAb (*bottom*). *B*, platelets were stimulated with 3 μg/ml CRP for the time indicated. Immunoprecipitated SLP-76 was analyzed on 10% SDS-PAGE and immunoblotted using anti-phosphotyrosine mAb 4G10. Membranes were stripped, and equal loading was checked by immunoblotting with the anti-SLP-76 mAb (*bottom*). *C*, platelets were stimulated with 3 μg/ml CRP for 90 s or left untreated. Proteins were immunoprecipitated with an anti-SLP-76 mAb covalently linked to Protein A-Sepharose, and proteins were separated on 10% SDS-PAGE and immunoblotted using the anti-phosphotyrosine mAb 4G10. Membranes were stripped and immunoblotted with the anti-Lyn polyclonal antibody (*middle*) and using the anti-SLP-76 mAb (*bottom*).

rosine-phosphorylated band of approximately 55 kDa (Fig. 1*C*) that was sometimes resolved as a doublet. This band was hidden by the IgG heavy chain band under standard immuno-

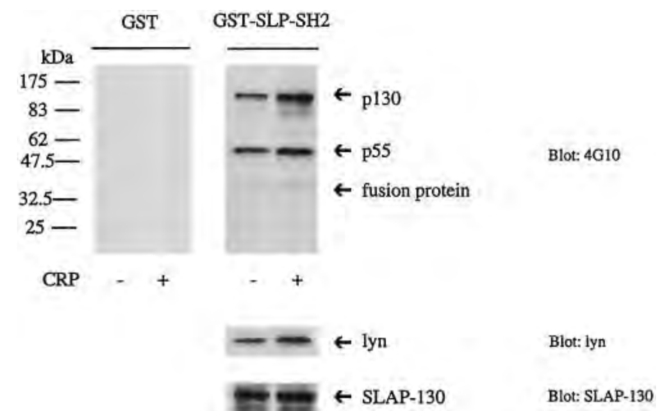

FIG. 2. **Lyn and SLAP-130 associate to the SH2 domain of SLP-76.** Platelets were stimulated with 3 μg/ml CRP for 90 s. The precleared lysate was incubated with 5 μg of GST-SLP-SH2 or GST alone (control). Precipitated proteins were resolved on 10% SDS-PAGE and immunoblotted using the anti-phosphotyrosine mAb 4G10. Membranes were stripped and immunoblotted using the anti-Lyn polyclonal antibody (*middle panel*) and using the anti-SLAP-130 antiserum (*bottom panel*).

precipitation conditions. The 55-kDa band was present in resting platelets, and its level of tyrosine phosphorylation increased with stimulation of platelets by CRP. It was identified as the tyrosine kinase Lyn by immunoblotting (Fig. 1*C*). There was a small increase in association with SLP-76 following CRP stimulation, although this was less than the increase in tyrosine phosphorylation. Immunoblotting for Grb2 revealed the presence of a similar level of the adapter protein co-immunoprecipitating with SLP-76 in resting and CRP-stimulated platelets (not shown). This is likely to be mediated through the SH3 domain of Grb2 as previously shown in FcγRIIA-stimulated platelets (15).

SLP-76 Is Associated with Lyn and SLAP-130 through Its SH2 Domain—We used a fusion protein containing the SH2 domain of SLP-76, GST-SLP-SH2, to locate the binding site of Lyn and the component(s) of the 130-kDa protein. GST alone, used as control, did not bind any tyrosine-phosphorylated proteins (Fig. 2). GST-SLP-SH2 precipitates two major tyrosine-phosphorylated proteins of 55 and 130 kDa (Fig. 2) in resting platelets. Stimulation of platelets by CRP strongly increased the degree of tyrosine phosphorylation of 130-kDa protein that is precipitated by GST-SLP-SH2, whereas the level of phosphorylation of the 55-kDa protein underwent a small increase. Reprobing revealed Lyn and SLAP-130 as components of these bands, respectively (Fig. 2). There was a small increase in the association of Lyn and SLAP-130 in CRP-stimulated lanes (Fig. 2). Association of SLAP-130 to SLP-76 under basal conditions was also reported in T cells (8). Minor tyrosine-phosphorylated proteins of 90 and 75 kDa were also associated with GST-SLP-SH2 in CRP-stimulated platelets. The 75-kDa band was not identified as Syk or Btk by immunoblotting, although either or both may be below the detection sensitivity of the antibodies.

SLP-76 Tyrosine-phosphorylated in Vitro by a Member of the Src Kinase Family—Comparison of autoradiographs of kinase assays of immunoprecipitated SLP-76 before and after KOH treatment indicated the absence of serine/threonine kinase co-immunoprecipitating with SLP-76 in resting and CRP-stimulated platelets (not shown). Three major bands of 130, 75, and 55 were phosphorylated in SLP-76 immunoprecipitates from resting platelets (Fig. 3*A*). The three bands of 130, 75, and 55 kDa correspond to the major tyrosine-phosphorylated bands observed in SLP-76 immunoprecipitates when immunoblotted for phosphotyrosine (Fig. 3*A*). The level of tyrosine phosphorylation of the 130-, 75-, and 55-kDa bands increased dramatically in SLP-76 immunoprecipitates from CRP-stimulated platelets.

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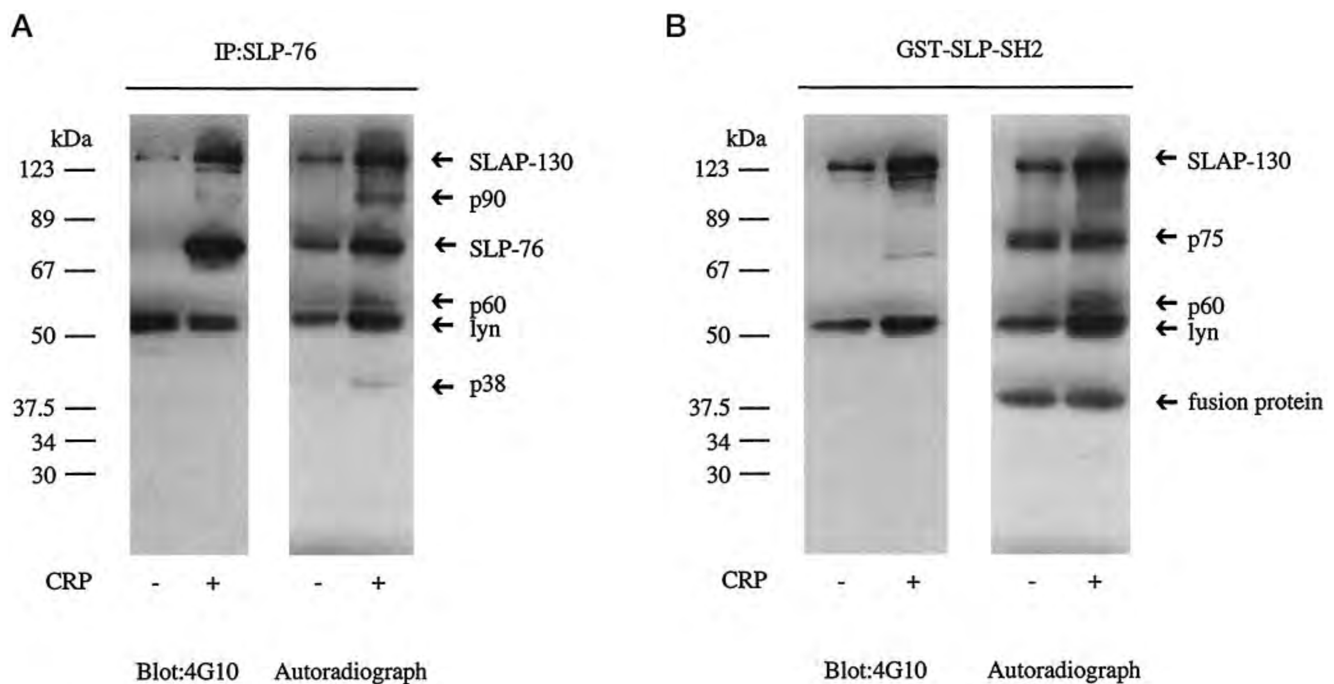

FIG. 3. ***In vitro* tyrosine kinase activity is detected in SLP-76 immunoprecipitates and GST-SLP-SH2 precipitates.** *A*, SLP-76 was immunoprecipitated from resting and stimulated platelets with 3 μg/ml CRP for 90 s. *B*, 5 μg of GST-SLP-SH2 were used for protein precipitation from lysate of resting or CRP-stimulated platelets. Immunoprecipitated and precipitated proteins were submitted to *in vitro* kinase assay and separated on 10–18% gradient SDS-PAGE and transferred to PVDF membranes. Membranes were immunoblotted using the anti-phosphotyrosine mAb 4G10. Membranes were then incubated in 1 M KOH for 1 h at 55 °C before autoradiography.

CRP stimulation also induced the appearance of three other minor tyrosine-phosphorylated bands of 90, 60, and 38 kDa following kinase assay. On a longer exposure, a doublet of 13/11.5 kDa could also be seen in CRP-stimulated samples (not shown). The 90-kDa band was also seen by anti-phosphotyrosine immunoblotting in Fig. 3*A*, while the 60-kDa band could be seen in a longer exposure. The 75-kDa band was identified as SLP-76 by immunoblotting. The remaining proteins were identified through sequential immunoprecipitation. Following *in vitro* kinase assay, the proteins co-immunoprecipitating with SLP-76 were dissociated by boiling in the presence of 2% SDS. Supernatant was diluted down to 0.1% SDS, and proteins from the supernatant were immunoprecipitated with specific antibodies. Components of the 13/11.5-, 55-, 60-, 90-, and 130-kDa radiolabeled bands were identified as FcR γ-chain (Fig. 4*A*), Lyn (Fig. 4*B*), Fyn (Fig. 4*C*), Vav (Fig. 4*D*), and SLAP-130 (Fig. 4*E*), respectively. A more prominent association with Fyn was seen in some studies as illustrated in Fig. 5. The weak band corresponding to Vav is likely to reflect both a low level of binding and the fact that it serves as a poor substrate in the *in vitro* kinase assay. Neither Syk nor PLC-γ_2 were detected in these studies, suggesting that they do not associate with SLP-76 or that PLC-γ_2 is not a substrate in the *in vitro* kinase assay.

A kinase assay was also performed on proteins precipitated with GST-SLP-SH2. This showed a similar profile of kinase activity as SLP-76 immunoprecipitates, except for the presence of a 38-kDa protein, an uncharacterized doublet of 75 kDa and the lack of SLP-76 (Fig. 3*B*). The tyrosine-phosphorylated band of 38 kDa represented the fusion protein that was labeled during the process. Bands of 130, 75, and 53/56 kDa were tyrosine-phosphorylated under basal conditions. The bands of 130 and 53/56 kDa correspond to SLAP-130 and Lyn, respectively, and both proteins were found to undergo a further increases in phosphorylation following stimulation (Fig. 3*B*). A weaker band of 60 kDa could be visualized by immunoblotting for tyrosine phosphorylation on longer exposures in CRP-stimulated platelets (Fig. 3*B*). This band has a similar electrophoretic mobility to Fyn. The 75-kDa doublet displayed a similar level of tyrosine phosphorylation in resting and CRP-stimulated platelets.

Immunoprecipitation of SLP-76, followed by kinase assay, was performed on platelet lysates using a milder detergent, Brij 96 (compared with Nonidet P-40). This revealed a marked increase in *in vitro* tyrosine phosphorylation of FcR γ-chain (Fig. 5) in CRP stimulated samples. The intensity of the band corresponding to Lyn in Brij 96 immunoprecipitation is lower than with Nonidet P-40, in agreement with the decrease of Lyn as shown by immunoblotting with anti-Lyn antibody (not shown). A similar result is seen for Fyn. The reduction in the level of these two kinases is in contrast to the increase in tyrosine phosphorylation of the FcR γ-chain, suggesting that the latter is a consequence of a greater amount of protein in the immunoprecipitate.

SLP-76 co-immunoprecipitates with two members of the Src kinase family, Lyn and Fyn, either or both of which could mediate the increase in tyrosine phosphorylation observed in the *in vitro* kinase assays. In order to investigate this, PP1, an inhibitor specific to Src kinases (34), was added to the kinase assay. In the presence of 10 μM PP1, *in vitro* tyrosine phosphorylation of SLP-76 and its co-immunoprecipitated proteins was abrogated under basal and CRP-stimulated conditions in Nonidet P-40 or Brij 96 lysates (Fig. 5).

Tyrosine Phosphorylation of SLP-76 Is Abolished in Syk-deficient Platelets—The identity of the kinases underlying phosphorylation of SLP-76 was investigated in knock-out mouse platelets. Immunoprecipitation studies showed that SLP-76 is phosphorylated in mouse platelets and that it associates with the same profile of tyrosine-phosphorylated proteins as seen with human SLP-76 (not shown). The increase in tyrosine phosphorylation of SLP-76 by CRP was not altered in Lyn- or Fyn-deficient mouse platelets (Fig. 6, *A* and *B*). *In vitro* phosphorylation of SLP-76, following immunoprecipitation of these samples, was dramatically reduced in Lyn-deficient

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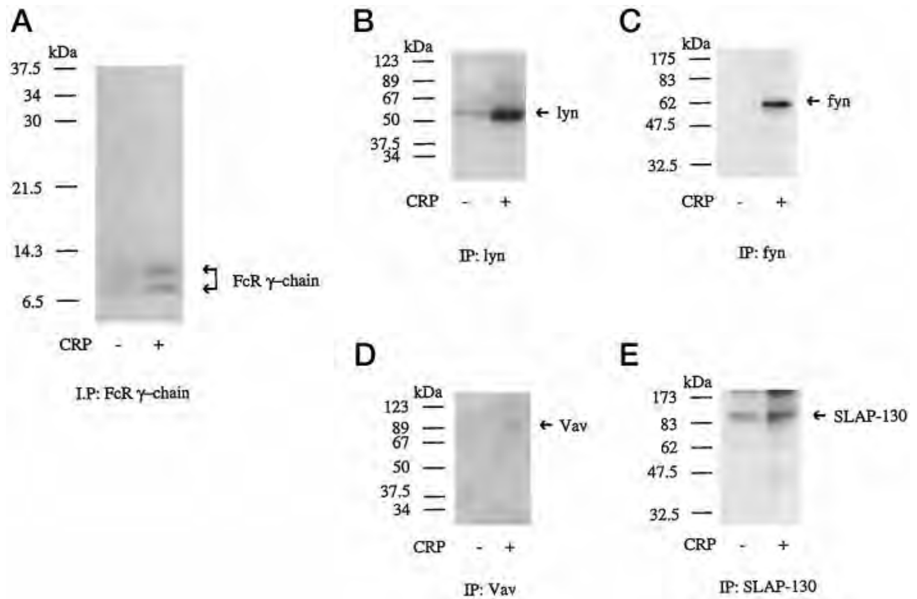

FIG. 4. **FcR γ-chain, Lyn, Fyn, Vav, and SLAP-130 co-immunoprecipitate with SLP-76 following CRP stimulation.** SLP-76 was immunoprecipitated as described in the legend of Fig. 3. Following an *in vitro* kinase assay, immunoprecipitated proteins were parted in lysis buffer containing 2% SDS. Proteins were heated, and subsequently the SDS concentration of the supernatant was reduced to 0.1% by the addition of Nonidet P-40 lysis buffer. Lysates were used for immunoprecipitation of FcR γ-chain (*A*), Lyn (*B*), Fyn (*C*), Vav (*D*), and SLAP-130 (*E*). Proteins were analyzed on 10% SDS-PAGE, expect for FcR γ-chain immunoprecipitation, which was resolved on 10–18% gradient SDS-PAGE. SDS-polyacrylamide gels were dried and exposed to autoradiography.

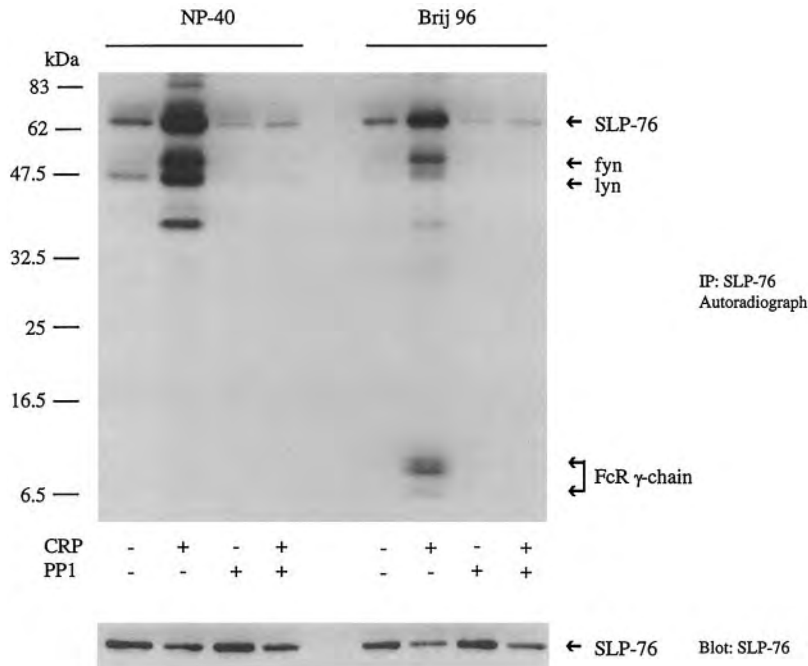

FIG. 5. **Effect of PP1 on *in vitro* kinase assay performed on SLP-76 immunoprecipitated from resting or CRP-stimulated platelets lysed in Nonidet P-40- or Brij 96-containing buffer.** Stimulation of platelets with Tyrode-Hepes buffer or 3 μg/ml CRP was stopped after 90 s by the addition of Nonidet P-40 lysis buffer or Brij 96 lysis buffer. SLP-76 was immunoprecipitated and submitted to an *in vitro* kinase assay. When indicated, kinase assay was performed in the presence of 10 μM PP1. Proteins were resolved on 10–18% gradient SDS-PAGE and transferred to PVDF membranes. Membranes were treated with 1 M KOH for 1 h at 55 °C and submitted to autoradiography (*upper panel*). Equal loading was checked by immunoblotting the membranes using the anti-SLP-76 mAb (*bottom panel*).

platelets (Fig. 6*A*) but hardly altered in Fyn-deficient platelets (Fig. 6*B*). This suggests that *in vitro* tyrosine phosphorylation is mainly caused by Lyn but that this kinase is unlikely to be responsible for mediating *in vivo* phosphorylation of SLP-76 following stimulation by CRP.

The role of Syk in SLP-76 tyrosine phosphorylation was carried out by the study of platelets from Syk-deficient mice. In platelets of control mice, SLP-76 exhibited an increase in tyrosine phosphorylation following CRP stimulation, which was abrogated in CRP-stimulated platelets from Syk-deficient mice (Fig. 7). An increase in tyrosine phosphorylation of the 130-kDa protein upon CRP activation could also be seen with longer exposures, and this was also lost in the Syk-depleted cells (data not shown).

SLP-76 possesses three N-terminal tyrosine phosphorylation sites, Tyr^{113}, Tyr^{128}, and Tyr^{145}. This N-terminal region (amino acids 103–154) was expressed as a GST fusion protein (GST-Tyr-WT) and was used as a substrate in kinase assays performed on Lyn, Fyn, and Syk immunoprecipitates. A fusion protein with the three tyrosine phosphorylation sites mutated to phenylalanine (GST-3Tyr-Mut) was used as a control. Two proteins of 75 and 32 kDa were tyrosine-phosphorylated in the Syk kinase assays, corresponding to Syk and GST-Tyr-WT, respectively (Fig. 8*A*). A small increase in Syk autophosphorylation and tyrosine phosphorylation of the fusion protein was observed in stimulated conditions relative to basal level. A similar level of tyrosine phosphorylation of the two proteins was observed in the presence of PP1 (not shown). No tyrosine phosphorylation of GST-3Tyr-Mut was detected in the Syk kinase assay from resting and CRP-stimulated platelets (Fig. 8*A*). This indicates that tyrosine phosphorylation of GST-Tyr-WT by Syk is specific to one or more of Tyr^{113}, Tyr^{128}, and Tyr^{145}. In contrast, GST-Tyr-WT was weakly phosphorylated by Lyn and Fyn, whereas both kinases underwent dramatic autophosphorylation (Fig. 8, *B* and *C*), which was abolished in the presence of PP1 (not shown). GST-3Tyr-Mut was not tyrosine-phosphorylated in the Lyn and Fyn kinase assays. This indicates that tyrosine phosphorylation of the N-terminal ty-

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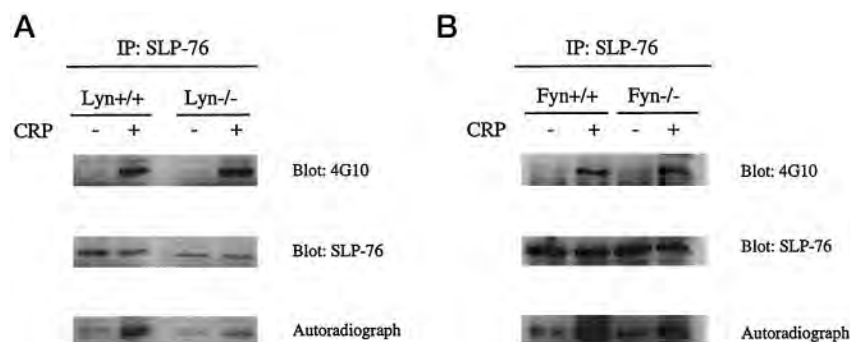

FIG. 6. ***In vivo* tyrosine phosphorylation of SLP-76 was not altered in Lyn- or Fyn-deficient platelets, but *in vitro* tyrosine phosphorylation of SLP-76 was reduced in Lyn-deficient platelets.** Platelets from control or Lyn −/− (*A*) or Fyn −/− (*B*) mice were stimulated with CRP for 90 s. The reaction was stopped by the addition of Nonidet P-40 lysis buffer. SLP-76 was immunoprecipitated from these lysates and submitted to *in vitro* kinase assay. Proteins were resolved on 10% SDS-PAGE and immunoblotted using the anti-phosphotyrosine mAb 4G10 (*A* and *B*, *upper part*). Membranes were stripped and reprobed using the anti-SLP-76 mAb (*A* and *B*, *middle part*). Autoradiographs of the membranes are shown in the *bottom part* of *A* and *B*.

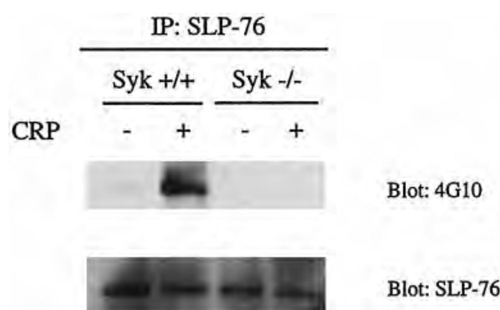

FIG. 7. **Tyrosine phosphorylation of endogenous SLP-76 is dependent on Syk activity.** SLP-76 was immunoprecipitated from control wild-type or Syk-deficient platelets that were resting or were stimulated with 3 μg/ml CRP for 90 s. Immunoprecipitated proteins were separated on 10–18% gradient SDS-PAGE and immunoblotted using the anti-phosphotyrosine mAb 4G10 (*upper panel*). Membranes were stripped and reprobed using the anti-SLP-76 mAb (*bottom panel*).

rosine-rich region of SLP-76 is mediated by Syk with only a very minor contribution from Lyn and Fyn.

Tyrosine Phosphorylation of both PLC-γ_2 and SLAP-130 Is Lost in SLP-76-deficient Mouse Platelets—The role of SLP-76 in tyrosine phosphorylation of PLC-γ_2 and SLAP-130 was investigated in SLP-76-deficient platelets. A similar profile of tyrosine phosphorylation was observed in basal and CRP-stimulated samples from wild-type (+/+) and heterozygous (+/−) platelets, whereas there was a marked decrease in phosphorylation of proteins of 130 and 75 kDa in the SLP-76-deficient (−/−) cells (not shown). The reduction in the 75-kDa protein is likely to be due to the absence of SLP-76, which migrates in this area. The remaining increase in phosphorylation of this band is likely to be due to phosphorylation of Syk, which co-migrates with SLP-76. This was confirmed by immunoprecipitation of the kinase (Fig. 9*A*). The 130-kDa band migrates in the region of SLAP-130 and PLC-γ_2. Phosphorylation of PLC-γ_2 was dramatically reduced in response to CRP in SLP-76-deficient platelets (Fig. 9*B*), although a residual increase could be seen on a longer exposure (not shown). SLAP-130 was tyrosine-phosphorylated in resting platelets, and the level of phosphorylation increased following stimulation by CRP. The level of phosphorylation of SLAP-130 under basal conditions was not altered in the SLP-76-deficient platelets, whereas the increase induced by CRP was abolished (Fig. 9*C*).

The functional consequence of the reduction in PLC-γ_2 phosphorylation was monitored through measurement of intracellular Ca^{2+} in Fura-2/AM-loaded platelets. CRP was unable to elevate Ca^{2+} in SLP-76-deficient platelets (Fig. 10*A*) in contrast to the robust response in control cells. Ca^{2+} mobilization in response to the G protein-coupled receptor agonist thrombin was unaltered (Fig. 10*B*) in SLP-76-deficient platelets, suggesting that SLP-76 plays a crucial function downstream of immunoreceptor tyrosine-based activation motif-containing receptor.

DISCUSSION

Here we report that FcγRIIA, CRP, and collagen stimulate dramatic tyrosine phosphorylation of SLP-76 in contrast to the G protein-coupled receptor agonist thrombin. This suggests that this adapter protein has a specific role in immunoreceptor tyrosine-based activation motif-mediated signaling. This is consistent with the absence of other reports of phosphorylation of SLP-76 by G protein-coupled receptor agonists.

Tyrosine phosphorylation of SLP-76 is one of the earliest of the events following CRP stimulation and is sustained for up to 10 min. This indicates that SLP-76 may be involved in initial events of the signal transduction pathway induced by CRP. This is consistent with the fact that tyrosine phosphorylation of SLP-76 was maintained in the presence of the Ca^{2+} chelator bis(*O*-aminophenoxy)-*N*,*N*,*N′*,*N′*-tetraacetic acid and a protein kinase C antagonist Ro 31-8220, a combination designed to inhibit events downstream of PLC-γ_2, following CRP stimulation.

SLP-76 was reported to be tyrosine-phosphorylated by ZAP-70 or Syk in T cells and rat basophilic leukemia cells (9, 14), respectively. Consistent with this, tyrosine phosphorylation of SLP-76 induced by CRP was abrogated in Syk-deficient platelets. However, we were not able to detect *in vivo* association between Syk and SLP-76 following immunoprecipitation, suggesting an indirect, unstable, or weak interaction between the two proteins.

SLP-76 contains three N-terminal tyrosine phosphorylation sites including two consensus sequences (pYESP; where pY represents phosphotyrosine) for association to the SH2 domain of Vav. SLP-76 also contains a proline-rich region that associates with the SH3 domain of Grb2 and an SH2 domain that associates to tyrosine-phosphorylated proteins. Immunoprecipitation of SLP-76 revealed a marked association with the Src kinase Lyn and a phosphotyrosyl protein of 130 kDa. One component of this 130-kDa phosphoprotein was identified as SLAP-130 through sequential immunoprecipitation following kinase assay. This strategy also revealed a lower level of binding of other proteins with SLP-76, namely Vav, Fyn, and FcR γ-chain. SLP-76 has been reported to co-immunoprecipitate with SLAP-130 and an uncharacterized protein of 62 kDa, as well as a serine/threonine kinase in rat basophilic leukemia cells (14) and T cells (7, 8). No evidence for association of the latter two proteins was found in the present study. However, SLAP-130, Lyn, and Fyn were found to interact with the SH2

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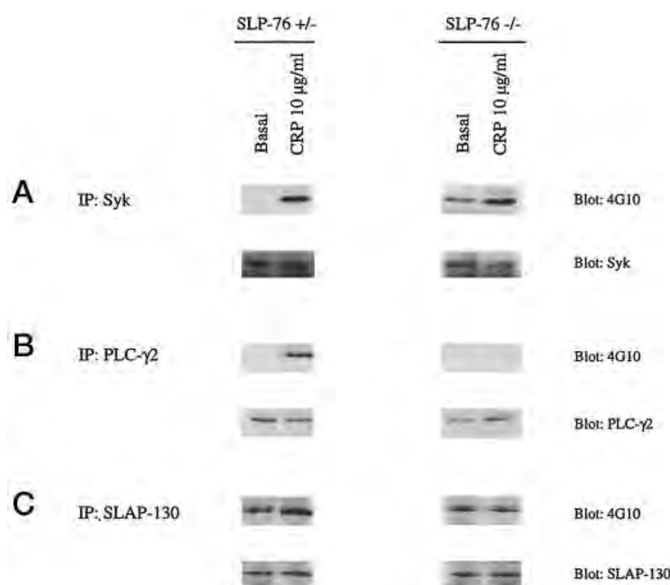

FIG. 9. **Tyrosine phosphorylation of PLC-γ_2 and SLAP-130 is inhibited following CRP stimulation of SLP-76-deficient platelets.** Syk (*A*), PLC-γ_2 (*B*), or SLAP-130 (*C*) were immunoprecipitated from resting or CRP-stimulated control (SLP-76 +/−) and SLP-76-deficient (SLP-76 −/−) mice platelets. Proteins were separated on 10% SDS-PAGE, electroblotted to PVDF, and immunoblotted using the anti-phosphotyrosine mAb 4G10 (*A–C*, *upper part*). Membranes were stripped and reprobed using the anti-Syk rabbit antiserum (*A*, *bottom part*), the anti-PLC-γ_2 polyclonal antibody (*B*, *bottom part*), and the anti-SLAP-130 rabbit antiserum (*C*, *bottom part*).

domain of SLP-76 expressed as a fusion protein. It is unclear whether the association of FcR γ-chain also occurs through this region, because radiolabeled incomplete GST fusion protein products co-migrated in this region of the gel. Vav did not associate with the SH2 domain of SLP-76, consistent with an interaction occurring between the SH2 domain of Vav and the N-terminal tyrosine-phosphorylated sites as shown in stimulated T cells (2–5).

Kinase assays performed on immunoprecipitated SLP-76 from CRP-stimulated platelets lysed with the mild detergent Brij 96 exhibited a stronger tyrosine phosphorylation of FcR γ-chain than in Nonidet P-40 lysates following CRP stimulation. This is in contrast with the lower level of co-immunoprecipitating Lyn and Fyn found in Brij 96 lysates. The interaction with the FcR γ-chain could be direct or indirect, since Fyn and Lyn were recently reported to associate with FcR γ-chain irrespective of stimulation by collagen (35). In the latter case, the increase in the level of FcR γ-chain in Brij 96 suggests either that the interaction is more stable in Brij 96 compared with Nonidet P-40 or that there is a selective solubilization of a pool of Lyn and/or Fyn associated with FcR γ-chain. In Nonidet P-40, SLP-76 could co-immunoprecipitate at least two pools of Src tyrosine kinases, a pool associated with FcR γ-chain and a second pool possibly associated with downstream events. Lyn and Fyn are likely to tyrosine-phosphorylate FcR γ-chain in the

FIG. 8. **Syk, but not Lyn and Fyn, *in vitro* phosphorylates Tyr^{113}, Tyr^{128}, and Tyr^{145} from SLP-76.** Syk (*A*), Lyn (*B*), and Fyn (*C*) were immunoprecipitated under basal or CRP-stimulated conditions and subjected to an *in vitro* kinase assay. 5 μg of GST-Tyr-WT, GST-3Tyr-Mut, or GST alone were added in the kinase assay. Kinase reactions were analyzed on 10% SDS-PAGE and electroblotted on PVDF membranes. Ser/Thr phosphorylation was removed by treating the membranes with 1 M KOH at 55 °C for 1 h. The *upper part* of each *panel* shows an autoradiograph of the kinase assay. Membranes were immunoblotted using anti-Syk (*A*, *middle part*), anti-Lyn (*B*, *middle part*), and anti-Fyn (*C*, *middle part*) polyclonal antibodies. Fusion protein equal loading was checked by immunoblotting with anti-GST mAb (*A–C*, *lower part*).

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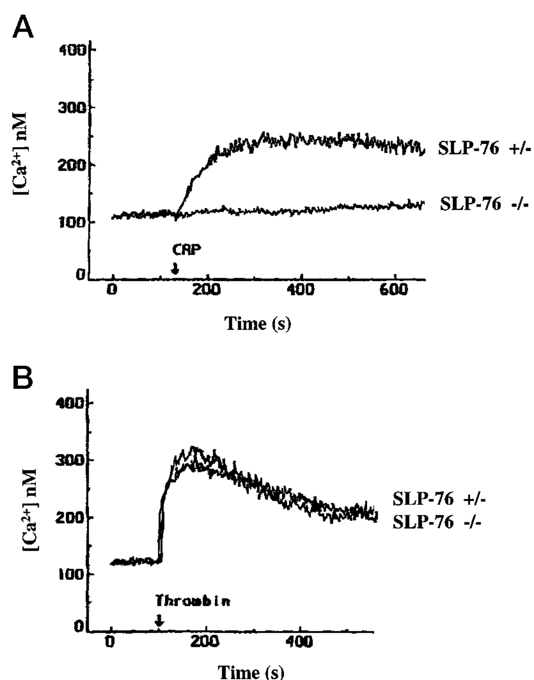

FIG. 10. **Ca^{2+} mobilization is inhibited in SLP-76-deficient platelets stimulated by CRP.** Mouse platelets were loaded with Fura-2/AM dye. Platelets were left unstimulated for 2 min and then stimulated with 3 μg/ml CRP (*A*) or 100 nM thrombin (*B*). Calculation of the cytosolic Ca^{2+} concentration from the fluorescence ratio (340/380 nm) was performed by the use of a calibration curve with Ca^{2+} standards. The *arrowheads* indicate the addition of agonist.

in vitro kinase assay, since the addition of PP1, the Src kinase inhibitor, abolished tyrosine phosphorylation.

In vitro tyrosine phosphorylation of SLP-76 was also abrogated in the presence of PP1 in both Nonidet P-40 and Brij 96 lysates following CRP stimulation. Lck was reported to be able to tyrosine-phosphorylate Tyr^{423}/Tyr^{426} within the SH2 domain of SLP-76, but this does not correlate with physiological mapping of tyrosine phosphorylation sites (Tyr^{113}, Tyr^{128}, and Tyr^{145}) of SLP-76 following TCR stimulation (9). Lyn and Fyn may also tyrosine-phosphorylate of SLP-76 on Tyr^{423}/Tyr^{426}, since the fusion protein for the SH2 domain of SLP-76 becomes tyrosine-phosphorylated *in vitro* as shown in Fig. 3. This was investigated further through the study of Lyn- and Fyn-deficient platelets. Tyrosine phosphorylation of SLP-76 in platelets from either knock-out mice was not altered when compared with control mice. However, *in vitro* phosphorylation was reduced in Lyn knock-out mice, suggesting this is the predominant active kinase in the assays. Reduction of *in vitro* tyrosine phosphorylation of SLP-76 but no change of *in vivo* phosphorylation strengthens the idea that Lyn and Fyn are not involved in phosphorylation of Tyr^{113}, Tyr^{128}, and Tyr^{145}. This was confirmed by very weak *in vitro* phosphorylation of a fusion protein containing these three tyrosine phosphorylation sites by immunoprecipitated Lyn or Fyn. In contrast, Syk displayed a much greater ability to phosphorylate this fusion protein in comparison with Lyn and Fyn, suggesting that Syk may phosphorylate this region *in vivo*.

The inability to detect *in vivo* association of Syk and SLP-76 suggests that other protein may bring the adapter molecule to the vicinity of the tyrosine kinase. One possibility is that this role is fulfilled by the T cell adapter protein LAT, which undergoes tyrosine phosphorylation in platelets and forms a complex with Grb2 and SLP-76 (15). A novel possibility is that SLP-76 is brought to Syk via binding to either Fyn or Lyn associated with the FcR γ-chain. A direct role of Lyn and Fyn in this way cannot be ruled out through the analysis of Lyn and Fyn knock-out platelets because of possible redundancy between members of the Src kinase family.

In this study, we show that tyrosine phosphorylation of PLC-γ_2 and Ca^{2+} mobilization are almost fully inhibited in response to CRP. This is consistent with the observations of Clements *et al.*[2] that aggregation and tyrosine phosphorylation of PLC-γ_2 in SLP-76-deficient platelets by collagen is abrogated. A similar result was observed with TCR-mediated tyrosine phosphorylation of PLC-γ_1 in T cells lacking SLP-76 (11). Tyrosine phosphorylation of PLC-γ_1 and PLC-γ_2 has also been shown to be downstream of tyrosine phosphorylation of Blnk in B cells (36). Tyrosine phosphorylation of Blnk following B cell stimulation is thought to recruit PLC-γ isoforms to Blnk, enabling phosphorylation by Syk, co-localized with Blnk. A similar model could apply for tyrosine phosphorylation of PLC-γ_2 in CRP-stimulated platelets. Residual tyrosine phosphorylation of PLC-γ_2 could be seen in CRP-stimulated SLP-76-deficient platelets, indicating the existence of a second, minor pathway leading to tyrosine phosphorylation of PLC-γ_2. This cannot be attributed to Blnk, since this is not expressed in platelets.[3] This might occur through a direct interaction between Syk and PLC-γ_2, similar to the interaction seen in B-cells (21, 37) or indirectly through LAT as suggested for residual phosphorylation of PLC-γ_1 in T cells (11).

SLAP-130 associated with the SH2 domain of SLP-76 in platelets. The function of SLAP-130 is not known. The relationship of the association between the SH2 domain of SLP-76 and SLAP-130 was investigated in SLP-76-deficient platelets. SLAP-130 was tyrosine-phosphorylated in resting platelets, and this level of phosphorylation was unaltered in SLP-76-deficient cells. The increase of tyrosine phosphorylation of SLAP-130 induced by CRP-stimulation, however, was inhibited in the absence of SLP-76. This suggests that tyrosine phosphorylation of SLAP-130 is regulated through two pathways, one SLP-76-independent pathway corresponding to phosphorylation in resting platelets and a SLP-76-dependent pathway involved in the increase of phosphorylation following CRP stimulation. These observations place SLAP-130 downstream of SLP-76 in CRP-induced signaling.

In conclusion, tyrosine phosphorylation of SLP-76 is downstream of tyrosine phosphorylation of Syk in CRP-stimulated platelets and upstream of phosphorylation of SLAP-130 and PLC-γ_2. SLP-76 co-immunoprecipitates with the tyrosine kinases Lyn and Fyn, and this interaction may be important for upstream as well as for downstream events. This work confirms SLP-76 as an important link between Syk activation and PLC-γ_2 regulation, although further work is required to establish a complete understanding of this pathway, including the role of SLAP-130.

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CELL BIOLOGY AND METABOLISM:

Tyrosine Phosphorylation of SLP-76 Is Downstream of Syk following Stimulation of the Collagen Receptor in Platelets

Barbara S. Gross, Jong Ran Lee, James L. Clements, Martin Turner, Victor L. J. Tybulewicz, Paul R. Findell, Gary A. Koretzky and Steve P. Watson

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