The Journal of Immunology

Syk- and Lyn-Dependent Phosphorylation of Syk on Multiple Tyrosines Following B Cell Activation Includes a Site That Negatively Regulates Signaling

Lakhu M. Keshvara, Christina C. Isaacson, Thomas M. Yankee, Radmila Sarac, Marietta L. Harrison and Robert L. Geahlen

J Immunol 1998; 161:5276-5283; ;
http://www.jimmunol.org/content/161/10/5276

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Syk- and Lyn-Dependent Phosphorylation of Syk on Multiple Tyrosines Following B Cell Activation Includes a Site That Negatively Regulates Signaling[1]

Lakhu M. Keshvara, Christina C. Isaacson, Thomas M. Yankee, Radmila Sarac, Marietta L. Harrison, and Robert L. Geahlen[2]

The Syk protein tyrosine kinase is an essential component of the B cell Ag receptor signaling pathway. Syk is phosphorylated on tyrosine following B cell activation. However, the sites that are modified and the kinases responsible for these modifications have yet to be determined. To approach this problem, we used a mapping strategy based on the electrophoretic separation of peptides on alkaline polyacrylamide gels to identify the tryptic phosphopeptides derived from metabolically labeled Syk. In this work, we report that Syk from activated B cells is phosphorylated principally on six tyrosines: one located between the tandem SH2 domains (Tyr^{130}); three in the linker region (Tyr^{317}, Tyr^{342}, and Tyr^{346}); and two in the catalytic domain (Tyr^{519} and Tyr^{520}). The linker region sites are the primary targets of the Src family protein tyrosine kinase, Lyn, and include a site that negatively (Tyr^{317}) regulates receptor signaling. Efficient phosphorylation of the catalytic domain and inter-SH2 domain tyrosines is catalyzed primarily by Syk itself, but only occurs to an appreciable extent in cells that express Lyn. We propose that these sites are phosphorylated following the binding of Syk to immunoreceptor tyrosine-based activation motif. ***The Journal of Immunology*****, 1998, 161: 5276–5283.**

Syk is a 72-kDa protein tyrosine kinase that participates in the transduction of signals initiated by the ligation of multiple hemopoietic cell surface receptors. These include Ag receptors, integrins, collagen receptors, and the Fc receptors FεRI, FcγRI, FcγRII, and FcγRIII (1–13). The importance of Syk to receptor-mediated signaling in hemopoietic cells is underscored by the signaling defects that adversely affect B cell differentiation, mast cell activation, and platelet activation in mice that lack Syk (5, 14, 15). In B cells, the pathway leading to the activation of Syk is initiated by the phosphorylation of a pair of tyrosines residing within a conserved motif, the ITAM[3] (immunoreceptor tyrosine-based activation motif) (16), located on the cytoplasmic domains of the receptor components Ig-α and Ig-β (17, 18). ITAM phosphorylation is initiated by the aggregation of surface Ig and is thought to be catalyzed by members of the Src family of protein tyrosine kinases. When phosphorylated, this domain functions as a docking site for the recruitment of Syk to the receptor: the interaction occurring between the ITAM phosphotyrosines and the tandem pair of SH2 domains located in the amino-terminal half of Syk (19). The binding of Syk to phospho-ITAMs results in both a relocalization of the kinase to the site of the aggregated receptor and an increase in its intrinsic kinase activity (20).

Much less is known of how Syk participates in the propagation of downstream signals following its activation. One important clue has been the observation that Syk becomes phosphorylated on tyrosine subsequent to receptor cross-linking (21, 22). These phosphorylations are characterized by an increase in the intrinsic activity of Syk (1, 22), a shift in its electrophoretic mobility when analyzed by SDS-PAGE, a change in receptor affinity (23), and the creation of docking sites for the binding of Syk-interacting proteins such as Lyn, Lck, PLC-γ, and Vav (24–27). While the importance of many individual tyrosines on Syk has been suggested through the use of site-directed mutants, no studies have fully addressed the more complicated issue of which tyrosines are actually phosphorylated under conditions that lead to the activation of Syk in intact B cells and which kinases are responsible for catalyzing these phosphorylations. The primary candidates for the kinases responsible for Syk phosphorylation are Syk itself and one or more members of the Src family.

Previously, we demonstrated that the incubation of purified Syk in vitro with ATP leads to the covalent modification of multiple tyrosines (28). In the present study, we have combined metabolic labeling and peptide mapping approaches to identify the tyrosine residues on Syk that become phosphorylated in response to the activation of B cells. Our results indicate that Syk is phosphorylated on multiple tyrosines in response to either receptor engagement or inducers of oxidative stress. The phosphorylation of specific sites on Syk is dependent on the catalytic activities of both Syk and Lyn. These tyrosines include residues in the inter-SH2 domain region and within the catalytic loop, which are phosphorylated primarily by Syk itself, and residues within the linker region that connects the tandem SH2 domains to the catalytic domain, which are the preferred Lyn-dependent phosphorylation sites. This includes a site (Tyr^{317}) that negatively regulates Syk function.

Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907

Received for publication April 29, 1998. Accepted for publication July 13, 1998.

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.

[1] This work was supported by National Institutes of Health Grant CA37372 awarded by National Cancer Institute. T.M.Y. was supported by National Cancer Institute Training Grant CA09634.

[2] Address correspondence and reprint requests to Dr. Robert L. Geahlen, Department of Medicinal Chemistry and Molecular Pharmacology, Hansen Life Sciences Research Building, Purdue University, West Lafayette, IN 47907.

[3] Abbreviations used in this paper: ITAM, immunoreceptor tyrosine-based activation motif; GST, glutathione *S*-transferase; NF-AT, nuclear factor of activated T cells; PLC, phospholipase C.

0022-1767/98/$02.00

Materials and Methods

Cells, Abs, and reagents

Wild-type, Syk$^-$, and Lyn$^-$ chicken DT40 B cells (29) were generously provided by Dr. Tomohiro Kurosaki (Kansai Medical University, Moriguchi, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% chicken serum, 50 μM 2-ME, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. Preparation of a cDNA for the expression of Myc epitope-tagged Syk using the pGEM/EPB vector (30) was described previously (31). cDNAs for the expression of site-directed mutants were constructed using the Transformer mutagenesis kit (Clontech, Palo Alto, CA). All mutations were confirmed by sequencing. Syk$^-$ or Lyn$^-$ DT40 B cells were transfected with 25 μg of the various linearized Syk-Myc DNA-containing plasmids and 2.5 μg of p3′SS (Stratagene, La Jolla, CA) by electroporation using a Cell-Porator (Life Technologies, Gaithersburg, MD) at 300 V, 330 μF. Stable transfected cell lines were selected in hygromycin (2 mg/ml). The 9E10 anti-Myc hybridoma cell line was obtained from American Type Culture Collection (Manassas, VA). Rabbit anti-phosphotyrosine (1) and anti-Syk (23) antisera were prepared by Purdue University Cancer Center Antibody Production Facility (West Lafayette, IN). Goat anti-chicken IgM Abs were obtained from Bethyl Laboratories (Montgomery, TX). The synthetic peptide ADENYYK was prepared by Purdue Cancer Center Peptide Synthesis Facility. Cloning, expression, and isolation of the GSTp42.5 form of Syk were as described (28). The NF-AT-luciferase reporter construct was a gift of Anjana Rao, Harvard University (Boston, MA).

Metabolic labeling

A total of 2.5×10^6 DT40 cells was preincubated in 15 ml of phosphate-free RPMI 1640 for 1 h and then incubated for an additional 2 h in the presence of 5 mCi of [^{32}P]orthophosphate (DuPont/NEN, Boston, MA). Cells were collected, washed, and resuspended in PBS. Where indicated, cells were activated for 15 min at 4°C by the addition of affinity-purified goat anti-chicken IgM (50 μg/ml). In some experiments, cells were activated instead by treatment for either 20 min with pervanadate (final concentration of 0.1 mM sodium orthovanadate and 0.5 mM H_2O_2) at 37°C or 10 min with 10 mM H_2O_2 at 37°C. Cells were lysed in buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Brij 96, 5 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates were centrifuged at 15,000 $\times$ *g* for 10 min at 4°C. The supernatants were precleared by incubation with protein A-Sepharose for 1 h at 4°C. Syk was immunoprecipitated from the unbound fraction by the addition of 20 μl of protein A-Sepharose that had been previously incubated with 5 μl of mouse ascites fluid containing the anti-Myc epitope mAb. The immune complexes were washed eight times with RIPA buffer (150 mM NaCl, 10 mM Tris/HCl, pH 7.2, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS), dissociated in SDS-sample buffer, and separated by SDS-PAGE. The separated proteins were then electrophoretically transferred to nitrocellulose membranes and detected by autoradiography.

To obtain in vitro autophosphorylated Syk, the epitope-tagged protein was immunoprecipitated from lysates of Syk-expressing DT40 cells with anti-Myc epitope Abs, as described above. The immune complexes were washed twice with lysis buffer, followed by two washes with 25 mM HEPES, pH 7.5, and 1 mM sodium orthovanadate, and then incubated in vitro for 30 min (or for the times indicated) at 30°C in 25 mM HEPES, pH 7.4, 10 mM $MnCl_2$, 5 mM *p*-nitrophenylphosphate, 5 mM ATP, and 100 μCi of [γ-^{32}P]ATP. Phosphoproteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by autoradiography.

Phosphopeptide mapping

Phosphoproteins were excised from nitrocellulose membranes and digested with trypsin essentially as described (32). In brief, membrane pieces were first incubated in 0.5% polyvinylpyrrolidone (PVP-10; Sigma, St. Louis, MO), 100 mM acetic acid for 30 min at 37°C. After extensive washing with H_2O, membranes were incubated for 2 h at 37°C with 10 μg L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) in 50 mM NH_4HCO_3 and then for an additional 2 h with freshly added 10 μg of trypsin. Samples were lyophilized and resuspended in alkaline PAGE sample buffer containing 0.125 M Tris/HCl, pH 6.8, 6 M urea, and a small amount of bromphenol blue (33). The tryptic phosphopeptides were resolved by electrophoresis on an alkaline 40% polyacrylamide gel, as described (33). Samples were electrophoresed at 180 V until the blue tracking dye had migrated to R_f = 0.5. Phosphopeptides were detected by autoradiography.

The synthetic peptide ADENYYK was phosphorylated in vitro using GSTp42.5 immobilized on glutathione-agarose (28) at a peptide concentration of 1 mM for 2.5 min at 30°C in buffer containing 10 mM $MnCl_2$, 5 mM *p*-nitrophenylphosphate, 25 mM HEPES, pH 7.4, 1 μM ATP, and 5 μCi of [γ-^{32}P]ATP. The reaction mixture containing the phosphopeptide was resolved by alkaline PAGE. The generation of tryptic phosphopeptides from in vitro phosphorylated GSTp42.5 and their separation by HPLC was as described previously (28).

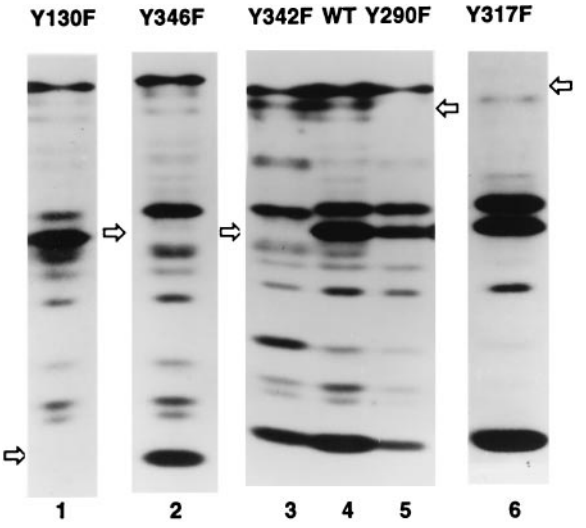

FIGURE 1. Characterization of phosphopeptides derived from autophosphorylated Syk and Syk mutants. Tryptic phosphopeptides were generated from in vitro autophosphorylated Syk mutants, separated by alkaline-PAGE, and detected by autoradiography. The mutants utilized were Syk(Y130F) (*lane 1*), Syk(Y346F) (*lane 2*), Syk(Y342F) (*lane 3*), Syk(WT) (*lane 4*), Syk(Y290F) (*lane 5*), and Syk(Y317F) (*lane 6*). The numbering system used is based on the sequence of murine Syk. Open arrows indicate the relevant phosphopeptides missing from the maps of mutant kinases.

Measurement of NF-AT promoter activity

Syk$^-$DT40 cells (5×10^6) were transfected with vectors containing the various Syk mutants (20 μg) along with NF-AT-luciferase (10 μg) by electroporation, as described above. Cells were harvested 40 h following transfection, plated at a density of 1×10^6/ml, and activated with anti-IgM Abs or with a combination of PMA (50 ng/ml) and ionomycin (1.0 μM) for 6 h at 37°C. Luciferase activity was determined using the luciferase assay system kit (Promega, Madison, WI) and measured on a Lumat LB 9501 luminometer (EG&G Wallac, Wellesley, MA).

Results

Syk-derived tryptic phosphopeptides can be resolved by alkaline polyacrylamide gel electrophoresis

To develop a convenient procedure for the analysis of sites of tyrosine phosphorylation on Syk, we investigated the use of alkaline 40% polyacrylamide gels for the separation of tryptic phosphopeptides (33, 34). We first examined phosphopeptides derived from Syk(WT), a form of murine Syk with a Myc epitope tag at the extreme carboxyl terminus expressed in a Syk$^-$DT40 B cell (31). Syk(WT) was recovered from cell lysates with anti-Myc epitope Abs, incubated with [γ-^{32}P]ATP to allow autophosphorylation to occur, and then digested with trypsin. The tryptic phosphopeptides were separated electrophoretically to generate the phosphopeptide map shown in Fig. 1, *lane 4*. A complete digest of autophosphorylated Syk(WT) yielded a series of phosphopeptides that were well resolved by one-dimensional alkaline PAGE.

To identify the major tryptic phosphopeptides, codons in the Syk(WT) cDNA specifying several of the known in vitro sites of tyrosine phosphorylation (28) were mutated to encode phenylalanine. The resulting Myc-tagged Syk mutants were expressed in

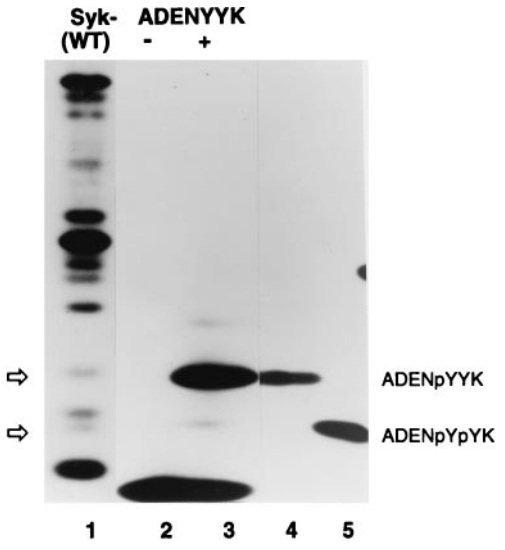

FIGURE 2. Characterization of Tyr^{519}- and Tyr^{520}-containing phosphopeptides. Tryptic phosphopeptides were generated from in vitro autophosphorylated Syk(WT) (*lane 1*) and separated by alkaline PAGE. The peptide ADENYYK was incubated with immobilized GSTp42.5 and [γ-^{32}P]ATP, electrophoresed on an alkaline polyacrylamide gel, and detected by autoradiography (*lane 3*). The sample in *lane 2* represented an identical reaction conducted in the absence of peptide. The peptides ADENpYYK and ADENpYpYK were isolated by HPLC from a tryptic digest of in vitro autophosphorylated GST-Syk and separated by alkaline PAGE (*lanes 4* and *5*).

Syk^-DT40 B cells, and stable cell lines were generated. The kinases were immunoprecipitated with anti-Myc epitope Abs, phosphorylated in vitro with [γ-^{32}P]ATP, and digested with trypsin. The resulting phosphopeptides were separated by alkaline PAGE and detected by autoradiography. Examples of these analyses are shown in Fig. 1 (*lanes 1*, *2*, *3*, *5*, and *6*). These studies positively identified peptides containing phosphotyrosines at positions 130 (*lane 1*), 342 (*lane 2*), 346 (*lane 3*), 290 (*lane 5*), and 317 (*lane 6*).

The migration position of phosphopeptides containing Tyr^{519} and Tyr^{520} was confirmed using a synthetic peptide corresponding in sequence to the tryptic fragment containing these residues (ADENYYK). This peptide was phosphorylated in vitro, using as a catalyst a truncated form of Syk expressed in insect cells as a fusion protein coupled to glutathione *S*-transferase (GSTp42.5) (28). The resulting phosphopeptides were analyzed by alkaline gel electrophoresis and are shown in Fig. 2. This reaction resulted primarily in the phosphorylation of ADENYYK on a single tyrosine with only small amounts of the doubly phosphorylated peptide being observed. These peptides comigrated with HPLC-purified and sequenced phosphopeptides corresponding to singly (*lane 4*) and doubly (*lane 5*) phosphorylated ADENYYK derived from in vitro autophosphorylated GSTp42.5 (28).

The complete pattern of tryptic phosphopeptides derived from autophosphorylated Syk(WT) and separated by alkaline PAGE is shown in Fig. 3*A*. The identity of each of the phosphopeptides on the map that were analyzed as described above is indicated. The location of each of these sites on the Syk molecule (Fig. 3*B*) and the sequence of each of these tryptic peptides (Fig. 3*C*) are also indicated.

Syk is phosphorylated on multiple tyrosines following B cell activation

A stable line of Syk^-DT40 B cells transfected to express Syk(WT) was used for the initial characterization of Syk phosphorylation in intact cells. Cells were preincubated in the presence of [^{32}P]orthophosphate, and then either remained untreated or were activated by treatment with anti-IgM Abs, pervanadate, or H_2O_2. Syk(WT) was then immunoprecipitated with anti-Myc epitope Abs. The resulting immune complexes were washed extensively, separated by SDS-PAGE, and transferred to nitrocellulose membranes. As shown in Fig. 4*A*, Syk(WT) from both untreated and activated cells was a phosphoprotein. The phosphate content of Syk(WT) increased following activation. However, as shown previously, Syk(WT) contains little or no phosphotyrosine in the absence of activation (31).

To characterize the major sites of Syk tyrosine phosphorylation, the recovered Syk(WT) proteins were digested with trypsin, and the resulting phosphopeptides were analyzed by alkaline PAGE. As shown in Fig. 4*B*, proteolysis of Syk(WT) recovered from unstimulated cells generated four prominent phosphopeptides, indicating that Syk is phosphorylated on multiple serine and/or threonine residues in the absence of activation. Additional phosphopeptides were obtained from a tryptic digest of Syk(WT) recovered from B cells activated by treatment with anti-IgM Abs (Fig. 4*B*), pervanadate, or H_2O_2 (Fig. 4*C*). These represented sites of tyrosine phosphorylation previously identified from the study of in vitro autophosphorylated Syk. Phosphorylation was observed on peptides corresponding to Tyr^{130}, Tyr^{317}, Tyr^{342}, Tyr^{346}, Tyr^{519}, and Tyr^{520}.

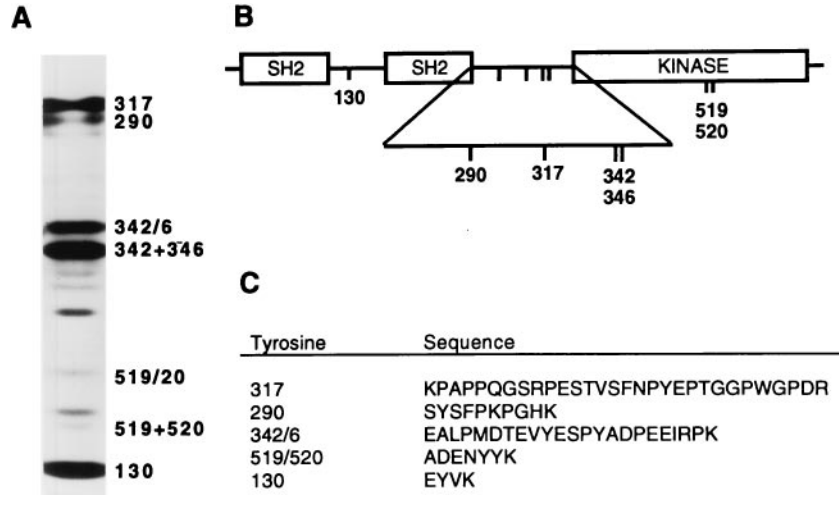

FIGURE 3. Summary of tryptic peptides generated from autophosphorylated Syk. *A*, A one-dimensional map showing tryptic phosphopeptides derived from in vitro autophosphorylated Syk(WT) separated by alkaline PAGE. The locations of the phosphorylated tyrosines in each phosphopeptide are indicated. *B*, Schematic representation of the relative locations of the major sites of autophosphorylation on Syk. *C*, Amino acid sequences of tryptic peptides derived from Syk(WT).

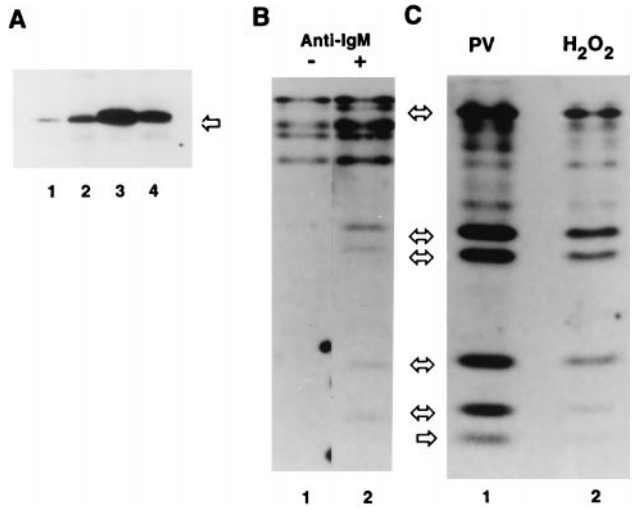

FIGURE 4. Identification of the in vivo sites of Syk phosphorylation. *A*, Syk(WT) was immunoprecipitated with anti-Myc epitope Abs from Syk$^-$, Syk(WT)-expressing DT40 cells preincubated with [^{32}P]orthophosphate and then treated without (*lane 1*) or with anti-IgM (*lane 2*), pervanadate (*lane 3*), or H_2O_2 (*lane 4*). The resulting immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and exposed to x-ray film. *B*, Tryptic phosphopeptides were generated from Syk(WT) recovered from metabolically labeled untreated (*lane 1*) or anti-IgM-activated (*lane 2*) cells, separated by alkaline PAGE, and detected by autoradiography. *C*, Tryptic phosphopeptides were generated from Syk(WT) recovered from metabolically labeled DT40 B cells activated by treatment with pervanadate (*lane 1*) or H_2O_2 (*lane 2*). The arrows indicate the major phosphotyrosine-containing peptides that are phosphorylated following activation.

Linker region tyrosines are phosphorylated in the absence of Syk catalytic activity

The phosphorylation of Syk on tyrosine in intact cells occurs most likely as a combination of autophosphorylation and phosphorylation by receptor-associated, Src family kinases. To determine which site or sites on Syk could be phosphorylated in the absence of Syk autophosphorylating activity (and in the absence of endogenous Syk), a catalytically inactive Syk mutant (Syk(K396R)) was prepared in which Arg replaced an essential Lys in the active site. Syk$^-$DT40 B cells were established that expressed the epitope-tagged Syk(K396R) protein. These were incubated in the presence of [^{32}P]orthophosphate with or without stimulation with anti-IgM Abs. The metabolically labeled proteins were immunoprecipitated with anti-Myc epitope Abs (Fig. 5*A*) and subjected to tryptic phosphopeptide mapping (Fig. 5*B*). In the absence of activation, Syk(K396R) was extensively phosphorylated. Immunoblotting experiments using Abs directed against phosphotyrosine indicated that the protein was not phosphorylated on tyrosine (data not shown). Upon receptor engagement, however, Syk(K396R) did become phosphorylated on tyrosine. As shown in Fig. 5*B*, the prominent sites of tyrosine phosphorylation were Tyr^{317}, Tyr^{342}, and Tyr^{346}.

To further characterize the phosphorylation of Syk(K396R), anti-Myc epitope immune complexes were prepared from Brij 96 lysates of Syk$^-$DT40 cells expressing high levels of Syk(K396R) and were incubated in vitro in buffer containing [γ-^{32}P]ATP. The resulting phosphoproteins were separated by SDS-PAGE and transferred to nitrocellulose. Interestingly, Syk(K396R) became phosphorylated in the immune complex, and this phosphorylation was enhanced by receptor engagement (Fig. 5*A*). To determine the sites that were labeled, the band corresponding to Syk(K396R) was digested with trypsin to generate a phosphopeptide map. As shown in Fig. 5*C*, Syk(K396R) was phosphorylated predominantly on Tyr^{317}, Tyr^{342}, and Tyr^{346}. These results indicated that an active protein tyrosine kinase coimmunoprecipitated with the catalytically inactive Syk(K396R) that could catalyze its phosphorylation upon the addition of [γ-^{32}P]ATP. Lyn, which is the only Src family kinase expressed in DT40 B cells, was a likely candidate. To explore this, Syk(K396R) was expressed in a Lyn$^-$DT40 B cell line that lacks any detectable Src family kinase activity (29). Anti-Myc epitope immune complexes prepared from this cell line were devoid of any significant tyrosine kinase activity (Fig. 5*A*). Thus, Lyn appeared to preferentially phosphorylate Syk on hinge region Tyr^{317}, Tyr^{342}, and Tyr^{346} both in vitro and in vivo.

To explore an absolute requirement for Lyn for Syk phosphorylation, Syk(WT) was expressed in Lyn$^-$DT40 B cells, which were then treated with or without H_2O_2. Peptide maps of metabolically labeled Syk(WT) recovered from treated cells revealed a low level of tyrosine phosphorylation occurring only at sites corresponding to Tyr^{317}, Tyr^{342}, and Tyr^{346} (Fig. 5*D*).

Phosphorylation of Syk on Tyr^{317} retards its electrophoretic mobility on SDS-PAGE

The tyrosine phosphorylation of Syk in vitro or in vivo typically results in a shift in its electrophoretic mobility on SDS-PAGE. To identify the site responsible for this mobility shift, Syk(WT) was immunoprecipitated from Syk(WT)-expressing DT40 B cells with anti-Myc epitope Abs and autophosphorylated in vitro with [γ-^{32}P]ATP. The resulting phosphoprotein migrated as a doublet on SDS-PAGE (Fig. 6*A*). The upper and lower phosphoprotein bands were excised separately and subjected to tryptic peptide mapping, as described above. As shown in Fig. 6*B*, only the peptide map generated from the upper band exhibited the phosphopeptide containing Tyr^{317}.

The substitution of Phe for Tyr^{317} also affected the mobility on SDS-PAGE of Syk phosphorylated in intact cells. Syk(WT) immunoprecipitated with anti-Myc epitope Abs from Syk(WT)-expressing Syk$^-$DT40 B cells treated with pervanadate (Fig. 6*A*) or H_2O_2 (not shown) exhibited a reduced electrophoretic mobility. In contrast, Syk(Y317F) showed little change in mobility. Phosphopeptide mapping of metabolically labeled Syk(Y317F) from peroxide-treated cells revealed the expected loss of the Tyr^{317}-containing phosphopeptide (Fig. 6*C*). However, the remaining sites of tyrosine phosphorylation (Tyr^{130}, Tyr^{342}, Tyr^{346}, Tyr^{519}, and Tyr^{520}) were unaffected by the mutation.

Mutation of Tyr^{317} to Phe enhances Ag receptor signaling

While the linker region Tyr^{342} has been reported previously as a binding site for the SH2 domains of Vav and PLC-γ (15, 31), a role for Tyr^{317} has yet to be explored. To examine this question, Syk$^-$DT40 cell lines were transiently transfected with cDNAs expressing Syk(WT) or Syk(Y317F) along with a NF-AT reporter construct. Cells lacking Syk failed to stimulate NF-AT activity following receptor cross-linking (Fig. 7). Expression of Syk(WT) restored signaling. Syk(Y317F), expressed at a level comparable with that of Syk(WT) (Fig. 7), exhibited a greatly enhanced ability to stimulate the B cell receptor-dependent induction of NF-AT activity (Fig. 7).

Discussion

The receptor-mediated activation of Syk in hemopoietic cells is invariably associated with an increase in its state of tyrosine phosphorylation. However, little is known regarding the complete repertoire of sites on Syk that are modified following receptor engagement. To approach this problem, we first identified those

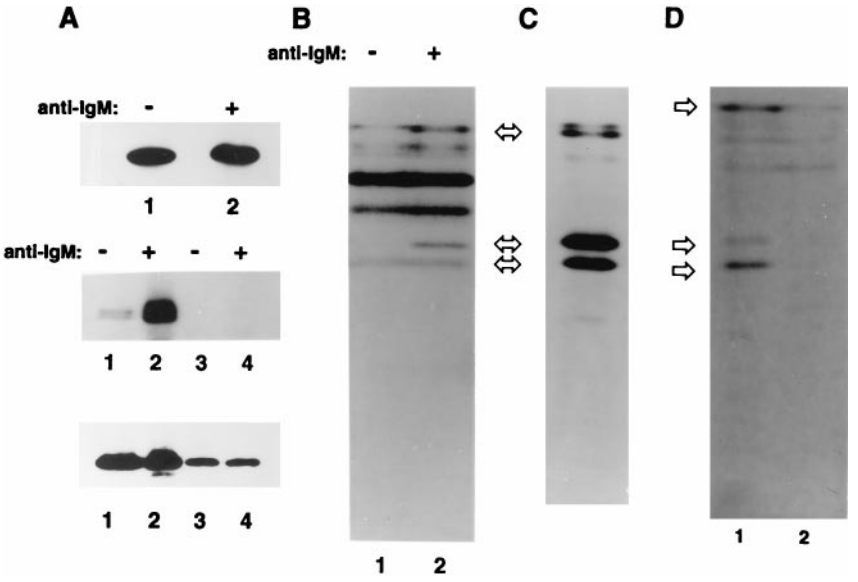

FIGURE 5. Phosphorylation of Syk by Lyn in vitro and in vivo. *A*, Syk(K396R) was immunoprecipitated from Syk$^-$, Syk(K396R)-expressing DT40 cells preincubated with [^{32}P]orthophosphate (*top panel*) and treated without (*lane 1*) or with (*lane 2*) activating anti-IgM Abs. The resulting immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and exposed to x-ray film. Alternatively, Syk(K396R) was immunoprecipitated from untreated (*lanes 1* and *3*) or anti-IgM-treated (*lanes 2* and *4*) Syk$^-$, Syk(K396R)-expressing (*lanes 1* and *2*) or Lyn$^-$, Syk(K396R)-expressing (*lanes 3* and *4*) DT40 cells. The immune complexes were incubated with [γ-^{32}P]ATP, separated by SDS-PAGE, and exposed to x-ray film (*middle panel*) or immunoblotted with anti-Syk Abs (*bottom panel*). *B*, Tryptic phosphopeptides were generated from Syk(K396R) recovered from metabolically labeled untreated (*lane 1*) or anti-IgM-activated (*lane 2*) DT40 cells, separated by alkaline PAGE, and detected by autoradiography. The open arrows indicate the migration positions of the major phosphotyrosine-containing peptides. *C*, Tryptic phosphopeptides were generated from Syk(K396R) immunoprecipitated from Syk$^-$, Syk(K396R)-expressing DT40 cells and phosphorylated in vitro, as described in *A*, and separated by alkaline PAGE. *D*, Tryptic phosphopeptides were generated from Syk(WT) immunoprecipitated from Lyn$^-$, Syk(WT)-expressing DT40 cells prelabeled with [^{32}P]orthophosphate and activated by treatment with (*lane 1*) or without (*lane 2*) H_2O_2.

tyrosines on Syk that could be modified by a Syk-catalyzed autophosphorylation reaction (28). These analyses identified a series of tryptic phosphopeptides containing 10 distinct tyrosines that could be phosphorylated upon prolonged incubation of an expressed GST-Syk fusion protein with [γ-^{32}P]ATP. These phosphopeptides, which varied in size from 4 to 30 amino acids, could not be completely resolved in a single HPLC run (28) and, in our hands, were not well resolved by conventional two-dimensional thin-layer electrophoresis/thin-layer chromatography. To separate the phosphopeptides, we adopted an alternative procedure, alkaline PAGE, which separates acidic peptides on the basis of charge and size (33, 34). This technique is particularly well suited to the analysis of tryptic phosphopeptides, which generally contain only a single positively charged amino acid and carry a net negative charge at pH 9 (34). In the specific case of Syk, the tryptic phosphopeptides representing the major sites of tyrosine phosphorylation varied sufficiently in mass and charge that they could be completely resolved in a single one-dimensional separation. By comparing the migration positions of the phosphopeptides derived from autophosphorylated Syk with those of purified and sequenced phosphopeptides (28), and through the use of phosphorylated synthetic peptides and site-directed mutants, we could develop a standard peptide map useful for the analysis and identification of Syk phosphorylation sites that are modified in vitro or in vivo in response to a variety of stimuli (Figs. 1–3).

Using this one-dimensional mapping approach, we mapped the major tyrosine residues on Syk that are phosphorylated in intact cells in response to external stimuli such as anti-IgM Abs or oxidizing agents (pervanadate or H_2O_2). These stimuli produce comparable patterns of protein tyrosine phosphorylation of cellular proteins that are all dependent on the expression of cell surface Ag receptors with intact cytoplasmic ITAMs (35). However, the extent of phosphorylation resulting from the treatment of B cells with pervanadate or H_2O_2 is generally greater than that seen with anti-IgM Abs. We found that the tyrosines on Syk that are phosphorylated in activated B cells are the tyrosines that also are phosphorylated in an in vitro autophosphorylation reaction. The ability of Syk to autophosphorylate on these presumably physiologically relevant sites most likely explains why the aggregation of a chimeric CD16-Syk molecule alone can signal in T cells (36). However, not all of the autophosphorylation sites that can be identified in vitro also become modified in vivo. The sites of autophosphorylation that we have been unable to detect in vivo, which include Tyr^{358} or any of the carboxyl-terminal tyrosines (623, 624 or 625), are sites that are among the slowest to be modified during autophosphorylation (31) and seem unlikely to play an important role in the receptor-mediated activation of Syk.

The tyrosines that are phosphorylated in vivo are present in multiple locations throughout the Syk molecule (Fig. 3) at sites that have been implicated as important mediators of Syk function in hemopoietic cells. Tyr^{130} is located between the tandem SH2 domains at a site implicated in the regulation of the Syk-receptor interaction (31). The ability of Syk to bind the Ag receptor and to participate in receptor-mediated signaling is enhanced by the replacement of Tyr^{130} with Phe and abrogated by its replacement with Glu (31). Phosphorylation at this site would be expected to lead to the dissociation of Syk from the receptor ITAMs. Tyr^{130} does not appear to be a major site of phosphorylation modified in response to receptor cross-linking (Fig. 4*B*), but is phosphorylated in cells treated with protein tyrosine phosphatase inhibitors such as pervanadate and H_2O_2 (Fig. 4*C*). Thus, it is possible that Tyr^{130} is phosphorylated, but only transiently, following receptor engagement. Alternatively, Tyr^{130} might be phosphorylated primarily in response to agents such as pervanadate and H_2O_2 that can activate

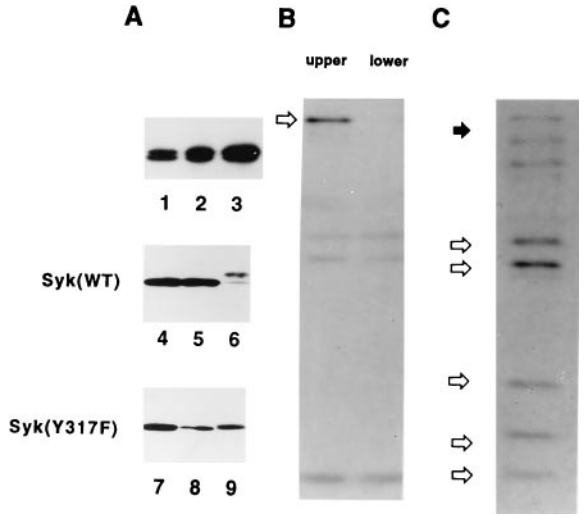

FIGURE 6. Mapping of Tyr^{317} as the site responsible for the shift in electrophoretic mobility of phosphorylated Syk. *A*, Syk(WT) was autophosphorylated in vitro with [γ-^{32}P]ATP for 1 (*lane 1*), 3 (*lane 2*), or 5 (*lane 3*) min, separated into slower and faster migrating forms by SDS-PAGE, and detected by autoradiography. Anti-Myc epitope immune complexes were prepared from lysates of Syk$^-$DT40 B cells expressing either Syk(WT) (*lanes 4–6*) or Syk(Y317F) (*lanes 7–9*), separated by SDS-PAGE, and immunoblotted with anti-Syk Abs. Cells had been treated before lysis without (*lanes 4* and *7*) or with anti-IgM (*lanes 5* and *8*) or pervanadate (*lanes 6* and *9*). *B*, Tryptic phosphopeptides were generated from the *upper* (*lane 1*) or *lower* (*lane 2*) of the two differentially migrating forms of autophosphorylated Syk(WT) (see *A*, *upper panel*). The arrow marks the migration position of the peptide containing Try^{317}. *C*, Tryptic phosphopeptides were generated from Syk(Y317F) immunoprecipitated from Syk, Syk(Y317F)-expressing DT40 cells prelabeled with [^{32}P]orthophosphate and activated by treatment with H_2O_2. The migration positions of the major phosphopeptides are indicated by the open arrows. The closed arrow indicates the normal migration position of the phosphopeptide containing Tyr^{317}, which is missing from this map.

Syk in the absence of B cell receptor aggregation (35). Since the placement of a negatively charged amino acid such as Glu at this site results in an enzyme with an elevated basal catalytic activity (31), the phosphorylation of Tyr^{130} could provide an intriguing alternative mechanism for the activation of Syk in the absence of receptor cross-linking.

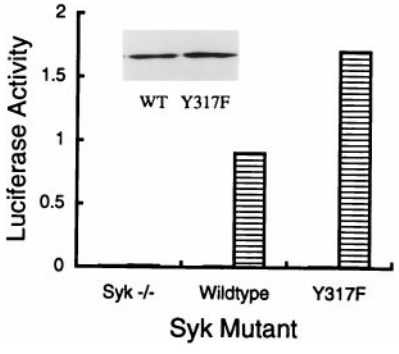

FIGURE 7. Activation of NF-AT by Syk(WT) and Syk(Y317F). Syk$^-$DT40 cells transiently transfected with vectors containing Syk(WT) or Syk(Y317F) cDNA and an NF-AT-luciferase expression plasmid either remained unstimulated (solid bars) or were activated with anti-IgM Abs (striped bars). Relative luciferase activity is reported as activity produced by anti-IgM treatment divided by the activity produced in response to stimulation with PMA + ionomycin. The relative level of Syk(WT) and Syk(Y317F) expressed in the population of transfected cells was determined by Western blotting with anti-Syk Abs (*inset*).

Tyr^{342} and Tyr^{346} are located in the linker region that separates the tandem SH2 domains from the catalytic domain. This region of Syk and the corresponding $Tyr^{315/319}$ region of ZAP-70 have been implicated as docking sites for SH2 domain-containing molecules based on the location of the sites, the sequence of amino acids surrounding the phosphotyrosines, and studies with site-directed mutants. However, these sites had not been demonstrated previously to be phosphorylated in vivo. The importance of these residues as docking sites is indicated by studies showing that the substitution of both Tyr^{342} and Tyr^{346} with Phe blocks the ability of a CD8$^-$Syk chimera to bind PLC-γ1 (37), and the substitution of Syk Tyr^{342} or ZAP-70 Tyr^{315} with Phe blocks their ability to bind to Vav (25, 38). In the case of ZAP-70, this severely disrupts signaling (38). Therefore, these sites are likely to be important, positive regulators of Syk function.

Phosphorylation of the linker region Tyr^{317} is responsible for the mobility shift of tyrosine-phosphorylated Syk frequently observed on SDS-polyacrylamide gels. This suggests that phosphorylation at this site may cause a conformational change in the protein. Since the sequence of the tetrapeptide surrounding Tyr^{317} (NPYE) strongly predicts a β-turn (39), it is possible that the disruption of this turn through phosphorylation of the tyrosine could account for this altered mobility. The substitution of Tyr^{317} with Phe also results in a form of the kinase with an enhanced ability to induce NF-AT activity following receptor cross-linking (Fig. 7). Thus, it would appear that the phosphorylation of Tyr^{317} serves as a negative regulatory event, perhaps acting as a feedback signal to down-regulate Syk activity. This Tyr is located in a region of Syk roughly equivalent to that of Tyr^{292} of ZAP-70, which is also phosphorylated in activated T cells (40). In ZAP-70, Tyr^{292} serves also as a negative regulatory site, and its replacement with Phe results in a form of the kinase with an enhanced ability to participate in receptor-mediated signaling when expressed in either T or B cells (41, 42). Tyr^{292} has recently been shown, when phosphorylated, to be the site of interaction between Zap-70 and the PTB domain of Cbl (43), a negative regulator of Syk (44). Although the amino acid sequences in these regions of Syk (VSFNPpYEPTGG) and ZAP-70 (LNSDGpYTPEPE) have diverged, Syk Tyr^{317} still fits the determined consensus for a Cbl interaction site (43).

Tyr^{519} and Tyr^{520} are located in the activation loop within the Syk catalytic domain and are homologous to ZAP-70 Tyr^{492} and Tyr^{493}. These are sites whose phosphorylation is important for the participation of each of these kinases in receptor-mediated signaling pathways. For ZAP-70, the critical residue for phosphorylation is Tyr^{493}, since its replacement with Phe results in a loss of function, while the replacement of Tyr^{492} with Phe enhances signaling (41, 42, 45–47). For Syk, the replacement of both Tyr^{519} and Tyr^{520} with Phe also reduces the receptor-mediated stimulation of protein tyrosine phosphorylation (19, 48). A reduction in protein tyrosine phosphorylation in transfected COS cells is also observed when either Tyr^{519} or Tyr^{510} is replaced with Phe, suggesting that the phosphorylation of both sites might be required for maximal activity (49). The phosphorylation of Tyr^{519} and Tyr^{520} also creates a docking site on Syk for the SH2 domain of Lck, and this interaction is thought to be important for signaling (24, 49).

The phosphorylation sites on Syk can be divided into two primary classes: 1) those that are preferentially phosphorylated by Lyn and do not require the catalytic activity of Syk; and 2) those that require the activities of both Syk and Lyn, but are not directly phosphorylated by Lyn. The linker region tyrosines, 317, 342, and 346, are the principal sites on Syk phosphorylated by the Lyn

kinase both in vivo and in vitro. The finding of a preferential phosphorylation of Syk by Lyn at sites located within the linker region rather than within the catalytic domain was somewhat unexpected since the Lck kinase preferentially phosphorylates ZAP-70 at Tyr^{493}, which is equivalent to Syk Tyr^{520} and is located within the activation loop (41). It is consistent, however, with other observations in both B cells and mast cells that have indicated that Syk is phosphorylated in *trans* by other tyrosine kinases primarily on sites outside the catalytic domain (48, 50). However, it is well established that Syk and ZAP-70 show fundamental differences in their intrinsic activities when expressed in B and T cells, with Syk being much less dependent on associated Src family kinase activity (51–53). This difference may be reflected, in part, by how they interact with Src family members and how important the phosphorylation of activation loop tyrosines is to changes in catalytic activity. It is also formally possible that a tyrosine kinase other than Lyn, but one that is dependent for activity on the expression of Lyn, is responsible for catalyzing the phosphorylation of the linker region sites. It is important to note that the contributions of Lyn-dependent phosphorylations on the coupling of Syk to downstream signaling pathways such as NF-AT induction are greatly dependent on the sites that are being modified. Some phosphorylations are likely to be stimulatory (Tyr^{342} and/or Tyr^{346}), while others are inhibitory (Tyr^{317}). Thus, the consequences of coclustering Lyn-associated receptors with the Ag receptor on Syk-mediated signaling may well be dependent on the exact location of the sites that are being phosphorylated.

The phosphorylation of Tyr^{130}, Tyr^{519}, and Tyr^{520} only occurs to a discernible extent when a catalytically active form of Syk is expressed in cells containing endogenous Lyn. These sites are not appreciably phosphorylated by Lyn, but are refractory to phosphorylation when Syk(WT) is expressed in Lyn^- cells (Fig. 5*D*). This may reflect a requirement for a Lyn-catalyzed phosphorylation of receptor ITAMs to create docking sites for the recruitment of Syk to the Ag receptor. The recruitment and binding of Syk to the Ag receptor ITAMs could then stimulate Syk-catalyzed autophosphorylation reactions that lead to the modification of Tyr^{130}, Tyr^{519}, and Tyr^{520}. The phosphorylated Ag receptor would act as a scaffold for the recruitment and proper positioning of Syk molecules to allow enhanced intermolecular phosphorylations to occur, as has been proposed previously for ZAP-70 (54). A recent model for Syk activation in RBL-2H3 mast cells proposed an initial Lyn-catalyzed phosphorylation of activation loop tyrosines, which would then initiate a cascade of Syk-catalyzed intermolecular phosphorylations that would account for the bulk of Tyr^{519} and Tyr^{520} phosphorylation (50). Our results cannot rule out a direct contribution of Lyn to a low-level phosphorylation of Tyr^{519} and Tyr^{520}.

Finally, the presence of phosphoserine or phosphothreonine on Syk isolated from metabolically labeled cells raises the possibility that Syk might also be regulated by protein-serine/threonine kinases. The kinase or kinases responsible for this phosphorylation have not yet been identified. However, since Syk has been reported to coimmunoprecipitate with protein kinase C μ (55), this enzyme is a potential candidate. Studies to identify the sites of serine/threonine phosphorylation on Syk are currently under way.

Acknowledgments

We thank Dr. Tomohiro Kurosaki for the generous gifts of the Syk^- and Lyn^- DT40 cell lines, and Dr. Anjana Rao for the NF-AT-Luc reporter plasmid.

References

1. Hutchcroft, J. E., M. L. Harrison, and R. L. Geahlen. 1992. Association of the 72-kDa protein tyrosine kinase PTK72 with the B cell antigen receptor. *J. Biol. Chem. 267:8613.*
2. Gotoh, A., H. Takahira, R. L. Geahlen, and H. E. Broxmeyer. 1997. Cross-linking of integrins induces tyrosine phosphorylation of the proto-oncogene product Vav and protein tyrosine kinase Syk in human factor-dependent myeloid cells. *Cell Growth Differ. 8:721.*
3. Lin, T. H., C. Rosales, K. Mondal, J. B. Bolen, S. Haskill, and R. L. Juliano. 1995. Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocytic cells. *J. Biol. Chem. 270:16189.*
4. Yan, S. R., M. Huang, and G. Berton. 1997. Signaling by adhesion in human neutrophils. *J. Immunol. 158:1902.*
5. Poole, A., J. M. Gibbins, M. Turner, M. J. Vanvugt, J. G. J. Vandewinkel, T. Saito, V. L. J. Tybulewicz, and S. P. Watson. 1997. The Fc receptor γ-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J. 16:2333.*
6. Hutchcroft, J. E., R. L. Geahlen, G. G. Deanin, and J. M. Oliver. 1992. FcεR1-mediated tyrosine phosphorylation and activation of a 72 kDa protein tyrosine kinase, PTK72, in RBL-2H3 rat tumor mast cells. *Proc. Natl. Acad. Sci. USA 89:9107.*
7. Benhamou, M., N. J. P. Ryba, H. Kihara, H. Nishikata, and R. P. Siraganian. 1993. Protein-tyrosine kinase $p72^{syk}$ in high affinity IgE receptor signaling: identification as a component of pp72 and association with the receptor γ chain after receptor aggregation. *J. Biol. Chem. 268:23318.*
8. Chan, A. C., N. S. C. van Oers, A. Tran, L. Turka, C.-L. Law, J. C. Ryan, E. A. Clark, and A. Weiss. 1994. Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in T cell antigen receptor signaling. *J. Immunol. 152:4758.*
9. Couture, C., G. Baier, A. Altman, and T. Mustelin. 1994. $p56^{lck}$-independent activation and tyrosine phosphorylation of $p72^{syk}$ by T-cell antigen receptor/CD3 stimulation. *Proc. Natl. Acad. Sci. USA 91:5301.*
10. Clark, E. A., S. J. Shattil, M. H. Ginsberg, J. Bolen, and J. S. Brugge. 1994. Regulation of the protein tyrosine kinase $pp72^{syk}$ by platelet agonists and the integrin $\alpha_{IIb}\beta_3$. *J. Biol. Chem. 269:28859.*
11. Agarwal, A., P. Salem, and K. C. Robbins. 1993. Involvement of $p72^{syk}$, a protein-tyrosine kinase, in Fcγ receptor signaling. *J. Biol. Chem. 268:15900.*
12. Darby, C., R. L. Geahlen, and A. D. Schreiber. 1994. Stimulation of macrophage FcγRIIIA activates the receptor-associated protein tyrosine kinase Syk and induces phosphorylation of multiple proteins including p95Vav and p62/GAP-associated protein. *J. Immunol. 152:5429.*
13. Kiener, P. A., B. M. Rankin, A. L. Burkhardt, G. L. Schieven, L. K. Gilliland, R. B. Rowley, J. B. Bolen, and J. A. Ledbetter. 1993. Cross-linking of Fcγ receptor I (FcγRI) and receptor II (FcγRII) on monocytic cells activates a signal transduction pathway common to both Fc receptors that involves the stimulation of p72 Syk protein tyrosine kinase. *J. Biol. Chem. 268:24442.*
14. Turner, M., P. J. Mee, P. S. Costello, O. Williams, A. A. Price, L. P. Duddy, M. T. Furlong, R. L. Geahlen, and V. L. J. Tybulewicz. 1995. Perinatal lethality and a block in the development of B cells in mice lacking the tyrosine kinase $p72^{syk}$. *Nature 378:298.*
15. Cheng, A. M., B. Rowley, W. Pao, A. Hayday, J. B. Bolen, and T. Pawson. 1995. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature 378:303.*
16. Cambier, J. C. 1995. New nomenclature for the Reth motif (or ARH1/TAM/ARAM/YXXL). *Immunol. Today 16:110.*
17. Flaswinkel, H., and M. Reth. 1994. Dual role of the tyrosine activation motif of the Ig-α protein during signal transduction via the B cell antigen receptor. *EMBO J. 13:83.*
18. Law, D. A., V. W.-F. Chan, S. K. Datta, and A. L. DeFranco. 1993. B cell antigen receptor motifs have redundant signalling capabilities and bind the tyrosine kinases PTK72, Lyn and Fyn. *Curr. Biol. 3:645.*
19. Kurosaki, T., S. A. Johnson, L. Pao, K. Sada, H. Yamamura, and J. C. Cambier. 1995. Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. *J. Exp. Med. 182:1815.*
20. Rowley, R. B., A. L. Burkhardt, H.-G. Chao, G. R. Matsueda, and J. B. Bolen. 1995. Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Igα/Igβ immunoreceptor tyrosine activation motif binding and autophosphorylation. *J. Biol. Chem. 270:11590.*
21. Hutchcroft, J. E., M. L. Harrison, and R. L. Geahlen. 1991. B lymphocyte activation is accompanied by phosphorylation of a 72 kDa protein-tyrosine kinase. *J. Biol. Chem. 266:14846.*
22. Yamada, T., T. Taniguchi, C. Yang, S. Yasue, H. Saito, and H. Yamamura. 1993. Association with B-cell-antigen receptor with protein-tyrosine kinase $p72^{syk}$ and activation by engagement of membrane IgM. *Eur. J. Biochem. 213:455.*
23. Peters, J. D., M. T. Furlong, D. J. Asai, M. L. Harrison, and R. L. Geahlen. 1996. Syk, activated by cross-linking the B-cell antigen receptor, localizes to the cytosol where it interacts with and phosphorylates α-tubulin on tyrosine. *J. Biol. Chem. 271:4755.*
24. Couture, C., M. Deckert, S. Williams, F. O. Russo, A. Altman, and T. Mustelin. 1996. Identification of the site in the Syk protein tyrosine kinase that binds the SH2 domain of Lck. *J. Biol. Chem. 271:24294.*
25. Deckert, M., S. Tartare-Deckert, C. Couture, T. Mustelin, and A. Altman. 1996. Functional and physical interactions of Syk family kinases with the Vav proto-oncogene product. *Immunity 5:591.*

26. Sidorenko, S. P., C.-H. Law, K. A. Chandran, and E. A. Clark. 1995. Human spleen tyrosine kinase p72Syk associates with the Src-family kinase p53/56Lyn and a 120-kDa phosphoprotein. *Proc. Natl. Acad. Sci. USA 92:359.*
27. Sillman, A. L., and J. G. Monroe. 1995. Association of $p72^{syk}$ with the *src* homology-2 (SH2) domains of PLCγ1 in B lymphocytes. *J. Biol. Chem. 270:11806.*
28. Furlong, M. T., A. M. Mahrenholz, K.-H. Kim, C. L. Ashendel, M. L. Harrison, and R. L. Geahlen. 1997. Identification of the major sites of autophosphorylation of the murine protein-tyrosine kinase Syk. *Biochim. Biophys. Acta 1355:177.*
29. Takata, M., H. Sabe, A. Hata, T. Inazu, Y. Homma, T. Nukada, H. Yamamura, and T. Kurosaki. 1994. Tyrosine kinase Lyn and Syk regulate B cell receptor-coupled Ca^{2+} mobilization through distinct pathways. *EMBO J. 13:1341.*
30. Dildrop, R., A. Ma, K. Zimmerman, E. Hsu, A. Tesfaye, R. DePinho, and F. W. Alt. 1989. IgH enhancer-mediated deregulation of N-*myc* gene expression in transgenic mice: generation of lymphoid neoplasias that lack c-*myc* expression. *EMBO J. 8:1121.*
31. Keshvara, L. M., C. Isaacson, M. L. Harrison, and R. L. Geahlen. 1997. Syk activation and dissociation from the B-cell antigen receptor is mediated by phosphorylation of tyrosine-130. *J. Biol. Chem. 272:10377.*
32. Luo, K., T. R. Hurley, and B. M. Sefton. 1991. Cyanogen bromide cleavage and proteolytic peptide mapping of proteins immobilized to membranes. *Methods Enzymol. 201:149.*
33. West, M. H. P., R. S. Wu, and W. M. Bonner. 1984. Polyacrylamide gel electrophoresis of small peptides. *Electrophoresis 5:133.*
34. Dadd, C. A., R. G. Cook, and C. D. Allis. 1993. Fractionation of small tryptic phosphopeptides by alkaline PAGE followed by amino acid sequencing. *Biotechniques 14:266.*
35. Wienands, J., O. Larbolette, and M. Reth. 1996. Evidence for a preformed transducer complex organized by the B cell antigen receptor. *Proc. Natl. Acad. Sci. USA 93:7865.*
36. Kolanus, W., C. Romeo, and B. Seed. 1993. T cell activation by clustered tyrosine kinases. *Cell 74:171.*
37. Law, C.-L., K. A. Chandran, S. P. Sidorenko, and E. A. Clark. 1996. Phospholipase C-γ interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. *Mol. Cell. Biol. 16:1305.*
38. Wu, J., Q. Zhao, T. Kurosaki, and A. Weiss. 1997. The Vav binding site (Y315) in Zap-70 is critical for antigen receptor-mediated signal transduction. *J. Exp. Med. 185:1877.*
39. Chou, P. Y., and G. D. Fasman. 1974. Prediction of protein conformation. *Biochemistry 13:222.*
40. Watts, J. D., M. Affolter, D. L. Krebs, R. L. Wange, L. E. Samelson, and R. Aebersold. 1994. Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. *J. Biol. Chem. 269:29520.*
41. Kong, G., M. Dalton, J. B. Wardenburg, D. Straus, T. Kurosaki, and A. C. Chan. 1996. Distinct tyrosine phosphorylation sites in Zap-70 mediate activation and negative regulation of antigen receptor function. *Mol. Cell. Biol. 16:5026.*
42. Zhao, Q., and A. Weiss. 1996. Enhancement of lymphocyte responsiveness by a gain-of-function mutation of Zap-70. *Mol. Cell. Biol. 16:6765.*
43. Lupher, M. L., S. Y. Zhou, S. E. Shoelson, L. C. Cantley, and H. Band. 1997. The Cbl phosphotyrosine-binding domain selects a D(N/D)XpY motif and binds to the Tyr(292) negative regulatory phosphorylation site of Zap-70. *J. Biol. Chem. 272:33140.*
44. Ota, Y., and L. E. Samelson. 1997. The product of the proto-oncogene c-Cbl: a negative regulator of the Syk tyrosine kinase. *Science 276:418.*
45. Chan, A. C., M. Dalton, R. Johnson, G. Kong, T. Wang, R. Thomas, and T. Kurosaki. 1995. Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. *EMBO J. 14:2499.*
46. Mege, D., V. Dibartolo, V. Germain, L. Tuosto, F. Michel, and O. Acuto. 1996. Mutation of tyrosines 492/493 in the kinase domain of Zap-70 affects multiple T-cell receptor signaling pathways. *J. Biol. Chem. 271:32644.*
47. Wange, R. L., R. Guitian, N. Isakov, J. D. Watts, R. Aebersold, and L. E. Samelson. 1995. Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. *J. Biol. Chem. 270:18730.*
48. Li, H.-L., M. S. Forman, T. Kurosaki, and E. Puré. 1997. Syk is required for BCR-mediated activation of p90Rsk, but not p70S6k, via a mitogen-activated protein kinase-independent pathway in B cells. *J. Biol. Chem. 272:18200.*
49. Couture, C., S. Williams, N. Gauthier, P. Tailor, and T. Mustelin. 1997. Role of Tyr518 and Tyr519 in the regulation of catalytic activity and substrate phosphorylation by Syk protein-tyrosine kinase. *Eur. J. Biochem. 246:447.*
50. El-Hillal, O., T. Kurosaki, H. Yamamura, J.-P. Kinet, and A. M. Scharenberg. 1997. Syk kinase activation by a Src kinase-initiated activation loop phosphorylation chain reaction. *Proc. Natl. Acad. Sci. USA 94:1919.*
51. Chu, D. H., H. Spits, J. F. Peyron, R. B. Rowley, J. B. Bolen, and A. Weiss. 1996. The Syk protein tyrosine kinase can function independently of CD45 or Lck in T cell antigen receptor signaling. *EMBO J. 15:6251.*
52. Latour, S., L. M. L. Chow, and A. Veillette. 1996. Differential intrinsic enzymatic activity of Syk and Zap-70 protein-tyrosine kinases. *J. Biol. Chem. 271:22782.*
53. Zoller, K. E., I. A. Macneil, and J. S. Brugge. 1997. Protein tyrosine kinases Syk and Zap-70 display distinct requirements for Src family kinases in immune response receptor signal transduction. *J. Immunol. 158:1650.*
54. Neumeister, E. N., Y. Zhu, S. Richard, C. Terhorst, A. C. Chan, and A. S. Shaw. 1995. Binding of ZAP-70 to phosphorylated T-cell receptor ζ and η enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. *Mol. Cell. Biol. 15:3171.*
55. Sidorenko, S. P., C.-L. Law, S. J. Klaus, K. A. Chandran, M. Takata, T. Kurosaki, and E. A. Clark. 1996. Protein kinase C μ (PKC-μ) associates with the B cell antigen receptor complex and regulates lymphocyte signaling. *Immunity 5:353.*