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Conformational Changes Induced in the Protein Tyrosine Kinase $p72^{syk}$ by Tyrosine Phosphorylation or by Binding of Phosphorylated Immunoreceptor Tyrosine-Based Activation Motif Peptides

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A critical event in signaling in immune cells is the interaction of Syk or ZAP-70 protein tyrosine kinases with multisubunit receptors that contain an ~18-amino-acid domain called the immunoreceptor tyrosine-based activation motif (ITAM). Tyrosine-phosphorylated Syk from activated cells was in a conformation different from that in nonstimulated cells as demonstrated by changes in immunoreactivity. The addition of tyrosine-diphosphorylated ITAM peptides resulted in a similar conformational change in Syk from nonactivated cells. The peptides based on FcεRIγ were more active than those based on FcεRIβ. In vitro autophosphorylation of Syk was dramatically enhanced by the addition of the diphosphorylated ITAM peptides. The conformational change and the enhanced autophosphorylation required the presence of both phosphorylated tyrosines on the same molecule. These conformational changes in Syk by tyrosine phosphorylation or binding to diphosphorylated ITAM could be critical for Syk activation and downstream propagation of intracellular signals.

The binding of antigen by T cells, B cells, and immunoglobulin-bearing basophils or mast cells initiates a cascade of intracellular signals that results in cell activation. The ligand binding domain of the receptors on these cells lacks intrinsic tyrosine kinase activity but is associated with transducing subunits that contain the cytoplasmic motif $(D/E)X_2YX_2LX_{6-8}YX_2(L/I)$, which is critical for cell activation (43, 47, 58). This motif, called the immunoreceptor tyrosine-based activation motif (ITAM), is present in the β and γ chains of FcεRI, in the ζ subunit of the T-cell receptor complex, and in immunoglobulin β (Igβ) and Igα of the B-cell receptor (10, 12). Chimeric proteins with the cytoplasmic domain of ζ or FcεRIγ linked to the extracellular and transmembrane domains of other proteins have been used for signal transduction studies (17, 22, 24, 34, 45). Aggregation of these chimeric molecules by antibodies to their extracellular domains results in cell activation (21, 44). After receptor aggregation, the Syk/ZAP-70 protein tyrosine kinases associate with these receptors and are critical for the downstream activation signals (6, 10, 12, 13, 19, 31).

The Syk/ZAP-70 family of protein tyrosine kinases has two tandem Src homology 2 (SH2) domains in the N-terminal half that interact with specific phosphorylated tyrosine residues in other proteins (6, 14, 28, 30, 37, 55, 57). It is postulated that aggregation of these ITAM-containing receptors results in their phosphorylation on the tyrosine residues in the ITAMs probably because of a Src family protein tyrosine kinase (23). The tyrosine-phosphorylated ITAM then becomes the binding site for the Syk/ZAP-70 family of molecules (32). Although there is some association of Syk/ZAP-70 in the absence of receptor stimulation, the predominant interaction is with receptors that are activated and therefore tyrosine phosphorylated (13). For example, in the RBL-2H3 rat mast cell line aggregation of FcεRI results in the activation of Lyn and in the tyrosine phosphorylation of the β and γ subunits of the receptor. Because of SH2-mediated interactions, there is increased association of Lyn with FcεRIβ and the recruitment of Syk by the tyrosine-phosphorylated γ subunit of the receptor (6, 28, 48).

The Syk/ZAP-70 tyrosine kinases that are bound to the ITAMs are usually tyrosine phosphorylated and activated (13, 14, 16). In transient transfection experiments, the activation of Syk/ZAP-70 required the presence of the Src family kinase (23). It is therefore postulated that the Src family kinase may tyrosine phosphorylate the Syk/ZAP-70 and induce activation (23). Here we present evidence that the binding of Syk to a diphosphorylated ITAM peptide results in a conformational change and in an increase in kinase activity. This change is likely involved in the downstream propagation of tyrosine phosphorylation signals (4, 5, 7).

MATERIALS AND METHODS

Materials. Protein A-agarose, aprotinin, Triton X-100, and ATP were obtained from Sigma (St. Louis, Mo.). The materials for electrophoresis were purchased from NOVEX (San Diego, Calif.), polyvinylidene difluoride transfer membrane was purchased from Millipore (Bedford, Mass.), and the sources of other materials not indicated in this section were as described previously (6).

Antibodies. Two peptides based on the rat $p72^{syk}$ deduced amino acid sequence were synthesized with an additional Cys residue at either the carboxy or the N terminus to allow coupling to proteins. Antibodies to an internal sequence, anti-SykI, were prepared by immunizing rabbits with the EPTGGAWGPDR GLC peptide coupled to keyhole limpet hemocyanin as described previously (6). This peptide sequence is between the second Syk SH2 and kinase domains. The anti-SykC antibodies were prepared with the peptide CAVELRLRNYYYDV VN, which corresponds to the carboxy-terminal amino acids (39). The IgG fraction of the antibodies was purified by GammaBind Plus Sepharose (Pharmacia LKB, Uppsala, Sweden). The anti-SykI antibodies were also affinity purified as described previously (6). All other antibodies have been described previously (3, 25, 53). The affinity-purified polyclonal rabbit antiphosphotyrosine antibodies were coupled to agarose beads as recommended by the manufacturer.

Synthesis of peptides. Peptides based on the sequence of the β and γ subunits of FcεRI (9, 29) were synthesized by solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 430A synthesizer. Phosphotyrosine residues were incorporated by using Fmoc-Tyr(PO_3H_2)OH (40). Cleavage of the peptides from resins and removal of side chains protecting groups were by treatment with trifluoroacetic acid–phenol–thianisole–1,2-ethanedithiol–H_2O (82.5:5:5:2.5:5). The crude peptides were purified by preparative reverse-phase high-pressure liquid chromatography using a Vydac C_8 column with a gradient of

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H_2O and acetonitrile containing 0.05% trifluoroacetic acid. The purity and the mass of the peptides were confirmed by analytical reverse-phase high-pressure liquid chromatography and mass spectroscopy, respectively.

Cell culture and activation. The RBL-2H3 cells were maintained as monolayer cultures, and FcεRI-mediated activation was as described previously (2, 5). The cells were stimulated either with antigen after overnight culture in the presence of antigen-specific IgE or with anti-FcεRIα antibodies (monoclonal antibody BC4). After stimulation for the indicated times, the monolayers were rinsed once with ice-cold phosphate-buffered saline and solubilized in lysis buffer (10 mM Tris [pH 7.5], containing 1.0% Triton X-100, 1 mM Na_3VO_4, 150 mM NaCl, 50 μg of leupeptin per ml, 0.5 U of aprotinin per ml, 2 mM pepstatin A, 1 mM phenylmethylsulfonyl fluoride). The cells were scraped from the plates, the lysates were centrifuged for 15 min at 16,000 × *g* at 4°C, and the postnuclear supernatants were used for these experiments.

Immunoprecipitation. Lysates from 10^7 cells in 1.0 ml were precleared by mixing for 1 h at 4°C with normal rabbit IgG prebound to protein A coupled to agarose beads. The lysates were then added to 5 μg of anti-Syk antibody that had been preincubated with 15 μl of protein A-agarose beads. After gentle rotation at 4°C for 90 min, the beads were washed three times with wash buffer (lysis buffer with Triton X-100 decreased to 0.5%) and once with 150 mM NaCl–50 mM Tris (pH 7.5), and the proteins were eluted by boiling for 5 min with sample buffer as described previously (28). In some experiments, the synthetic peptides were added to the precleared lysates at the indicated concentrations.

Partial proteolysis. Partial proteolysis was as previously described (35). Briefly, cell lysates immunoprecipitated with anti-SykI antibody were washed three times with wash buffer without protease inhibitors and then once with protease buffer (1 mM $CaCl_2$, 0.5 mM Na_3VO_4, 10 mM Tris [pH 8.1]). The immunoprecipitates were resuspended in 10 μl of the protease buffer, 10 μl of trypsin or protease V8 was added to a final concentration of 100 μg/ml, and tubes were then incubated for 5 min at 25°C. The reaction was stopped by adding sample buffer and immediately boiling.

Immunoprecipitation and in vitro kinase assay. Syk was immunoprecipitated with anti-SykI antibodies prebound to protein A beads and washed as described above. They were further washed with kinase buffer (30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 10 mM $MgCl_2$, 2 mM $MnCl_2$) and resuspended in 1 ml of kinase buffer. Synthetic peptides were added at the indicated concentrations, and the reaction was initiated by the addition of ATP (5 μM, final concentration). After incubation at 4°C with gentle rotation, the tubes were centrifuged for 1 min at 16,000 × *g*, and the immunoprecipitates were resuspended in sample buffer and boiled for 5 min. The proteins were then analyzed by immunoblotting with antiphosphotyrosine and anti-Syk antibodies.

Immunoblotting. Samples from the immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electrotransferred to polyvinylidene difluoride membranes. Immunoblotting with the horseradish peroxidase-conjugated antiphosphotyrosine antibody PY-20 was as described previously (52). The antiphosphotyrosine antibodies were stripped from the membranes, and the membranes were reprobed with other antibodies as recommended by the manufacturer. For the immunoblotting with anti-Syk, anti-FcεRIγ, and FcεRIβ antibodies, the secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse antibody. In some experiments, horseradish peroxidase-conjugated protein A was used instead of a secondary antibody. In all blots, proteins were visualized by enhanced chemiluminescence (ECL Kit; Amersham) as previously described (52). Scanning densitometry was with a Pharmacia LKB Imagemaster.

RESULTS

Recognition of only some forms of Syk by a polyclonal antibody. To investigate conformational changes in Syk after cell activation, we used antibodies raised to different parts of the molecule. From lysates of stimulated RBL-2H3 cells, two different anti-Syk antibodies precipitated very different amounts of Syk protein although the signal with antiphosphotyrosine antibodies was similar (Fig. 1A). A polyclonal anti-Syk antibody (anti-SykC) raised to a peptide sequence in the COOH-terminal portion of the molecule precipitated very little Syk from nonstimulated cells but appeared only to precipitate tyrosine-phosphorylated Syk from activated cells (Fig. 1A). In contrast, antibodies to an internal sequence (anti-SykI) precipitated approximately equal amounts of Syk from both stimulated and nonstimulated cells. Although the anti-SykC immunoprecipitated very little Syk from nonstimulated cells, it recognized the denatured protein in immunoblots (Fig. 1B) and precipitated it after Syk had been denatured (Fig. 1B). The sera from five different rabbits immunized with the COOH-terminal peptide all produced antibodies with these characteristics. These results suggest that anti-SykC recognized a linear epitope that was masked prior to cell activation.

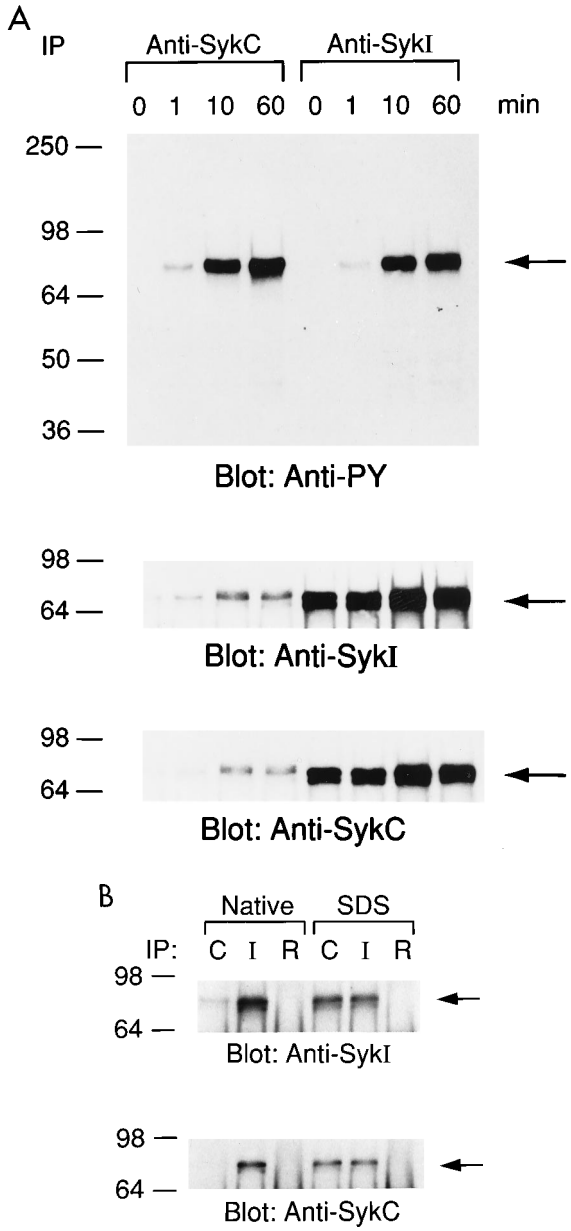

FIG. 1. Immunoprecipitation and immunoblotting with anti-Syk antibodies. (A) Immunoprecipitation from nonactivated or activated cell lysates. RBL-2H3 cells were incubated with IgE and then stimulated by addition of antigen. Cell lysates prepared at the indicated times after stimulation were precipitated with either anti-SykC or anti-SykI antibodies. The precipitated proteins were analyzed by immunoblotting with antiphosphotyrosine antibodies (anti-PY) or anti-SykI or anti-SykC antibodies. In four different experiments, there was 19% more Syk immunoprecipitated with anti-SykI from stimulated than from nonstimulated cells, whereas with anti-SykC there was 287% more Syk immunoprecipitated from stimulated cells. (B) Immunoprecipitation with anti-SykI and anti-SykC from lysates of nonstimulated cells that were either nontreated or incubated with 1% SDS for 10 min. Lysates were diluted 10-fold before immunoprecipitation with anti-SykC (C), anti-SykI (I), or normal rabbit IgG (R). Precipitated proteins were analyzed by immunoblotting; arrows indicate Syk. Molecular mass markers represent migration of prelabeled standards (in kilodaltons).

Tyrosine-phosphorylated Syk has a conformationally different form. Activation of RBL-2H3 cells by aggregation of their high-affinity IgE receptors (FcεRI) results in tyrosine phos-

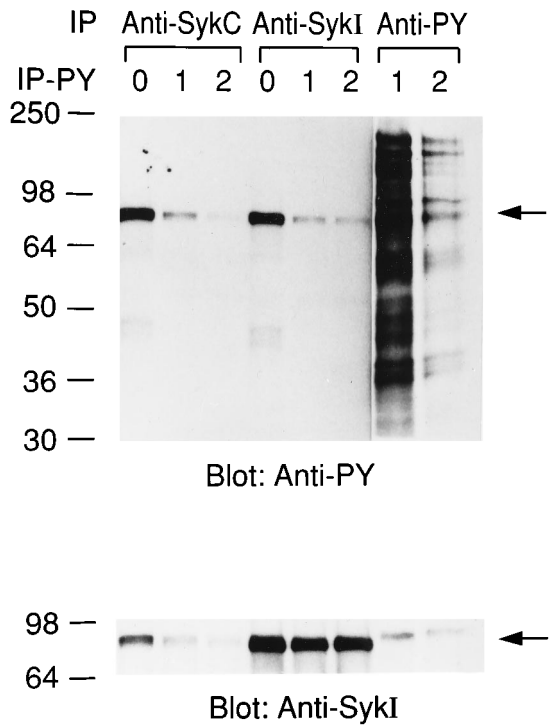

FIG. 2. Tyrosine-phosphorylated Syk was in a conformationally different form. Lysates from activated RBL-2H3 cells were immunoprecipitated with anti-Syk antibodies either directly (IP-PY, 0) or after depletion of tyrosine-phosphorylated proteins by passing once (IP-PY, 1) or twice (IP-PY, 2) over antiphosphotyrosine affinity beads. The immunoprecipitated proteins and the proteins eluted from the antiphosphotyrosine affinity beads were analyzed by immunoblotting with antiphosphotyrosine or anti-SykI antibodies. By densitometric analysis, 14% of Syk was tyrosine phosphorylated. Molecular mass markers represent migration of prelabeled standards (in kilodaltons).

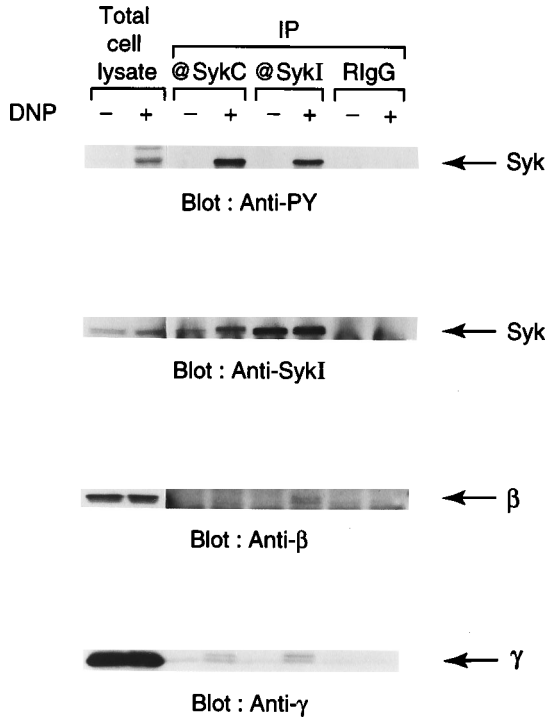

FIG. 3. Association of tyrosine-phosphorylated Syk with FcεRI. Lysates from nonactivated cells (DNP−) or activated cells (DNP+) were immunoprecipitated with anti-SykC (@SykC), anti-SykI (@SykI), or normal rabbit IgG (RIgG). The proteins were separated by SDS-PAGE, and the membrane was cut into three sections. The Syk part of the membrane was analyzed with antiphosphotyrosine (anti-PY), stripped, and then reblotted with anti-Syk antibodies. The two other parts of the membrane were blotted with anti-FcεRIβ and anti-FcεRIγ antibodies.

phorylation of Syk (6, 19, 38). Here in our experiments, the slower migration of the Syk precipitated by anti-SykC suggested that this antibody recognized the tyrosine-phosphorylated form of the molecule. Therefore, we tested whether tyrosine phosphorylation of Syk results in a conformational change that allows the anti-SykC antibody to recognize the protein (Fig. 2). Lysates from stimulated cells were depleted of tyrosine-phosphorylated proteins by two rounds of absorption with antiphosphotyrosine affinity beads. Although this resulted in a minor decrease in the Syk present in the lysates, it markedly depleted the Syk that was precipitated by anti-SykC. Even though anti-SykC immunoprecipitated Syk from activated cells, it did not bind to other tyrosine-phosphorylated proteins, nor was the precipitation of Syk inhibited by phenyl phosphate, an analog of phosphotyrosine. Therefore, anti-SykC recognizes Syk that has a different conformation after tyrosine phosphorylation whereas the anti-SykI binds Syk irrespective of its state of phosphorylation. Furthermore, only a very small fraction of total Syk is tyrosine phosphorylated after cell activation.

Association of tyrosine-phosphorylated Syk with FcεRI. Stimulation of cells results in the association of the Syk/ZAP-70 family of protein tyrosine kinases with the phosphorylated tyrosines on the ITAM motif of proteins (6, 13, 20). For example, both β and γ subunits of FcεRI are coprecipitated with Syk after receptor aggregation (6). In the present experiments, both anti-SykC and anti-SykI precipitated equal amounts of the γ subunit of FcεRI from lysates of stimulated cells but not from nonstimulated cells (Fig. 3). Although anti-SykC precipitated much less Syk than did anti-SykI, the amount of associated γ was approximately equal with both antibodies. There was also much less of the β subunit of FcεRI associated with Syk, and this was detected only in the anti-SykI immunoprecipitates. Therefore, only a small fraction of Syk was tyrosine phosphorylated, and most of that was associated with FcεRI.

Changes in the conformation of Syk induced by phosphorylated peptides. The interaction of Syk with tyrosine-phosphorylated receptor subunits could result in a conformational change that allowed precipitation with anti-SykC antibodies. Therefore, we tested several nonphosphorylated and tyrosine-phosphorylated synthetic peptides corresponding to the ITAMs of the β and γ subunits of FcεRI (Fig. 4A). When diphosphorylated γ ITAM peptide was added to the cell lysates, there was enhanced precipitation of Syk with the anti-SykC antibodies (Fig. 4B). This increase was apparent with lysates from both nonactivated and stimulated cells. Comparison of the antiphosphotyrosine with the anti-Syk immunoblots indicated that anti-SykC antibodies precipitated nonphosphorylated Syk after the addition of the diphosphorylated ITAM. However, the increased immunoprecipitation was not observed with the anti-SykI antibodies or with non-tyrosine-phosphorylated peptides. Therefore, interaction of diphosphorylated ITAM peptides with Syk induces a conformational change that allows the immunoprecipitation with anti-SykC antibodies.

Dose-response curves were used to further define the effect of the tyrosine-diphosphorylated γ ITAM peptide on the immunoprecipitation with anti-SykC antibodies (Fig. 5). From the lysates of nonactivated cells, there was a dose-dependent

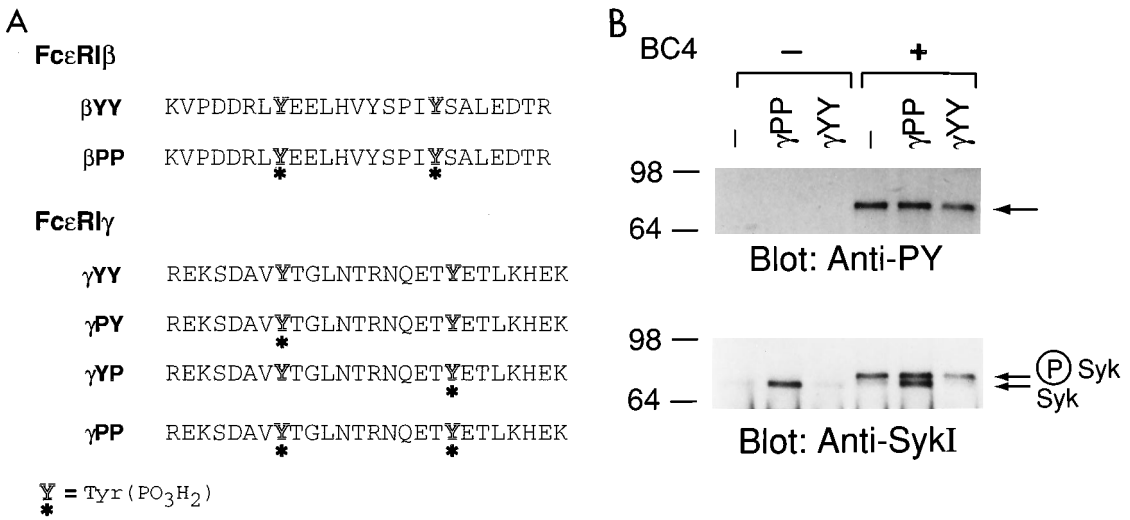

FIG. 4. Changes in the immunoprecipitation of $p72^{syk}$ in the presence of tyrosine-phosphorylated ITAM peptides. (A) Synthetic peptides corresponding to the ITAM of the β and γ subunits of FcεRI. The tyrosine residues in the ITAM motif are shadowed. In this nomenclature, γ refers to FcεRIγ and β refers to FcεRIβ. The βYY is unphosphorylated, whereas βPP is the diphosphorylated ITAM of FcεRIβ. The γYY is the unphosphorylated peptide; γPP is the same peptide with both tyrosines phosphorylated; γYP and γPY are monophosphorylated peptides with phosphorylation of either the first or the second tyrosine. (B) Cell lysates from nonactivated cells (BC4−) or anti-FcεRI monoclonal antibody BC4-stimulated cells (BC4+) were incubated with buffer or nonphosphorylated or tyrosine-phosphorylated ITAM peptides (at 1 μM) and then immunoprecipitated with anti-SykC. The immunoprecipitates were analyzed by immunoblotting with antiphosphotyrosine followed by anti-SykI antibodies.

enhancement of the amount of precipitated Syk. With lysates from activated cells, there were two populations of Syk molecules precipitated by anti-SykC. There was no change in the amount of tyrosine-phosphorylated Syk after the addition of the diphosphorylated γ ITAM peptide. In contrast, this peptide resulted in a dose-dependent immunoprecipitation of a faster-migrating non-tyrosine-phosphorylated Syk. Syk expressed in insect cells (in the absence of ITAM molecules) was tyrosine phosphorylated and could be immunoprecipitated with anti-SykC antibodies (28a). Therefore, Syk molecules that are tyrosine phosphorylated or that have bound ITAM peptides have similar conformations.

Previous studies have shown that there is selectivity in the interaction of phosphorylated ITAM peptides with SH2 domains. For example, Syk preferentially binds to the γ subunit of FcεRI but very weakly if at all to the β subunit (28, 48). As expected from previous results, the addition of the diphosphorylated β ITAM peptide produced only a very slight increase in the immunoprecipitation of Syk (Fig. 6). There were minimal effects with the monophosphorylated synthetic γ ITAM peptides. Thus, a conformational change of Syk is induced by the binding of diphosphorylated ITAM peptide.

Change in sensitivity of Syk to trypsin digestion by binding of diphosphorylated ITAM peptide or tyrosine phosphorylation. Altering the conformation of a protein changes its sensi-

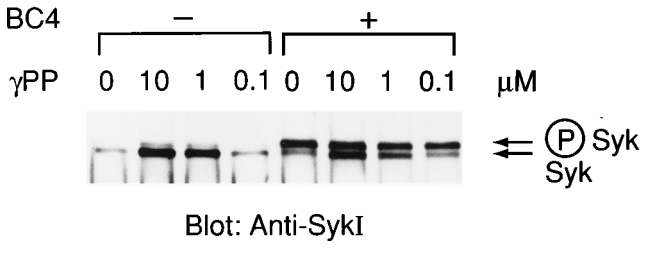

FIG. 5. Dose-response curves of the effect of the γPP peptides on immunoprecipitation with anti-SykC antibodies. The diphosphorylated γ ITAM peptide was added to cell lysates from nonactivated cells (BC4−) or cells activated with anti-FcεRI monoclonal antibody BC4 (BC4+), and then Syk was immunoprecipitated with anti-SykC antibodies. The precipitates were analyzed by immunoblotting with anti-SykI antibodies.

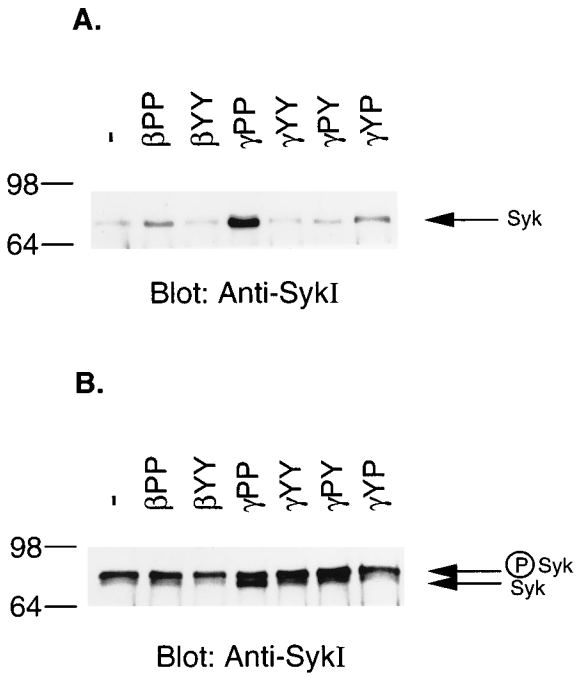

FIG. 6. The effect of different ITAM peptides on the immunoprecipitation of Syk. The peptides (final concentration, 1 μM) were added to cell lysates from nonstimulated (A) or IgE-stimulated (B) cells and then immunoprecipitated with anti-SykC antibodies. The precipitates were analyzed by immunoblotting with anti-SykI antibodies.

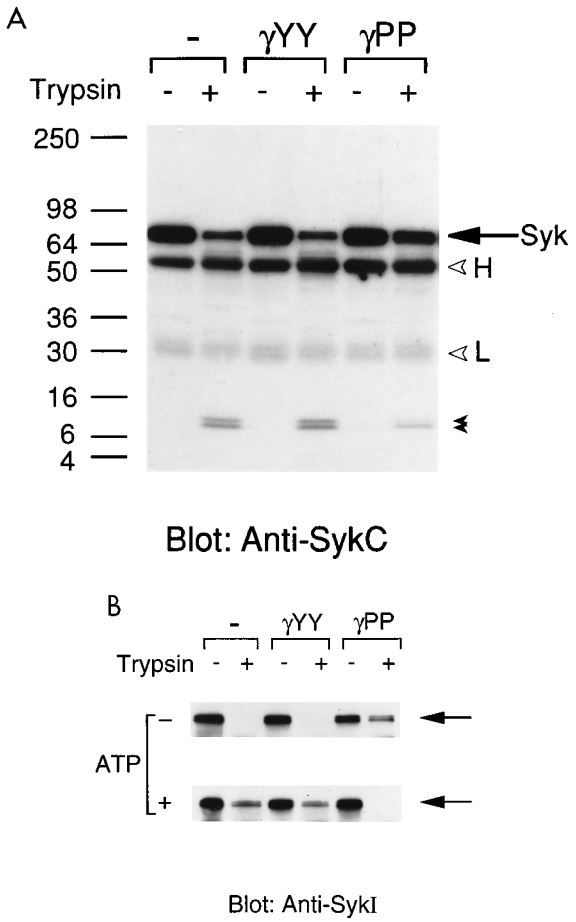

FIG. 7. Effect of ITAM peptide and tyrosine phosphorylation on trypsin digestion of Syk. (A) Lysates from nonstimulated cells were precipitated with anti-SykI coupled to Sepharose 4B beads in the presence or absence of 1 μM ITAM peptides. The washed precipitates were then incubated with trypsin (final concentration, 100 μg/ml) for 5 min at 25°C. The samples were analyzed by immunoblotting with anti-SykC. Open arrowheads indicate the IgG heavy and light chains (H and L); solid arrowheads point to the ~8-kDa Syk fragment. (B) Lysates of nonstimulated cells were precipitated with anti-SykI bound to protein A-agarose beads. Some of the sample was subjected to in vitro kinase reaction (ATP+) with 5 μM ATP for 1 h at 4°C. After washing, 1 nmol of ITAM peptides was added at 4°C for 10 min. After addition of trypsin (100 μg/ml, final concentration), tubes were incubated at 25°C for 5 min. Samples were analyzed by immunoblotting with anti-SykI. Arrows indicate Syk.

tivity to proteolytic cleavage (35). Therefore, we tested the effect of the conformation of Syk on its sensitivity to proteolytic digestion. Under different experimental conditions, the native form of Syk was more susceptible to digestion than when either it had bound diphosphorylated ITAM peptide or it was tyrosine phosphorylated (Fig. 7). For these experiments, lysates from nonstimulated cells were precipitated with anti-SykI in the presence or absence of ITAM peptides. There was rapid digestion of Syk by the addition of trypsin with the formation of a ~8-kDa doublet that was detected by anti-SykC (Fig. 7A). The presence of the diphosphorylated γ ITAM peptide inhibited Syk digestion and the appearance of the COOH-containing fragments. These results were confirmed by digestion of Syk by a different method for immunoprecipitation, again from lysates of nonstimulated cells (Fig. 7B, upper panel). Syk that was autophosphorylated in vitro without added ITAM was also more resistant to proteolytic digestion (Fig. 7B, lower panel). The proteolytic digestion of phosphorylated Syk was similar to that of nonphosphorylated Syk that was bound to diphosphorylated ITAM peptides. Interestingly, with the autophosphorylated Syk the addition of the phosphorylated ITAM peptide enhanced the tryptic digestion. Similar digestion of Syk was observed when protease V8 was used instead of trypsin (data not shown). The addition of the nonphosphorylated ITAM peptide did not change the digestion of Syk. Altogether, these results support the concept that the conformation of Syk is changed by binding of phosphorylated ITAM peptide or by tyrosine phosphorylation.

Diphosphorylated peptides enhance the autophosphorylation of Syk. We also tested whether the binding of the tyrosine-diphosphorylated γ ITAM peptide induced any change in the kinase activity of the Syk molecule. Syk was immunoprecipitated with the anti-SykI antibodies and subjected to immune complex kinase assays (Fig. 8). The slow autophosphorylation of Syk during this in vitro incubation was dramatically increased by the addition of the diphosphorylated ITAM peptides (Fig. 8A). This was apparent at 10 min of incubation and throughout the 60 min of incubation. In dose-response studies, the diphosphorylated γ ITAM was more active than the β ITAM peptide (Fig. 8B). The nonphosphorylated peptides had no effect, whereas the monophosphorylated γ ITAM peptides were much less effective than either of the two diphosphorylated ITAM peptides. There was no additive effect by the addition of the two monophosphorylated γ ITAM peptides.

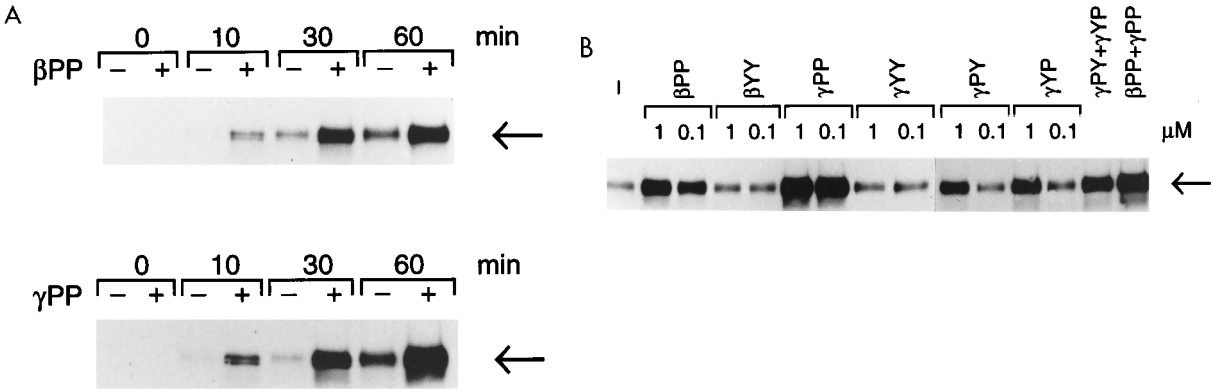

FIG. 8. The effect of ITAM peptides on enzymatic activity of Syk. For in vitro kinase assays, ITAM peptides were added to Syk immunoprecipitated with anti-SykI antibodies from nonactivated cells. The precipitates were analyzed by immunoblotting with antiphosphotyrosine and anti-SykI antibodies. (A) Time of in vitro incubation. The diphosphorylated ITAM peptides based on the β and γ subunits of FcεRI were at a final concentration of 1 μM. (B) The effect of different ITAM peptides on the autophosphorylation of Syk. Inocubation was for 60 min. There was a 1 μM concentration of each of the two phosphorylated peptides when two peptides were used. Arrows indicate Syk.

These results indicate that the conformational changes induced by the ITAM peptides are accompanied by enhanced kinase activity.

DISCUSSION

The data strongly indicate that anti-SykC antibodies recognize a conformationally different form of the Syk molecule. This form was induced either by tyrosine phosphorylation of Syk or by the binding of the diphosphorylated ITAM peptides. This conclusion is based on the following experimental observations. First, both anti-Syk antibodies recognized denatured Syk in immunoblots and both antibodies precipitated Syk if it was first denatured. Second, the anti-SykC antibodies failed to bind the non-tyrosine-phosphorylated native form of Syk, although both antibodies recognize the molecule after it is tyrosine phosphorylated. Third, in the presence of diphosphorylated ITAM peptides both antibodies recognized the Syk protein. Fourth, the sensitivity of Syk to trypsin digestion was changed by binding of diphosphorylated ITAM peptide or after autophosphorylation.

Although anti-SykC immunoprecipitated Syk from lysates of activated cells, it was not directed towards phosphotyrosine residues. First, it did not precipitate other tyrosine-phosphorylated proteins. Second, it precipitated Syk which was not tyrosine phosphorylated but had bound diphosphorylated ITAM molecules. Third, the precipitation was not inhibited by phenylphosphate, an analog of phosphotyrosine.

Protease digestion experiments further support the concept that Syk has several conformations. Thus, digestion of Syk was inhibited by diphosphorylated γ ITAM peptides and when Syk was tyrosine phosphorylated. This suggests that either binding of phosphorylated ITAM peptides or tyrosine phosphorylation results in similar changes in the conformation of Syk such that protease digestion sites are less accessible. However, as both manipulations resulted in an increase in the enzymatic activity of Syk, the kinase domain of the molecule must have easier access to its substrate. Nevertheless, addition of diphosphorylated ITAM to tyrosine-phosphorylated Syk resulted in a paradoxical increase in digestion by trypsin. This suggests that there are also differences in the conformation induced by ITAM and in that due to tyrosine phosphorylation. Thus, Syk appears to have multiple conformations.

Our experiments do not provide evidence for intramolecular interactions between different portions of the Syk molecule. Thus, glutathione *S*-transferase–fusion proteins of Syk that contained the first 269 amino acids (including both SH2 domains) did not precipitate Syk from cell lysates (28). Furthermore, a 40-kDa COOH fragment of Syk was precipitated with SykC from cell lysates left for 2 h at room temperature (55) but did not bind to glutathione *S*-transferase–Syk fusion proteins in membrane binding assays.

The addition of tyrosine-phosphorylated ITAM to Syk immunoprecipitated with anti-SykI resulted in an increased kinase activity and autophosphorylation. This suggests that the change in conformation induced by the binding of tyrosine-diphosphorylated ITAM by itself is enough to induce a change in the kinase activity of Syk and does not require the presence of another kinase. While these studies were in progress, Shiue et al. (49) and Rowley et al. (46) reported that the kinase activity of Syk was enhanced by diphosphorylated ITAM peptides. Our observations of a conformational change on the binding of the diphosphorylated ITAM peptides provide an explanation for these two reports. In immunoprecipitates with anti-SykC, addition of phosphorylated ITAM peptides did not enhance the in vitro kinase activity (reference 49 and unpublished observations). Furthermore, in anti-SykI immunoprecipitates, addition of anti-SykC antibody increased the autophosphorylation of Syk in the in vitro kinase reaction (unpublished observations). These results suggest that anti-SykC binds to and maintains Syk in its active conformation.

Tyrosine phosphorylation of platelet-derived growth factor receptor results in a conformational change as demonstrated by changes in immunoreactivity (8, 27). The tyrosine-phosphorylated residues then become sites for high-affinity interactions with SH2 domains on either the same or other proteins. Furthermore, intramolecular and intermolecular interactions mediated by SH2 or SH3 domains not only recruit specific partners in signaling cascades but also modulate the enzymatic activity of protein tyrosine kinases and phosphatases (1, 11, 15, 18, 26, 33, 41, 42, 50, 54). What is striking in the present results is the strong requirement for diphosphorylated peptides for both the conformational changes and the autophosphorylation of Syk. Similarly, there is activation of phosphatidylinositol 3′-kinase by binding of the SH2 domains of its p85α regulatory subunit to two phosphotyrosines 11 amino acids apart in the platelet-derived growth factor receptor (11, 15, 26, 41, 50).

In activated RBL-2H3 cells, Syk is tyrosine phosphorylated and also binds to the tyrosine-phosphorylated ITAM motifs of FcεRI. Therefore, both tyrosine phosphorylation and the binding to phosphorylated ITAM motifs could contribute to immunoprecipitation of Syk with anti-SykC antibodies. In analysis of the precipitates with anti-SykC, some Syk could be detected that was faster migrating and non-tyrosine phosphorylated. The amount of this increased in activated cells. The observation that it was precipitated with the anti-SykC suggests that this was nonphosphorylated Syk that was associated with tyrosine-phosphorylated subunits of FcεRI. Similarly in thymocytes and after anergic peptide stimulation of T cells, there is tyrosine-phosphorylated ζ with associated nonphosphorylated ZAP-70 (36, 51, 56). Although only a small fraction of Syk was tyrosine phosphorylated in activated cells, most of the tyrosine-phosphorylated Syk was associated with FcεRI. This could indicate that Syk is rapidly dephosphorylated if it is not associated with ITAM motifs.

Transfection experiments in COS cells suggest that activation of ZAP-70 requires three molecules; the ITAM-containing receptor domain, a Src family kinase, and the ZAP-70 kinase (23). The conformational change induced by binding of diphosphorylated ITAM suggests a model for the control of the function of the Syk/ZAP-70 protein tyrosine kinases. Phosphorylation of the two tyrosines in the ITAM by Src family kinases recruits Syk to immune receptors. The two SH2 domains of Syk form a bidentate interaction with diphosphorylated ITAM motifs, resulting in a conformational change in Syk. This conformational change results in tyrosine phosphorylation of Syk. The activated and tyrosine-phosphorylated Syk could then phosphorylate other substrates, such as phospholipase Cγ, and propagate downstream activation signals.

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