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syk kinase activation by a src kinase-initiated activation loop phosphorylation chain reaction

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ABSTRACT **Activation of the syk tyrosine kinase occurs almost immediately following engagement of many types of antigen receptors, including Fc receptors, but the mechanism through which syk is activated is currently unclear. Here we demonstrate that Fc receptor-induced syk activation occurs as the result of phosphorylation of the syk activation loop by both src family kinases and other molecules of activated syk, suggesting that syk activation occurs as the result of a src kinase-initiated activation loop phosphorylation chain reaction. This type of activation mechanism predicts that syk activation would exhibit exponential kinetics, providing a potential explanation for its rapid and robust activation by even weak antigen receptor stimuli. We propose that a similar mechanism may be responsible for generating rapid activation of other cytoplasmic tyrosine kinases, such as those of the Bruton tyrosine kinase/tec family, as well.**

Fc receptors are multisubunit receptors present on immunologic cells that are responsible for linking the humoral immune response with cellular immune effectors. These receptors do not possess intrinsic enzymatic activity; instead, their signal transduction pathways rely on activation of several families of nonreceptor tyrosine kinases (1–4). Among these kinases, the syk/zap70 family member syk has been shown to be activated following engagement of Fc receptors (5, 6) and to be critical for linking receptor engagement to many early downstream events including calcium mobilization, activation of the ras/mitogen-activating protein kinase pathway, and histamine release (7–10).

Regulation of the activity of tyrosine kinases is a critical issue for cells because tyrosine phosphorylation-mediated signaling pathways are utilized for the control of many cellular functions. The recent elucidation of the crystal structure of the catalytic core of the insulin receptor tyrosine kinase has provided an important insight into how tyrosine kinases are regulated (11). All known tyrosine kinases have a structure known as the activation loop that contains a roughly central tyrosine residue. In the insulin receptor tyrosine kinase structure, the activation loop lies in a groove between the catalytic lobe and the ATP binding lobe, with the central tyrosine residue occupying the catalytic site (11). Based on this structure, a pseudosubstrate inhibition mechanism for the regulation of tyrosine kinase activity has been proposed (11). In this mechanism, tyrosine phosphorylation of the activation loop central tyrosine moves the activation loop structure out of the active site, thereby allowing substrates access to the active site and ATP access to the ATP binding site with resulting "activation" of the catalytic activity of the tyrosine kinase. This mechanism has the important implication that understanding how activation loop tyrosine phosphorylation is generated is critical for understanding how the activity of a particular tyrosine kinase is controlled.

Because of syk's pivotal role in signaling by various types of antigen receptors (9, 12), the mechanism through which it is activated has been a focus of intense investigation. In support of the idea that syk activity is regulated through a pseudosubstrate inhibition mechanism, studies of B cell receptor signaling have shown that syk activation in this system is lyn-dependent and requires the presence of an intact activation loop (13, 14), although the relationship between activation loop tyrosine phosphorylation and syk activity was not addressed. However, *in vitro* studies have suggested that syk activation can be induced directly by binding of phosphopeptides to its tandem SH2 domains or by syk autophosphorylation at sites apparently other than within the activation loop (15), leaving the role or even the existence of receptor-induced syk activation loop tyrosine phosphorylation an open question. The studies described in this paper represent a detailed analysis of Fc receptor-induced syk tyrosine phosphorylation and activation. Our results suggest that tyrosine phosphorylation of the syk activation loop is required for Fc receptor-induced syk activation, and that it is generated as a result of phosphorylation by both src family kinases and other molecules of activated syk.

MATERIALS AND METHODS

Construction of Recombinant Vaccinia Viruses. Viruses driving expression of lynA, wild-type porcine syk, and truncated porcine syk (syk-T) have been described (9). Porcine syk cDNAs with point mutations at position 395 (lysine to arginine) or positions 518 and 519 (both tyrosines changed to phenylalanine) were constructed as described (12, 13). These cDNAs were subcloned into the pSC-65 vaccinia recombination plasmid, and recombinant vaccinia viruses were generated using standard techniques (16).

Cell Culture, Vaccinia Infections, Cell Stimulations, and Cell Lysis. 3T3E cells are NIH 3T3 cells that have been transfected with the α, β, and γ subunits of the high affinity IgE receptor (FcεRI) (9). 3T3E cells, RBL-2H3 cells, their cell culture methods, and all vaccinia infections were as described (9). Infected cells were stimulated by adding DNP(24–40)-HSA (Sigma) to a final concentration of 250 ng/ml in 1 ml total volume of DMEM/25 mM Hepes cell suspension for the time indicated. Cells were lysed in borate-buffered saline containing 0.5% Triton-X100, 2 mM sodium vanadate, 5 mM NaF, 5 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 5 μg/ml pepstatin.

Antibodies, Immunoprecipitations, and Western Immunoblotting. Monoclonal anti-2,4-dinitrophenyl IgE, rabbit polyclonal anti-IgE, and rabbit polyclonal anti-porcine syk were obtained and used as described (9). Monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology,

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Lake Placid, NY) was used for anti-phosphotyrosine immunoblotting. Polyclonal anti-syk recognizing both rat and porcine syk was purchased from Upstate Biotechnology. Immunoprecipitations, *in vitro* kinase assays, SDS/PAGE, and Western transfers were performed as described (9). Immunoblotting was performed according to the protocols supplied with the enhanced chemiluminescent detection kit (Amersham).

Peptide Mapping Analyses. 3T3E cells were infected with lyn virus and the indicated syk virus in phosphate-free DMEM with 25 mM Hepes medium for 30 min at 4°C, and then for 1 h at 37°C before labeling with [^{32}P]orthophosphate for 2 h at 37°C. Infected cells were harvested and then washed twice in phosphate-free DMEM with 25 mM Hepes before resuspension in 1 ml phosphate-free DMEM with 25 mM Hepes. Infected cells were then stimulated and lysed as above. syk was purified by incubating ^{32}P-labeled lysates with porcine anti-syk antiserum immobilized on protein A-Sepharose (Pharmacia) for 4–12 h. Beads were washed twice with lysis buffer, eluted with SDS/PAGE loading buffer, and bound protein was separated by SDS/PAGE, transferred to Immobilon polyvinylidene difluoride membranes (Millipore), and visualized by autoradiography. Tryptic digests of the bound protein, tryptic peptide oxidation, and anti-phosphotyrosine immunoprecipitation of the peptides were then performed using standard methods (17). Briefly, the polyvinylidene difluoride membranes were stained with Naphthol Blue Black (Sigma) to visualize protein corresponding to the radioactive band on the autoradiogram, the band was excised, blocked with 0.5% PVP-360 (polyvinylpyrrolidone; Sigma) for 30 min at 37°C, washed five times with H_2O and twice with 50 mM NH_4HCO_3, and digested with 10 μg L-l-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Sigma) in 50 mM NH_4HCO_3 for 4–12 h at 37°C followed by an additional cleavage with 10 μg TPCK-trypsin for 2 h to ensure complete cleavage. Tryptic peptides were then lyophilized, washed with H_2O and relyophilized three times in decreasing volumes of water, subjected to oxidation in performic acid (Sigma), again lyophilized, resuspended in NH_4HCO_3, and immunoprecipitated with anti-phosphotyrosine antibody 4G10 immobilized on protein A-Sepharose for 1 h at 0°C. Beads were washed twice with 50 mM NH_4HCO_3, and the bound peptides were eluted with two applications of pH 1.9 electrophoresis buffer. The portion of the tyrosine-phosphorylated tryptic peptides bound to the beads and subsequently eluted was designated the eluate fraction, while the rest of the total tryptic peptide population which did not bind (including all washes) was designated the supernatant fraction. The eluate fractions were lyophilized and directly resuspended in pH 1.9 electrophoresis buffer, while the supernatant fractions were lyophilized, washed twice with water, and resuspended in pH 1.9 electrophoresis buffer. Two-dimensional phosphoamino acid analyses of an aliquot of the eluate and supernatant from the wild-type syk anti-phosphotyrosine immunoprecipitation, and two-dimensional thin layer electrophoresis/thin layer chromatography of the eluates from all anti-phosphotyrosine immunoprecipitations were performed as described (17).

RESULTS

Generation of Recombinant Vaccinia Driving Expression of syk Mutants. To analyze Fc receptor-induced tyrosine phosphorylation and activation of syk, recombinant vaccinia viruses that drive expression of various porcine syk mutants were generated for use as signaling probes. The schematics of the syk constructs used for the construction of these viruses are outlined in Fig. 1*A*. Each mutant was constructed on a porcine syk backbone corresponding to the wild-type porcine syk. The syk-K mutant has a point mutation (lysine to arginine) within the ATP binding region at position 395 which eliminates its

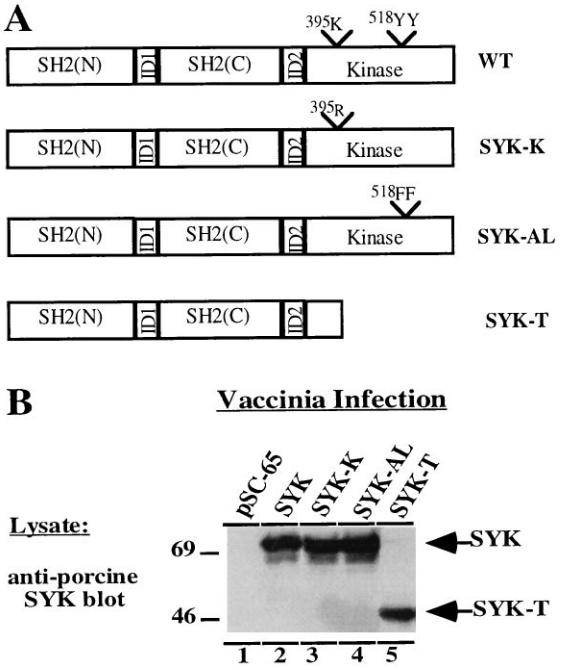

FIG. 1. Structure and expression of porcine syk mutants. (*A*) Schematic representation of syk cDNA constructs used for construction of recombinant vaccinia viruses. SH2(N), NH2 terminal Src homology 2 domain; SH2(C), COOH terminal Src homology 2 domain; ID1, interdomain region between the tandem SH2 domains; ID2, interdomain region between SH2(C) and the tyrosine kinase domain. The amino acid residues of wild-type syk that are mutated in the syk-K (Lys-395 to arginine) and syk-AL mutants (Tyr-518 and Tyr-519 to phenylalanine) are indicated. (*B*) Expression of various syk mutants in RBL-2H3 cells. RBL-2H3 cells were infected with the indicated viruses for 14 h. Cells were then lysed and analyzed by immunoblotting with an anti-porcine syk-specific antibody.

ability to bind ATP and so all of its tyrosine kinase activity. The syk-AL mutant has both tyrosines within its activation loop structure (positions 518 and 519 as indicated) mutated to phenylalanine. The syk-T mutant is truncated at position 395 so that it encompasses both SH2 domains and both interdomain regions, but is missing most of the tyrosine kinase domain including the active site and activation loop region (9). These viruses drive expression of appropriate proteins when used to infect RBL-2H3 cells (Fig. 1*B*) or a variety of other cell types (data not shown). It is important to note that since the virally expressed proteins are porcine, they are immunologically distinguishable from syk proteins of other species (such as rat syk) by using anti-porcine syk-specific antibodies for immunoblotting (as was done in Fig. 1*B*) or immunoprecipitation (as we do in several experiments below).

Probing of syk Phosphorylation Induced by Fc Receptor Engagement on RBL-2H3 Mast Cells. In our initial experiments, a model Fc receptor signaling system, the RBL-2H3 mast cell line, was probed by using two of these viruses to express varying levels of syk or syk-K (Fig. 2*A*). As can be seen, a 4-h infection produces no detectable increase in the amount of total syk detected by an antibody that recognizes both rat and porcine syk, while a 14-h infection produces a large increase in the total syk present (assuming that the antibody affinity for porcine and rat syk is similar, this would be an $\approx$20-fold increase as assessed by densitometric analysis; data not shown). Therefore, 4-h infection conditions allow us to sample the "phosphorylation environment" around the expressed proteins without substantially altering that environment. This produces experimental conditions that can differentiate whether a particular molecule of syk is phosphorylated by other kinase molecules (syk-K would become phosphory-

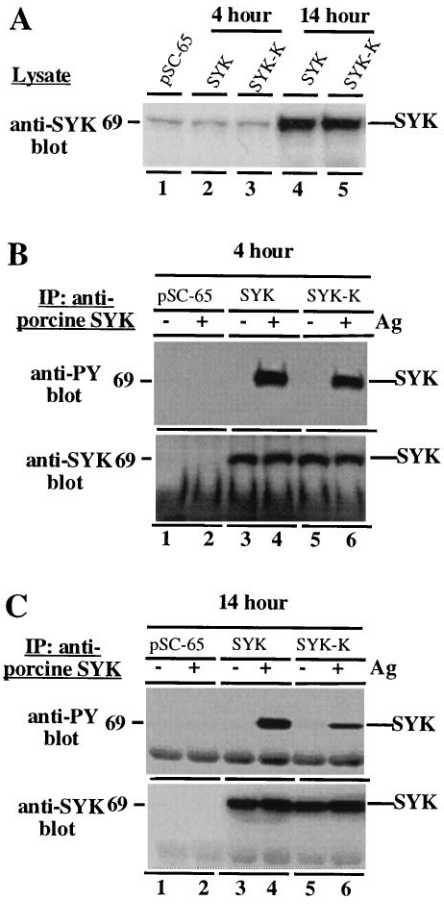

FIG. 2. syk tyrosine phosphorylation is generated primarily by syk transphosphorylation and phosphorylation by other kinases. (*A*) Relative expression of syk with 4-h or 14-h vaccinia infections. RBL-2H3 cells were infected with the indicated viruses for either 4 h (lanes 1–3) or 14 h (lanes 4 and 5). Cells were then lysed and analyzed by immunoblotting with an anti-syk antibody capable of recognizing both rat and porcine syk. This experiment was done as a pilot experiment to determine the expression time course of syk after infection. The experiments below were done separately using the conditions defined in this experiment. (*B*) Tracer expression of wild-type syk or syk-K results in similar levels of Fc receptor-induced tyrosine phosphorylation. RBL-2H3 cells were loaded with IgE, infected for 4 h with the indicated recombinant vaccinia viruses, stimulated or not with antigen, and lysed. (*Upper*) Lysates were subjected to precipitation with anti-porcine syk antibody and analyzed by anti-phosphotyrosine immunoblotting. (*Lower*) The blot was then stripped and reprobed by anti-syk immunoblotting to verify equivalent amounts of syk protein in each lane. (*C*) High level overexpression of wild-type syk or syk-K results in substantially reduced tyrosine phosphorylation of syk-K relative to wild-type syk. Identical experiment to above except that RBL-2H3 cells were infected for 14 h with the indicated recombinant vaccinia viruses. (*Upper*) Lysates were subjected to precipitation with anti-porcine syk antibody and analyzed by anti-phosphotyrosine immunoblotting. (*Lower*) The blot was then stripped and reprobed by anti-syk immunoblotting to verify equivalent amounts of syk protein in each lane.

lated) or autophosphorylates (syk-K would not become phosphorylated). On the other hand, 14-h infections provide experimental conditions in which the overexpressed kinase is quantitatively dominant, allowing us to test whether syk transphosphorylates (i.e., activated syk phosphorylates other molecules of syk, in which case syk-K would be expected to show little tyrosine phosphorylation), or is due completely to phosphorylation by other kinases (in which case syk-K would be tyrosine phosphorylated to the same level as wild-type syk).

The results of a 4-h experiment are shown in Fig. 2*B*. The use of a porcine syk-specific antibody for immunoprecipitation allows the state of phosphorylation of the virally expressed syk-K and wild-type porcine syk to be assessed independently of that of the endogenous rat syk. Under these conditions, the level of syk-K tyrosine phosphorylation is nearly identical to that of wild-type porcine syk. This suggests that only a very small amount of Fc receptor-induced syk tyrosine phosphorylation could be accounted for by intramolecular autophosphorylation, and it follows that the vast majority of syk tyrosine phosphorylation must necessarily be due to phosphorylation by other kinase molecules. The results of an experiment performed with a 14-h infection are shown in Fig. 2*C*. In this case, there is a clear reduction in the level of tyrosine phosphorylation of syk-K relative to wild-type syk. For the interpretation of this experiment it is important to note that (*i*) lyn activity is not differentially affected by the overexpression of active or inactive forms of syk—the "*in vivo*" activity of lyn toward known lyn substrates, the FcεRI FcRβ and FcRγ subunits—is similar after overexpression of wild-type syk or syk-T (9), and *in vitro* lyn activity after overexpression of wild-type syk or syk-K is identical (data not shown); and (*ii*) overexpression of wild-type syk or syk-T have the identical effect of reducing endogenous rat syk phosphorylation, demonstrating that wild-type syk overexpression is not causing hyperphosphorylation of syk molecules via hyperactivation of other kinase families (9). Therefore, the reduced phosphorylation of syk-K relative to wild-type syk observed under these conditions is best explained by a loss of syk transphosphorylation. However the differential here (an ≈5-fold reduction by densitometry; data not shown) is less than might be expected based on the degree of syk-K overexpression (≈20-fold as noted above) if all syk tyrosine phosphorylation were due to syk transphosphorylation. Therefore, these results further suggest that at least part of the detectable Fc receptor-induced syk phosphorylation may be due to other kinase families.

Analysis of Fc Receptor-Induced syk Tyrosine Phosphorylation and Activation in a Heterologous System. We have previously established a heterologous expression system in NIH 3T3 cells [designated the 3T3E reconstitution system (9)] for reconstitution of antigen-controlled interactions between tyrosine kinases and the high affinity IgE Fc receptor, FcεRI. The early antigen stimulated tyrosine kinase activation events that occur in this system are identical to those in native FcεRI signaling systems (9, 18). Because it contains no endogenous hematopoietic specific tyrosine kinases, the 3T3E reconstitution system provides an experimental environment in which the contributions of specific kinases to syk tyrosine phosphorylation and activation can be isolated and analyzed in detail.

The results from Fig. 2 suggested that syk tyrosine phosphorylation was due to both phosphorylation by other kinases and syk transphosphorylation. In the 3T3E system, receptor-induced syk activation is dependent on exogenous expression of a hematopoietic-specific src family kinase, such as lyn (9). Therefore our initial experiments in the 3T3E system focused on isolating the functional contributions of lyn and syk to Fc receptor-induced syk tyrosine phosphorylation (Fig. 3*A*). As previously described (9), receptor-induced phosphorylation of wild-type syk is greater in the presence of lyn than in its absence (compare lanes 2 and 8). Comparison of the level of Fc receptor-induced tyrosine phosphorylation of syk-K which occurs in the absence of lyn to that which occurs in the presence of lyn (compare lane 4 to lane 10) demonstrates that lyn is able to phosphorylate syk directly. The small amounts of phosphorylation [note that low levels of phosphorylation of syk-K and syk-AL in the absence of lyn are detectable on longer exposures (data not shown)] induced by Fc receptor stimulation in the absence of lyn expression are likely due to the presence of endogenous src in the 3T3E cells acting similarly to, but less efficiently than, lyn (9). The increased level of tyrosine phosphorylation of wild-type syk relative to syk-K in either the absence or presence of lyn (compare lane

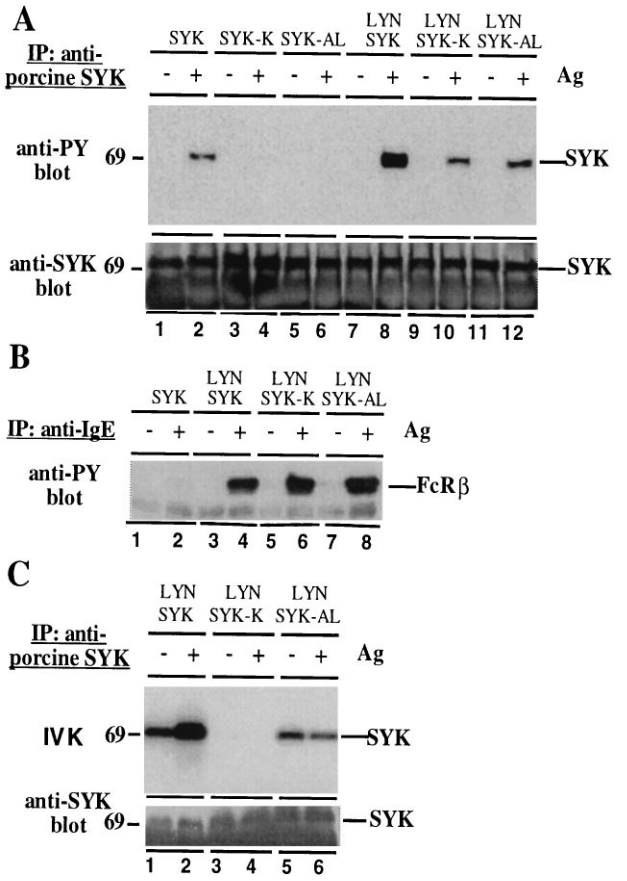

FIG. 3. Analysis of syk tyrosine phosphorylation and activation in 3T3E cells. (*A*) syk-K and syk-AL have decreased Fc receptor-induced tyrosine phosphorylation relative to wild-type syk. 3T3E cells were loaded with IgE, infected with the indicated combinations of viruses, triggered (+) or not (−) with antigen, and lysed. (*Upper*) Lysates were immunoprecipitated with rabbit polyclonal anti-porcine syk antibody and analyzed by immunoblotting with anti-phosphotyrosine antibody. (*Lower*) The blot was then stripped and reprobed by anti-syk immunoblotting to verify equivalent amounts of syk protein in each lane. Note that longer exposures of the anti-phosphotyrosine blot reveal very low levels of phosphorylation of syk-K and syk-AL in lanes 4 and 6, which is likely attributable to endogenous SRC. (*B*) lyn activity is not differentially affected by syk, syk-K, or syk-AL. 3T3E cells were loaded with IgE, infected with the indicated combinations of viruses, triggered (+) or not (−) with antigen, and lysed. Lysates were immunoprecipitated with rabbit polyclonal anti-IgE antibody to isolate phosphorylated Fc receptor complexes and analyzed by immunoblotting with anti-phosphotyrosine antibody. The band that runs just under FcRβ in all lanes is the light chain of IgE, which is detected by the peroxidase-conjugated anti-mouse secondary antibody used to develop the blot. (*C*) Fc receptor-induced tyrosine phosphorylation activates the tyrosine kinase activity of wild-type syk, but not the syk-AL mutant. Similar experiment to above, in that cells were infected with the indicated viruses, and then triggered, lysed, and immunoprecipitated as in *A*. However, in this experiment, each anti-porcine syk immunoprecipitate was split into two samples. One sample was analyzed by *in vitro* kinase assay (*Upper*), while the second sample was analyzed by anti-phosphotyrosine immunoblotting to confirm the expected pattern of syk tyrosine phosphorylation (this produced results identical to those in *A*; data not shown), and was then stripped and subjected to anti-syk immunoblotting to determine the relative amount of syk protein present in each assay (*Lower*).

2 to lane 4 and lane 8 to lane 10) is consistent with the results from Fig. 2 above. This result suggests either that syk and syk-K differentially regulate lyn (and src) activity such that reduced direct phosphorylation of syk-K by src kinases occurs, or that the syk-K mutation eliminates syk transphosphorylation. To determine whether lyn activity was being differentially affected by the various forms of syk, we analyzed the phosphotyrosine content of a known *in vivo* lyn substrate, the FcRβ subunit, when lyn was coexpressed with wild-type syk, syk-K, or syk-AL (Fig. 3*B*). The lack of phosphorylated FcRβ after receptor engagement when only wild-type syk is expressed (lane 2) compared with coexpression of lyn and wild-type syk (lane 4) confirms our previous demonstration that FcRβ phosphorylation is lyn-dependent (9). Comparison of lanes 4, 6, and 8 demonstrates that equivalent levels of phosphorylated FcRβ are generated by receptor engagement whether lyn is coexpressed with wild-type syk, syk-K, or syk-AL, indicating that lyn activity is not being differentially regulated by the various syk mutants. Therefore, the reduced phosphorylation of syk-K and syk-AL relative to wild-type syk cannot be accounted for by reduced lyn activity, and is necessarily due to a loss or reduction of syk transphosphorylation induced by the loss of syk kinase activity (syk-K) or disruption of the activation loop (syk-AL). Finally, because coexpression of Btk had no apparent effect on syk tyrosine phosphorylation in the presence or absence of lyn (data not shown), lyn (or other src family kinases) are likely the sole other major contributors (besides syk itself) to syk tyrosine phosphorylation. Together, these data directly demonstrate that syk tyrosine phosphorylation is a combination of direct phosphorylation by src kinases and syk transphosphorylation. Because lyn coexpression enhances antigen-dependent receptor tyrosine phosphorylation, recruitment of syk to phosphorylated receptors, and syk activation (9, 18), the enhanced syk tyrosine phosphorylation seen with lyn coexpression is best explained as the result of increased recruitment of syk to engaged receptors with resulting enhanced direct phosphorylation of syk by lyn and syk transphosphorylation.

The reduction of the tyrosine phosphorylation of syk-AL to a level similar to that of syk-K (Fig. 3*A*, compare lanes 2, 4, and 6 or lanes 8, 10, and 12) has two major implications: (*i*) it suggests that src kinases are able to phosphorylate syk at at least one major site outside the activation loop (although a less likely alternative possibility is that they somehow induce syk-AL to autophosphorylate), and (*ii*) that the syk-AL mutation is functionally equivalent to the elimination of syk activity. The latter would be consistent with a pseudosubstrate inhibition mechanism for syk activation in which phosphorylation of the syk activation loop is required for syk enzymatic activation, and syk enzymatic activation was required for syk transphosphorylation. Therefore, the role of the syk activation loop in syk activation was directly analyzed by coexpressing lyn and each syk mutant in 3T3E cells, followed by analysis by *in vitro* kinase assay to determine the activation state of each form of syk before and after Fc receptor engagement (Fig. 3*C*). As can be seen, Fc receptor-induced tyrosine phosphorylation of wild-type syk correlates with a marked enhancement of its activity, while syk-AL has basal activity nearly identical to that of wild-type syk and no increase in activity after Fc receptor engagement. As expected, syk-K had no activity. This result demonstrates that the syk activation loop is required for the enzymatic activation of syk, supporting the existence of a pseudosubstrate inhibition mechanism for regulating syk activity.

Tyrosine Phosphopeptide Mapping Analysis of syk Tyrosine Phosphorylation. The results from Figs. 2 and 3 establish the origin of syk phosphorylation as direct phosphorylation by src family kinases and syk transphosphorylation, and demonstrate the importance of the activation loop in the regulation of syk enzymatic activation. However, the above experiments leave open several important questions, including a definitive demonstration of syk activation loop phosphorylation, the origin of syk activation loop phosphorylation, and the possible existence of other phosphorylation sites. To begin to clarify these issues, we performed tyrosine phosphopeptide mapping studies of wild-type syk, syk-K, syk-AL, and syk-T (Fig. 4).

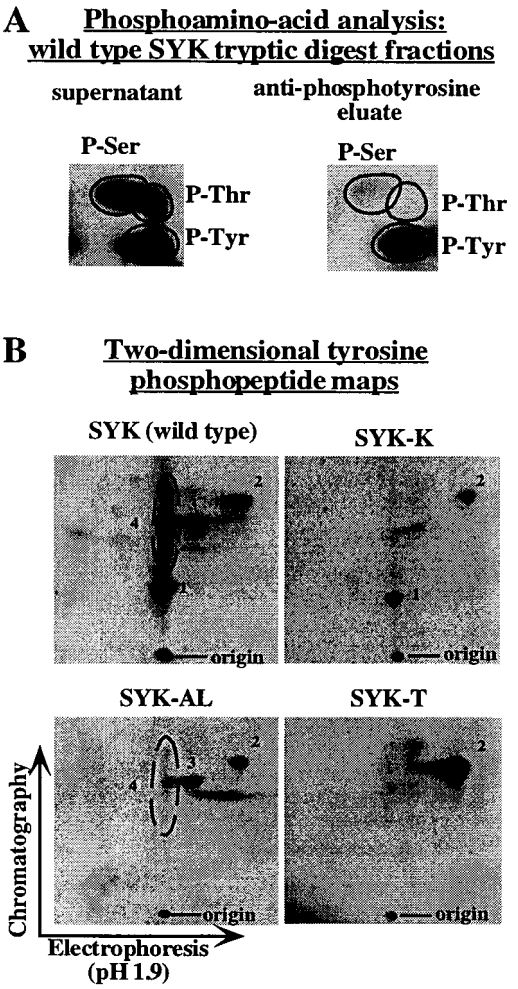

FIG. 4. Phosphopeptide analysis of syk tyrosine phosphorylation sites. Tryptic peptides derived from ^{32}P-labeled wild-type syk, syk-K, syk-AL, and syk-T were generated as described, and then immunoprecipitated with anti-phosphotyrosine to generate a population of bound peptides (eluate fraction) and unbound peptides (supernatant fraction). (*A*) Anti-phosphotyrosine immunoprecipitation of tryptic peptides effectively eliminates serine/threonine phosphorylated peptides from the bound peptides (eluate fraction). Phosphoamino acid analysis was performed on aliquots of the supernatant (*Left*) or eluate (*Right*) fractions from the anti-phosphotyrosine immunoprecipitation of wild-type syk tryptic peptides. (*B*) Two-dimensional phosphopeptide analyses of the eluate fractions. For all syk constructs, equal amounts of eluate fractions were subjected to two-dimensional phosphopeptide mapping. The set of spots inside the ellipse in the wild-type syk map are grouped together as "peptide 4" because over several experiments the labeling of the spots above and below the main spot was variable. We believe that these spots are isoforms of a single peptide generated by contaminating protease activity in the trypsin preparation as we have previously described (19). The increased intensity of peptide 2 phosphorylation in the syk-T map relative to the other maps is reproducible, but is of unclear origin.

These studies involved generating ^{32}P-labeled syk by purification of syk from Fc receptor-stimulated ^{32}P-labeled 3T3E cells, digestion of the labeled syk into tryptic peptides, and an anti-phosphotyrosine purification step for the purpose of isolating a portion of the tyrosine-phosphorylated tryptic peptides (eluate fraction) from the rest of the total tryptic peptide population [supernatant fraction (17, 20), and see *Materials and Methods*]. The supernatant and eluate fractions from the anti-phosphotyrosine purification step of wild-type syk were subjected to phosphoamino acid analysis to confirm the efficacy of the anti-phosphotyrosine purification step (Fig. 4*A*). As can be seen, the supernatant fraction contained phosphoserine, phosphothreonine, and phosphotyrosine, while the eluate from the anti-phosphotyrosine immunoprecipitation contained essentially only phosphotyrosine [a very low level of phosphoserine (<1.5% of the phosphotyrosine counts detected as assessed by densitometric analysis of the autoradiogram) was detectable with a longer exposure]. All eluates (wild-type syk, syk-K, syk-AL, and syk-T) were subjected to two-dimensional phosphopeptide mapping (Fig. 4*B*). Because the wild-type syk eluate contains >98.5% phosphotyrosine by phosphoamino acid analysis, spots containing >10% of the total counts detected can confidently be considered as tyrosine-phosphorylated peptides. Using this criteria with a densitometric analysis of the phosphopeptide map autoradiogram (data not shown), wild-type syk has four major tyrosine-phosphorylated peptides (labeled 1–4 on the wild-type syk map). Strikingly, only one of these peptides ("peptide 1") is completely absent from the maps of both syk-AL and syk-T (in all experiments and with all exposures, this was the only peptide completely absent from both of these maps). Although it is formally possible that the syk-AL and syk-T mutations could inhibit phosphorylation of peptide 1 due to conformational changes induced by the mutations, it is extremely unlikely that such a mechanism would completely eliminate this peptide from detection over multiple experiments and with very long exposures. Therefore, the lack of peptide 1 in the syk-T and syk-AL maps provides very strong evidence that this is a tyrosine-phosphorylated peptide derived from the syk activation loop. Because this peptide is present in the syk-K map, it follows that lyn is responsible for a portion of the syk activation loop phosphorylation that occurs during Fc receptor-mediated syk activation. However, the phosphorylation of peptide 1 is much more intense in wild-type syk than in syk-K (≈8-fold more by densitometry), indicating that syk transphosphorylation is the major contributor to syk activation loop phosphorylation. A second piece of information that the maps provide is the identification of peptide 2 as a lyn-dependent phosphorylation site that is located somewhere in the syk SH2 domain/interdomain regions (due to its presence at an equivalent intensity in wild-type syk, syk-K, syk-AL, and syk-T). The loss of most of the phosphorylation of "peptide 4" and the near complete (syk-K) or complete (syk-AL) loss of activation loop phosphorylation "peptide 1" account well for the magnitude of the reduction in overall tyrosine phosphorylation of the syk-K and syk-AL mutants relative to wild-type syk seen in Fig. 3.

DISCUSSION

A Proposed syk Activation Mechanism and Its Implications for syk Activation Kinetics. Taken together, the results described here demonstrate several characteristics of Fc receptor-induced syk tyrosine phosphorylation and activation that have important implications for the mechanism of Fc receptor-induced syk activation: (*i*) syk tyrosine phosphorylation is due primarily to direct phosphorylation by src family kinases and syk transphosphorylation; (*ii*) the syk activation loop is tyrosine phosphorylated, and this phosphorylation is generated by both src family kinases and syk transphosphorylation, with the majority due to syk transphosphorylation; (*iii*) a phosphorylateable syk activation loop is required for syk enzymatic activation; and (*iv*) syk enzymatic activation is required for the generation of a full level of syk tyrosine phosphorylation, including that of the activation loop. Taken together, these results suggest that syk is activated as a consequence of tyrosine phosphorylation within its activation loop by both src family kinases and other molecules of activated syk, with syk activated in this manner participating in the transphosphorylation of other molecules of syk at sites including, but not limited to, their activation loops.

The activation mechanism described above can be thought of as an activation loop phosphorylation chain reaction, with lyn (or other src family kinase) acting as the initiating trigger by generating the first few molecules of activated syk. A src kinase-initiated activation loop phosphorylation chain reaction process implies that syk is able to catalyze its own activation. Because syk is targeted to activated Fc receptors by its SH2 domains, self-catalyzed activation has the critical implication that the rate at which syk molecules become activated within a syk population recruited to a cluster of activated receptors will be dependent on the amount of activated syk present in that cluster. Situations in which the rate of increase of a particular parameter within a population is dependent on its own value have been the subject of extensive mathematical analysis, providing a pre-existing literature of deterministic and probabilistic models for predicting their behavior (reviewed in ref. 21). One common insight that these models bring to syk activation is the prediction that it will exhibit exponential kinetics; that is, once an activated syk molecule is introduced to a population of susceptible inactive syk molecules, the inactive syk molecules will be converted to active ones in an accelerating fashion until the supply of inactive syk is exhausted. Given syk's critical role in cell activation, exponential activation kinetics would provide a compelling explanation for how even relatively weak Fc receptor stimuli would be able to induce rapid and robust syk, and therefore cellular, activation.

Wider Implications. The syk activation mechanism can be considered to consist of two discrete events: (*i*) a receptor targeting event (mediated by the syk SH2 domains) to place syk in proximity to receptor-associated src family kinases, and (*ii*) a src family kinase-initiated activation loop phosphorylation chain reaction resulting in rapid activation of the receptor-targeted syk. We have recently shown that activation loop tyrosine phosphorylation of Bruton tyrosine kinase (Btk) is generated by a combination of the activities of both src family kinases and Btk, with Btk activity responsible for most of this phosphorylation (19, 20), and similar findings have been reported by another group (22). Intriguingly, there is some evidence that Btk requires targeting to a membrane/cytoskeleton fraction before its activation, suggesting that Btk may have to be placed in proximity to a src family kinase in order for it to become activated (23). If confirmed, this would suggest that the activation mechanisms of syk and Btk are functionally homologous sequences of events: membrane targeting to place them in proximity to src kinases followed by a src kinase-initiated activation loop phosphorylation chain reaction. Such a strong functional similarity between the activation mechanisms of these structurally distinct tyrosine kinases would suggest that src family kinase-initiated activation loop phosphorylation chain reactions might be a general strategy utilized by cells to produce rapid cytoplasmic tyrosine kinase activation in response to extracellular stimuli.

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