Syk Activation by the Src-family Tyrosine Kinase in the B Cell Receptor Signaling

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Summary

Signaling through the B cell antigen receptor (BCR) results in rapid increases in tyrosine phosphorylation on a number of proteins. The BCR associates with two classes of tyrosine kinase: Src-family kinase (Src-protein-tyrosine kinase [PTK]; Lyn, Fyn, Blk, or Lck) and Syk kinase. We have investigated the interaction between the Src-PTK and the Syk kinase in the BCR signaling. In contrast to wild-type B cells, BCR-mediated tyrosine phosphorylation of Syk and activation of its in vitro kinase activity were profoundly reduced in lyn-negative cells. The requirement of the Src-PTK to induce tyrosine phosphorylation and activation of Syk was also demonstrated by cotransfection of syk and src-PTK cDNAs into COS cells. These results suggest that the Src-PTK associated with BCR phosphorylates the tyrosine residue(s) of Syk upon receptor stimulation, enhancing the activity of Syk.

Materials and Methods

Cell Culture and DNA Transfection. COS-7 cells were cultured in DME containing 10% FCS. Wild-type and lyn-negative DT40 cells were cultured in RPMI 1640 supplemented with 10% FCS. Methods to establish lyn-negative DT40 cells were described in detail (23). Briefly, using gene targeting constructs including chicken genomic lyn and drug selection markers such as Neo, we created the lyn-negative cells by homologous recombination. Three alleles of lyn locus were sequentially disrupted and after isolating the three alleles targeted clone, we confirmed that this clone has incorporated a single copy of each construct. Transfection of lyn cDNA into the lyn-negative cells restored the normal BCR-mediated functions such as Ca2+ mobilization or overall tyrosine phosphorylation pattern (23). Mouse fyn cDNA (from R. Perlmutter, University of Washington, Seattle, WA) (24), human lyn cDNA (American Type Culture Collection, Rockville, MD) (25) were cloned into the pcEXV-3 vector. A point mutation in the ATP binding site of porcine syk cDNA (20) and lyn cDNA was created by polymerase chain reaction. Wild-type and kinase-negative syk cDNAs were cloned into the pcDL-SRc296 vector (26). DNA (15 μg each DNA/60-mm dish) was transfected into COS-7 cells using the calcium-phosphate method. For DNA transfection into DT40 cells, syk cDNA was cloned into the pApuuro vector (23). DNA (15 μg each DNA/60-mm dish) was transfected into wild-type and lyn-negative DT40 cells (23) by electroporation, and selected in the presence of puromycin (0.5 μg/ml). The expression of Syk in drug-resistant clones was analyzed by Western blotting.

Immunoprecipitation Analysis. DT40 cells were stimulated by a mAb, M4 (27), that recognizes chicken IgM (from C. Chen, Univer-
tyrosine phosphorylation on a number of proteins, whereas the induction of phosphorylation on many of these substrates was abolished in lyn-negative cells (23). Although endogenous Syk is expressed in DT40 cells, we transfected porcine syk cDNA into wild-type and lyn-negative cells in order to easily detect the expression of Syk. Wild-type and lyn-negative DT40 cells transfected with syk cDNA were stimulated with anti-BCR mAb M4, and immunoprecipitated with anti-Syk antibody which recognizes only porcine Syk. These immunoprecipitates were analyzed by antiphosphotyrosine mAb 4G10 and anti-Syk Ab (Fig. 1A). Syk was tyrosine phosphorylated upon BCR stimulation in wild-type DT40 cells, with very rapid kinetics. In contrast, the induction of tyrosine phosphorylation on Syk in lyn-negative cells was barely detected; overexposure of this blot showed the weak 4G10 reactive species of Syk by 3 min stimulation of BCR. These results showed that Lyn is primarily involved in the induction of tyrosine phosphorylation of Syk through BCR signaling in DT40 cells. The antiphosphotyrosine mAb reactive species of Syk migrated a little more slowly than anti-Syk Ab reactive one. This difference probably reflects the tyrosine phosphorylation of Syk.

Syk has been already shown to be activated by cross-linking of the BCR (21, 22). To examine whether this activation is dependent on Src-PTK, we carried out in vitro kinase assay of Syk in wild-type and lyn-negative DT40 cells after BCR stimulation. As shown in Fig. 1B, cross-linking of BCR stimulated autophosphorylation activity of Syk in wild-type cells, consistent with previous reports (21, 22). This rapid activation of Syk was not observed in lyn-negative cells. However, a 5-min stimulation of BCR on lyn-negative cells resulted in about a twofold increase of autophosphorylation activity of Syk. In wild-type DT40 cells, the dominant autophosphorylated Syk after BCR stimulation migrated a little more slowly than the major Syk molecule, suggesting that the antiphosphotyrosine mAb reactive Syk corresponds to the activated autophosphorylated molecule. These results suggest that Lyn-dependent phosphorylation increases the autophosphorylation activity of Syk through BCR stimulation in DT40 cells.

**Figure 1.** Stimulation of tyrosine-phosphorylation on Syk (A) and autophosphorylation activity of Syk (B) in wild-type and lyn-negative DT40 cells. (A) Cells (2 × 10^5/ml) were stimulated with mAb M4 (4 μg/ml) for indicated time, lysed by NP-40 buffer, and immunoprecipitated with anti-Syk Ab. Immunoprecipitates were electrophoresed on an 8% SDS-PAGE gel, transferred, and incubated with antiphosphotyrosine mAb 4G10 (top). After the filter was stripped, the same blot was reprobed with anti-Syk Ab (bottom). (B) Cells (2 × 10^6/ml) were stimulated with mAb M4 for indicated time, lysed by modified RIPA buffer, and immunoprecipitated with anti-Syk Ab. Immunoprecipitates were divided, and half of them were used for in vitro kinase assay (top). The remaining half were used for Western blotting with anti-Syk Ab (bottom). Samples were electrophoresed on 8% SDS-PAGE gels.
To provide more insights into the interaction between Src-PTK and Syk, we cotransfected the *src-PTK* and *syk* cDNAs into COS cells (Fig. 2). Tyrosine phosphorylation of Syk was only observed in the presence of Lyn, as judged by antiphosphotyrosine mAb reactivity. In contrast, the tyrosine phosphorylation of Lyn in the presence of Syk was not drastically changed by the presence of Syk; the increased tyrosine phosphorylation of Lyn in the presence of Syk is accounted for by the expression extent of Lyn (data not shown). Comparison of anti-Syk and antiphosphotyrosine blotting showed that the antiphosphotyrosine mAb reactive molecule is corresponding to the upper band of the anti-Syk Ab reactive species. To examine the specificity of each Src-PTK member in its capability of phosphorylation on Syk, cotransfection of *fyn* or *lck* with *syk* cDNAs was carried out. Similar to cotransfection with *lyn* and *syk* cDNAs, Syk was tyrosine phosphorylated in the presence of Fyn (Fig. 2) or Lck (data not shown), indicating no strict specificity for Src-PTK members.

To exclude the possibility that the observed tyrosine phosphorylation of Syk is due to the enhanced autophosphorylation activity induced by Src-PTK, cotransfection of kinase-negative *syk* and *lyn* cDNAs was carried out. The Syk kinase-negative mutant was also tyrosine phosphorylated in the presence of Lyn, suggesting that Lyn phosphorylates Syk directly, rather than affects the autophosphorylation activity of Syk. However, in the presence of Lyn, the extent of tyrosine phosphorylation of the wild-type Syk was about threefold higher than that of the kinase-negative one (Fig. 3, left). Thus, this observation suggests that tyrosine phosphorylation of Syk by Lyn may enhance Syk autophosphorylation or that Lyn may not only phosphorylate Syk, but also enhance Syk autophosphorylation activity through a phosphorylation-independent mechanism. Cotransfection experiments with kinase-negative *fyn* and *syk* cDNAs showed that kinase activity of Syk is essential for phosphorylation of Syk (Fig. 3, right).

To determine the change in Syk activity induced by Src-PTK in COS cells, we compared the autophosphorylation activity of Syk in the absence or presence of Src-PTKs. Fig. 4 shows that the autophosphorylation activity of Syk is increased by Lyn, or Fyn, and that most of this enhanced activity corresponds to the antiphosphotyrosine mAb reactive species of Syk (Fig. 4, upper band in anti-Syk blotting; bottom left). Under these conditions, the association of Lyn or Fyn with Syk could not be detected, as judged by the appearance of phosphate-labeled Lyn or Fyn. Autophosphorylation activity of Lyn or Fyn was not changed by the presence of Syk (data not shown). The overall tyrosine phospho proteins of the COS cell extract transfected with *syk* alone or cotransfected with *syk* and *lyn* cDNAs, was also analyzed with an antiphosphotyrosine mAb. The tyrosine phospho proteins of the COS cell extract cotransfected with *syk* and *lyn* cDNAs was significantly increased, compared with either alone. This induction of tyrosine phosphorylation of cellular proteins is attributed to the enhanced kinase activity of Syk induced by Lyn, since the tyrosine phospho proteins of the COS cell extract cotransfected with *lyn* and kinase-negative *syk* cDNAs.
Stimulation of phosphorylation activity of Syk by Src-PTK in COS cells. COS cells were transfected with indicated combinations of cDNAs. Transfected COS cells were lysed by modified RIPA buffer, and immunoprecipitated by anti-Syk Ab. Immunoprecipitates were divided, and half of them were used for in vitro kinase assay (top left). The remaining half were used for Western blotting with anti-Syk Ab (bottom left). Transfected cells were dissolved in NP-40 buffer, electrophoresed on an 8% SDS-PAGE gel (same amount of protein content per lane), blotted, and incubated with antiphosphotyrosine mAb 4G10 (right).

Analysis of wild-type and lyn-negative DT40 B cells revealed that the tyrosine phosphorylation of Syk through BCR stimulation requires Lyn in DT40 cells. However, these data do not distinguish the possibility that Syk is phosphorylated directly or indirectly by Lyn through BCR signaling. Kinase-negative Syk was tyrosine phosphorylated only in the presence of Lyn and kinase-negative Fyn was unable to phosphorylate Syk in COS cells, suggesting that Syk is directly phosphorylated by Src-PTK.

Even in the lyn-negative DT40 cells, autophosphorylation activity of Syk is about twofold stimulated by cross-linking of BCR, indicating that Syk is activated by receptor aggregation in the absence of Lyn to some extent. This result is consistent with the previous report that stimulation of the Syk chimera bearing a CD16 extracellular domain and a Syk kinase intracellular domain, induced protein tyrosine phosphorylation (29). However, in the wild-type DT40 cells, BCR-mediated autophosphorylation activity of Syk was more rapid and drastic. These results demonstrate that Lyn enhances the phosphorylation activity of Syk through BCR signaling in DT40 cells. The tyrosine phosphorylated species of Syk seems to have an activated autophosphorylation activity, judged by the gel mobility shift, implicating the correlation between phosphorylation of Syk and its activity. This is also supported by the observation that in COS cells, the tyrosine phosphorylated species of Syk induced by Lyn or Fyn has an increased autophosphorylation activity. In TCR signaling, it was already proposed that ZAP-70, which is a homologue of Syk, requires Lck or Fyn for synergistic induction of PTK activity (30). Our data using COS cells are consistent with the previous report that coexpression of Src-PTK with ZAP-70 leads to a remarkable increase in tyrosine phosphorylation (31, 32).

Although our results suggest that the phosphorylation of Syk by Src-PTK activates its kinase activity, it is still possible that Syk may be activated through another mechanism by Src-PTK. For example, the association of Src-PTK with Syk may enhance Syk activity. Thus, our observations provide the possibility that the activation of Syk through phosphorylation by Src-PTK is one of the mechanisms accounting for vast tyrosine phosphorylation through BCR stimulation.

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