Syk is a dual-specificity kinase that self-regulates the signal output from the B-cell antigen receptor

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Upon B-cell activation, the signaling subunits Ig-α and Ig-β of the B-cell antigen receptor become phosphorylated not only on tyrosines but also on serine residues. Using a specific antibody, we show that serine 197 (S197) in the cytoplasmic tail of Ig-α is phosphorylated upon B-cell antigen receptor activation, and that this modification inhibits the signal output of the B-cell antigen receptor. Surprisingly, we found that the well-known protein tyrosine kinase Syk (spleen tyrosine kinase) phosphorylates S197 on Ig-α, thus not only activating but also inhibiting signaling from the B-cell antigen receptor. This finding identifies Syk as a dual-specificity kinase and establishes a previously unexplored paradigm for the self-regulation of biological signaling processes.

The B-cell antigen receptor (BCR) comprises the membrane-bound Ig molecule and the Ig-α/Ig-β heterodimer, which function as the ligand-binding and signaling subunits, respectively. The cytoplasmic tails of Ig-α and Ig-β contain an immunoreceptor tyrosine-based activation motif (ITAM) (1, 2). Upon BCR activation, protein tyrosine kinases (PTK), such as the spleen tyrosine kinase (Syk) and the Src family kinase Lyn, become active and phosphorylate the ITAM tyrosines of Ig-α and Ig-β (3–5). Syk is a cytoplasmic PTK that carries two tandem N-terminal Src homology 2 (SH2) domains (6, 7). The phosphorylation of the two ITAM tyrosines of Ig-α or Ig-β creates docking sites for the tandem SH2 domains of Syk (8–10). This process allows Syk to bind to the BCR and to phosphorylate neighboring ITAM tyrosines, thus amplifying the signaling output of the BCR (11).

Syk not only phosphorylates the ITAM sequences of Ig-α and Ig-β, but also tyrosines on several other substrate proteins controlling signaling pathways downstream of the BCR. For example, by phosphorylating the coreceptor CD19 and the adaptor protein BCAP, Syk activates the phosphoinositide-3-kinase (PI3K) pathway that controls proliferation and survival of B cells (12–14). Another well-known substrate of Syk is the adaptor protein SH2 domain-containing leukocyte protein of 65 kDa (SLP-65) (also known as BLNK or BASH) (15–17). Upon phosphorylation of SLP-65 on several tyrosines, this adaptor protein organizes a signalosome that promotes Ca^{2+} response and the differentiation of developing B cells (18, 19). Signal transduction from the BCR also results in the activation of the ERK pathway, which can support both the proliferation and the differentiation of B cells (20).

Tyrosine phosphorylation is not the only posttranslational protein modification observed in activated B cells. In addition, many serine/threonine kinases (STK) are activated and can phosphorylate a multitude of protein substrates. Depending on the substrate, phosphorylation on serine/threonine (S/T) residues can have positive or negative effects on signal transduction (21–26).

The cytoplasmic sequence of Ig-α and Ig-β not only contains tyrosines but also S/T residues and it has been shown that some of the latter residues are phosphorylated in activated B cells (27, 28). Specifically, the Ig-α tail carries two serines that flank the second ITAM tyrosine Y193 and one threonine adjacent to the non-ITAM tyrosine Y204. We have previously mutated the serine and threonine residues of Ig-α and found an increase in tyrosine phosphorylation of the mutant Ig-α, suggesting that S/T phosphorylation inhibits the activation signals of the BCR (29). Here, we identify the inhibitory residue of Ig-α as S197 and show that this serine is indeed phosphorylated in activated B cells. Moreover, we found that Syk phosphorylates S197. This uniquely characterizes Syk as a dual-specificity kinase with opposing signaling functions on the BCR.

Results

Serine 197 Phosphorylation of Ig-α Inhibits BCR Signals. The cytoplasmic tail of Ig-α not only contains tyrosines but also two serines and one threonine as potential targets of phosphorylation (Fig. 1*A*). To investigate the function of these residues, we generated a mutant form of Ig-α (Ig-αAAV) in which the two serines and the threonine were substituted with alanine and valine, respectively (Fig. 1*A*). In addition to this triple mutant, we also generated a single point-mutant of Ig-α carrying an alanine instead of a serine at position 197 (Ig-αA) (Fig. 1*A*). Retroviral vectors expressing WT or mutant forms of Ig-α were introduced into ex vivo-cultured pro-B cells derived from bone marrow of *mb-1*$^{-/-}$ /B1-8-knock-in mice (30, 31). Because of the deletion of the *mb-1* gene, these cells do not produce Ig-α and express the μm heavy chain of the B1-8 antibody from a V_HDJ_H knock-in allele. Reconstitution of these pro-B cells with retroviral vectors coding for the λ light chain and a flag-tagged Ig-α results in the expression of a BCR that can specifically recognize the hapten 4-hydroxy-5-iodo-3-nitrophenyl-acetyl (NIP) (32, 33).

A flow cytometric analysis showed that cells expressing either WT or mutant Ig-α have similar amounts of the BCR on their surface (Fig. S1). The strength of BCR signaling can be monitored by the increase of the intracellular Ca^{2+} concentration in activated B cells (34). All Ig-α transfectants showed a Ca^{2+} influx upon stimulation of their BCR with either 1 (Fig. 1*B*) or 10 ng/mL (Fig. 1*C*) of NIP-BSA, whereas the BCR-negative recipient pro-B cells (control) do not respond to the stimulus (Fig. 1*B*). However, in comparison with B cells expressing Ig-αWT, those expressing the mutant forms of Ig-α had an increased Ca^{2+} response, suggesting that the mutant BCRs can transmit a stronger signal into the cytosol than the WT BCR. This conclusion was confirmed by a Western blot analysis that monitored the activation of the BCR proximal kinase Syk by a phospho-specific antibody detecting the autophosphorylation of Syk at Y630 (35). Antigen-stimulated B cells expressing the Ig-αAAV or Ig-αA mutants show a stronger and prolonged Syk phosphorylation than those expressing WT Ig-α (Fig. 1*D*). Furthermore, upon activation, B cells containing these Ig-α mutants have higher and more prolonged PKB/AKT phosphorylation (pS473) than cells carrying WT Ig-α (Fig. 1*E*).

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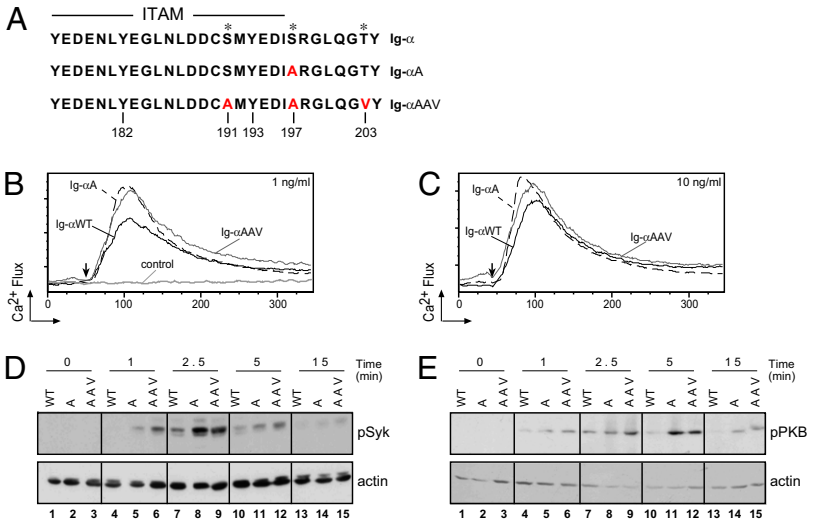

Fig. 1. Ig-α serine and threonine mutants exhibit increased BCR signaling compared with Ig-αWT. (*A*) The amino acid sequence of part of the mouse Ig-α cytoplasmic tail. The asterisks indicate the position of serine 191, serine 197, and threonine 203. The ITAM tyrosines are numbered (Y182 and Y193). The mutated amino acids are shown in red. The ITAM sequence is over-lined. (*B* and *C*) Following BCR antigen stimulation with NIP-BSA (1 and 10 ng/mL, as indicated), intracellular Ca^{2+} release was assessed in B cells carrying WT or mutant Ig-α. The control corresponds to Ig-α KO cells. Small arrows indicate the time points of NIP-BSA addition. (*D* and *E*) B cells containing Ig-α WT, Ig-αA or Ig-αAAV were activated with NIP-BSA (20 ng/mL) for the indicated time points. Western blot analysis of whole-cell lysates is shown with (*D*) an anti-pSyk (Y630) or with (*E*) an anti-pPKB (S473) antibody. Equal loading is shown with an antiactin antibody. Similar results were obtained in three independent experiments.

Because in these assays the BCR with an Ig-αA point mutation behaves similarly to the Ig-αAAV triple mutant, we focused our further analysis on the single mutant BCR. In a time-course experiment, we found that the total tyrosine phosphorylation response was always higher in the Ig-αA mutant than in Ig-αWT expressing B cells (Fig. 2*A*). Furthermore, upon antigen stimulation, the ERK phosphorylation was also stronger in the Ig-αA than in the Ig-αWT-expressing B cells (Fig. 2*B*). These data show that several signaling routes from the BCR are affected by the Ig-αA mutation, including the PI3K and the MAP kinase pathways (Figs. 1*E* and 2*B*).

To establish that Ig-α S197 is indeed phosphorylated, we generated a phospho-serine (pS197)-specific antibody. In a dot-blot assay, this antiserum only recognized the pS197 peptide but not the pY containing peptide (Fig. S2 *A* and *B*). To prove that the pS197-Ig-α antibody recognizes Ig-α only when S197 is phosphorylated, the pS197 peptide was or was not treated with alkaline phosphatase and analyzed by Western blot. The alkaline phosphatase-treated peptide was no longer recognized by the anti-pS197-Ig-α antibody, thus confirming the specificity of this antibody (Fig. S2*C*).

The anti-pS197 antiserum allowed us to monitor the kinetics of Ig-α S197 phosphorylation in activated B cells. In a Western blot analysis of NIP-BSA stimulated B cells, the phosphorylation of S197 is detected at 5 min, and decreased after 20 min of B-cell activation (Fig. 2*C*). No serine phosphorylation of Ig-α was detected in the total lysate of Ig-αA (S197A) and Ig-αD (S197D) mutants expressing B cells, again demonstrating the specificity of the anti-pS197 antibody (Fig. 2*C*).

Serine 197 of Ig-α Is Phosphorylated by Syk. The S2 *Drosophila* system allows to rebuild the BCR receptor complex and to study its interaction with signal transducing kinases (11, 36). In an attempt to find the kinase phosphorylating S197 of Ig-α, we co-expressed the BCR together with several STKs that are activated during BCR signaling, including PKCα, PKCδ, and PKB (Fig. S3). In this assay, none of these kinases phosphorylated the S197 residue either of Ig-αWT or of an Ig-αDD mutant, where the two ITAM tyrosines were replaced with negatively charged aspartic acids (D) residues (Fig. S3*A*). This Ig-αDD mutant was included in the S2 cell assay because it could mimic ITAM-phosphorylated Ig-α. The activity of these STKs was confirmed using the corresponding phospho-specific antibodies (Fig. S3*A*, third and fourth panels, lanes 3–8). As a control, we also tested the BCR-proximal PTK Syk in the S2 cell assay. Surprisingly, we found that coexpression of the BCR with Syk results not only in tyrosine but also in S197 phosphorylation (Fig. 3*A* and Fig. S3*A*). When the BCR was expressed alone, none of these residues were phosphorylated (Fig. 3*A*, lane 1). The mutant BCR containing Ig-αA was phosphorylated by Syk on tyrosines but no longer on S197 (Fig. 3*A*, lane 4). In this S2 assay, we also coexpressed SLP-65 because the phosphorylation of this adaptor protein is a reliable read-out for

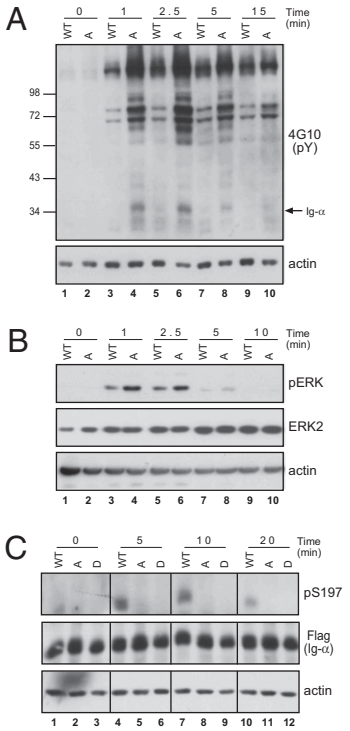

Fig. 2. Phosphorylation of S197 in the Ig-α cytoplasmic tail plays a negative role in BCR activation. B cells expressing Ig-αWT or Ig-αA were stimulated with 20 ng/mL NIP-BSA for the indicated times. Western blot analysis of total cellular lysates is shown with (*A*) antiphospho-tyrosine (4G10) and antiactin antibodies; (*B*) anti-pERK (T202/Y204), anti-ERK2 and anti-actin antibodies. (*C*) Ig-α KO B cells expressing Ig-αWT, Ig-αA or Ig-αD were activated with 20 ng/mL NIP-BSA for the indicated times and the whole-cell lysates were analyzed by immunoblot with antiphospho-S197 Ig-α (pS197), anti-Flag and antiactin antibodies. Blots are representative of three independent experiments.

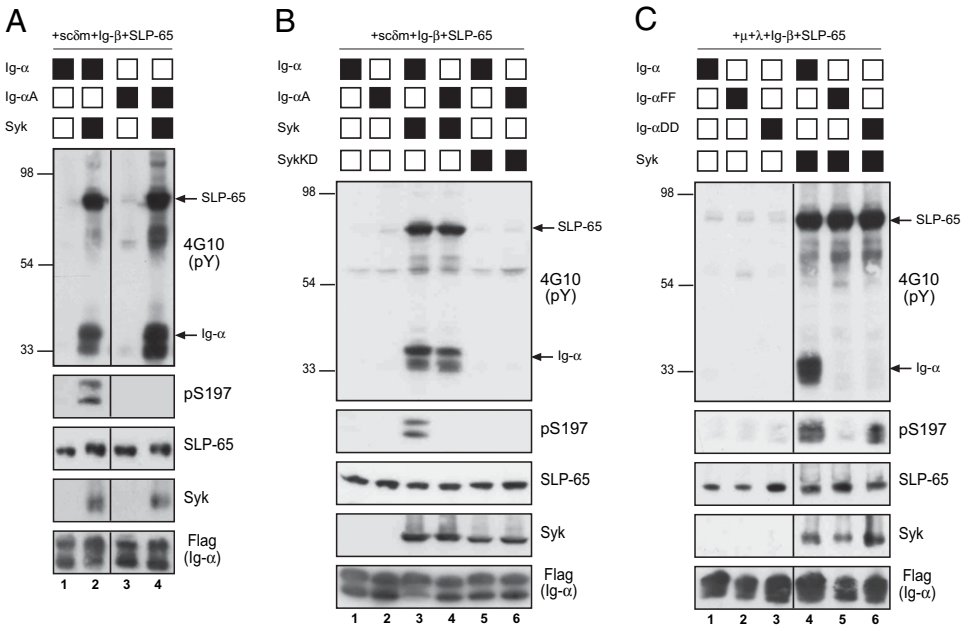

Fig. 3. Syk mediates Ig-α serine phosphorylation. (*A*) Whole-cell lysates from S2 *Drosophila* cells transiently expressing scδm together with Ig-β, SLP-65, Syk, and either Ig-αWT (lanes 1 and 2) or Ig-αA (lanes 3 and 4) were blotted with different antibodies as indicated. (*B*) S2 cells were transiently transfected with scδm, Ig-β, SLP-65, Syk (lanes 3 and 4), SykKD (kinase death) (lanes 5 and 6), and either Ig-α WT (lanes 1, 3, and 5) or Ig-αA (lanes 2, 4, and 6). Total-cellular lysates were immunoblotted with 4G10, anti-pS197, anti-SLP-65, anti-Syk and anti-Flag antibodies. (*C*) S2 cells containing BCR components (μ, λ, Ig-β, and SLP-65) together with Syk (lanes 4–6) and either Ig-αWT (lanes 1 and 4), Ig-αFF (lanes 2 and 5), or Ig-αDD (lanes 3 and 6) were lysed and blotted with the following antibodies: 4G10, anti-pS197, anti-SLP-65, anti-Syk, and anti-Flag antibodies. Comparable results were achieved in three independent experiments.

Syk activity (11). The coexpression of the BCR with a kinase dead mutant of Syk (SykKD) did not result in tyrosine or S197 phosphorylation of Ig-α (Fig. 3*B*, lanes 5 and 6). Thus, in the S2 system, the kinase activity of Syk is required for both the phosphorylation of ITAM tyrosines and of S197 of Ig-α.

To test whether or not the two ITAM tyrosines (Y182 and Y193) play a role in the process leading to S197 phosphorylation, we substituted these tyrosines with either phenylalanine (Ig-αFF) or aspartic acid (Ig-αDD). Clearly, S197 is no longer phosphorylated in a BCR carrying the Ig-αFF mutant, whereas a BCR with the Ig-αDD mutant still displays S197 phosphorylation (Fig. 3*C*, second panel, lanes 5 and 6). Thus, either the phosphorylation of the ITAM tyrosines or its replacement with negatively charged amino acids allow S197 phosphorylation. In S2 cells expressing BCR with Ig-α ITAM mutants, Syk is still activated as indicated by the strong SLP-65 phosphorylation (Fig. 3*C*, *Upper*, lanes 4–6). This was because of the presence of Ig-β carrying its own ITAM.

Syk as a Dual-Specificity Kinase. From the S2 experiments, it is not clear whether Syk is directly mediating or only indirectly influencing S197 phosphorylation. For example, it is possible that Syk helps to recruit a STK to the BCR by phosphorylating the ITAM

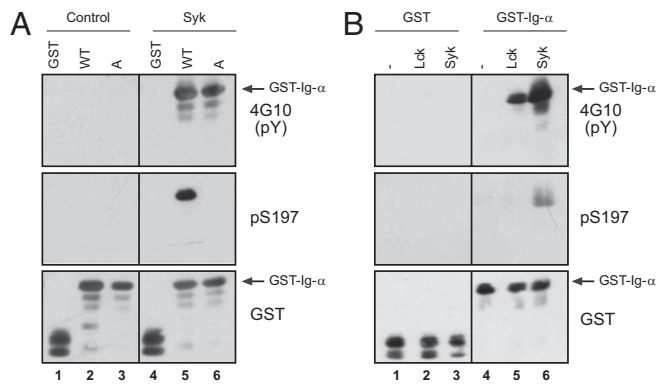

Fig. 4. The S197 of Ig-α is specifically phosphorylated by Syk. In vitro kinase assay. (*A*) Purified GST, GST-Ig-α (WT), or GST-Ig-αA (A) were incubated without or with recombinant GST-Syk. Ig-α phosphorylation was detected by immunoblot with 4G10 and anti-pS197 antibodies. (*B*) GST or GST-Ig-α were incubated either with GST-Lck (lanes 2 and 5) or with GST-Syk. No kinases were added in the controls (-). Phosphorylation was identified by immunoblotting with 4G10 and with anti-pS197 antibodies. The total amount of GST and GST-Ig-α proteins is shown with an anti-GST antibody. Comparable results were achieved in three independent experiments.

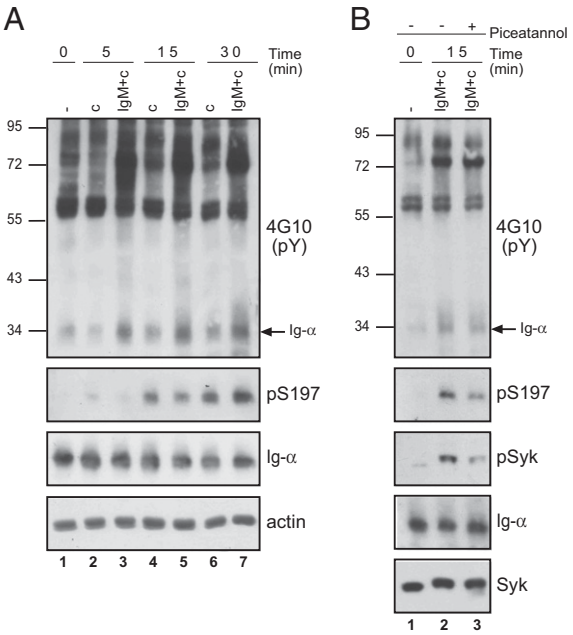

Fig. 5. Ig-α serine phosphorylation in primary B cells. (*A*) Splenic B cells were treated as indicated with 50 nM calyculin in the absence (c) or presence of 20 μg/mL anti-IgM (IgM+c). Total cellular lysates were analyzed by Western blot with 4G10, anti-pS197, anti-Ig-α and anti-actin antibodies. (*B*) Splenic B cells treated or not with Syk inhibitor piceatannol (50 μM) were activated with 50 nM calyculin and 20 μg/mL anti-IgM (IgM+c). Whole-cell lysates were immunoblotted with 4G10, anti-pS197, anti-pSyk (Y630), anti-Ig-α and anti-Syk antibodies. Blots are representative of three independent experiments.

tyrosines. To prove that Syk can directly phosphorylate S197, we performed an in vitro kinase assay where we incubated a purified GST-Syk fusion protein together with either GST, GST-Ig-αWT, or GST-Ig-αA (Fig. 4*A*). The phosphorylation state of the GST-Ig-α proteins was then analyzed by Western blotting using anti-pY (4G10) and anti-pS197 antibodies. In this assay, Syk phosphorylates the tyrosines and the S197 of the GST-Ig-αWT protein (Fig. 4*A*, lane 5) but only phosphorylates the tyrosines in the GST-Ig-αA mutant (Fig. 4*A*, lane 6). To demonstrate that additional kinases are not typically copurified with the GST-containing proteins produced in insect cells, another recombinant tyrosine kinase (GST-Lck) was examined for its ability to phosphorylate Ig-α. In an in vitro kinase assay, GST-Lck was only able to phosphorylate tyrosines of Ig-α (Fig. 4*B*, lane 5). Taken together, these results reveal that Syk is a dual-specificity kinase that not only phosphorylates tyrosine but also serine residues.

Syk Regulates Ig-α Serine Phosphorylation in Primary B Cells. To investigate Ig-α S197 phosphorylation in primary B cells, we stimulated splenic B cells with an anti-IgM antibody in the presence of the serine phosphatase inhibitor calyculin (c) (Fig. 5*A*). In these activated B cells, the Ig-α tyrosine phosphorylation has a faster kinetics than the S197 phosphorylation, suggesting that the later process relies on the former one (Fig. 5*A*, first and second panels).

To prove that Syk is the kinase involved in the Ig-α S197 phosphorylation in vivo, activated splenic B cells were pretreated with the Syk inhibitor piceatannol. Although the overall tyrosine phosphorylation was not grossly affected by the addition of the Syk inhibitor (Fig. 5*B*, *Upper*), piceatannol treatment decreased both the Ig-α S197 phosphorylation and the phosphorylation of Y630 in the C-terminal tail of Syk (Fig. 5*B*, second and third panels). Inhibition of Syk activity results in a reduction of Ig-α

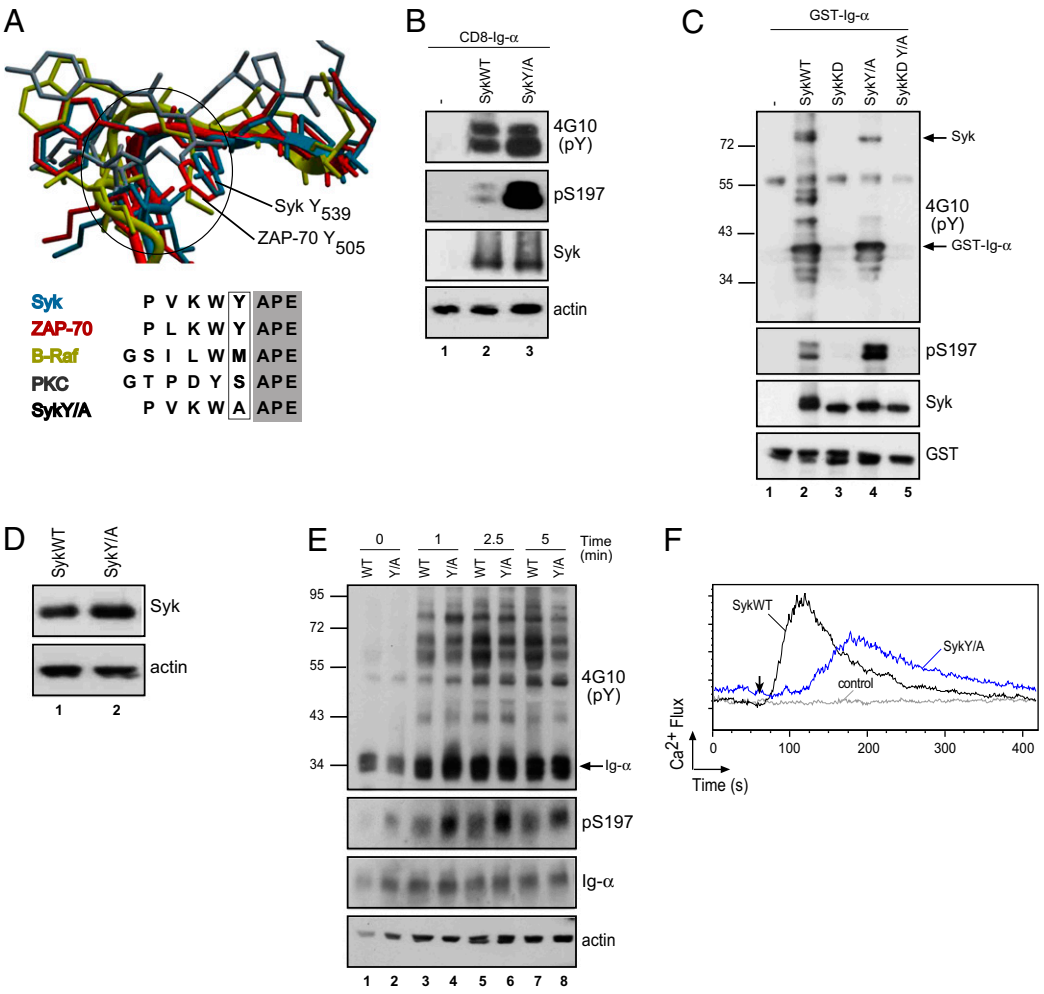

Fig. 6. Syk is a dual-specificity kinase. (*A*) Comparison of the activation loop from different kinases. The structural alignment for Syk (1xbc, blue), ZAP-70 (1u59, red), B-Raf (2fb8, yellow), and PKC (1xjd, gray) was done with PDB files using PyMOL software (39). The black circle indicates the activation loop kink. Y539 of Syk is depicted in the frame together with the corresponding amino acids of the indicated kinases. Shaded amino acids represent conserved residues inside the kinase activation loop. (*B*) Whole-cell lysates from S2 cells transiently transfected with CD8-Ig-α and either with SykWT or SykY/A were immunoblotted as indicated. Equal loading is shown with an anti-actin antibody. (*C*) SykWT, SykKD, SykY/A, and SykKDY/A proteins were expressed in S2 cells. After immunoprecipitation, the different kinases were used for the in vitro kinase assay by incubating them together with GST-Ig-α. Phosphorylation was detected by Western blot with the indicated antibodies (*Upper* and second panels). A loading control is shown with anti-Syk and anti-GST antibodies. (*D–F*) Syk deficient pre-B cells were transduced with SykWT or SykY/A and (*D*) analyzed for Syk expression by Western blot using anti-Syk antibody. Equal loading is shown with antiactin antibody. (*E*) Pre-B cells expressing either SykWT or SykY/A were activated with anti-IgM (20 μg/mL) for the indicated times. Western blot analysis of total cellular lysates was performed with 4G10, anti-pS197, anti-Ig-α, and anti-actin antibodies. (*F*) Following pre-BCR stimulation (anti-IgM 20 μg/mL), intracellular Ca^{2+} release was measured in Syk KO pre B-cells expressing either SykWT or SykY/A mutant. Syk KO pre B-cells were used as a control. Similar results were obtained in three independent experiments.

S197 phosphorylation, suggesting that Syk is also involved in this phosphorylation event in vivo.

A Syk Mutant with Increased Serine Phosphorylation Activity. To learn more about the molecular requirements for the serine phosphorylation activity of the dual-specificity kinase Syk, we compared the structure and the amino acid sequence of the substrate-binding loop of the PTKs Syk and ZAP-70 with that of the STKs B-Raf and PKC (Fig. 6*A*). The overall fold of this loop is quite similar between the two types of kinases. For example, they both have a bend at the C terminus of the substrate-binding loop that contains the highly conserved sequence alanine, proline, glutamic acid (APE). The conserved APE motif allows the alignment of different kinase sequences and reveals that the N-terminal region adjacent to the APE sequence is quite different between PTKs and STKs (Fig. 6*A*, *Lower*). For example, directly adjacent to the APE sequence, B-Raf and PKC both carry a nonaromatic amino acid (methionine and serine, respectively), whereas Syk and ZAP-70 contain a tyrosine (Y539 and Y505, respectively) at this position (Fig. 6*A*, *Upper*). To test whether the amino acid at this position influences the kinase activity, we substituted Y539 of Syk with alanine (SykY/A) (Fig. 6*A*). When tested in the S2 reconstitution system, the SykWT and SykY/A mutant proteins both phosphorylate the tyrosines of the CD8-Ig-α tail to a similar extent (Fig. 6*B*, *Upper*, lanes 2 and 3). However, phosphorylation of the S197 was clearly increased by the SykY/A mutant when compared with the SykWT protein (Fig. 6*B*, second panel, lanes 3 and 2). The increased ability of the SykY/A mutant to phosphorylate Ig-α S197 was also confirmed in an in vitro kinase assay (Fig. 6*C*, second panel). In this assay, the controls consisting of the catalytic dead versions of Syk and SykY/A resulted in no phosphorylation of GST-Ig-α (Fig. 6*C*, lanes 3 and 5). Furthermore, substitutions of Y539 with other amino acids such as threonine or methionine also increased the serine kinase activity of Syk (Fig. S4 *A* and *B*). We also mutated other amino acids of the substrate-binding loop but these mutations frequently resulted in either no alterations or in the loss of both the tyrosine and serine phosphorylation activity of Syk (Fig. S4 *A* and *C*). The finding that a point mutation in Syk resulted in an increased Ig-α serine phosphorylation is another indication that Syk is indeed a dual-specificity kinase.

We next asked whether the increased serine phosphorylation activity of the SykY/A mutant alters B-cell signaling. For this, we transduced Syk deficient pre-B cells with vectors coding either for SykWT or for the SykY/A mutant. Transfectants expressing similar amounts of Syk in the cytosol (Fig. 6*D*) and of pre-BCR on the surface (Fig. S5A) were stimulated and the total cellular lysates were analyzed for tyrosine and serine phosphorylation by Western blot (Fig. 6*E*). In comparison with pre-B cells expressing SykWT, cells expressing the SykY/A mutant responded to the pre-BCR stimulation with slightly reduced tyrosine phosphorylation, but increased Ig-α serine phosphorylation (Fig. 6*E*, first and second panels). Even more remarkable was the finding that the SykY/A-expressing pre-B cells displayed a reduced intracellular Ca^{2+} release in comparison with SykWT-expressing pre-B cells (Fig. 6*F*, blue line and black line). Conversely, Syk-deficient pre-B cells expressing a kinase dead version of Syk (SykKD) showed impaired Ca^{2+} influx upon activation, demonstrating the requirement of the Syk activity for pre-B cell response (Fig. S5*B*). These results once more show that the serine phosphorylation of Ig-α is controlled by the kinase Syk and that S197 phosphorylation negatively regulates BCR signaling.

SykY/A Mutant Has Impaired B-Cell Development. To examine weather the altered kinase activity of SykY/A has some influence on B-cell differentiation in vivo, we reconstituted Syk deficient pre-B cells with vectors coding either for GFP (control), SykWT-IRES-GFP, or for SykY/A-IRES-GFP. Pre-B cells were then sorted for GFP expression and i.v. transferred into *RAG-2*$^{-/-}$/$\gamma_c^{-/-}$ mice. The generation of B cells was assessed 9 d after injection. IgM$^+$ B cells (9%) expressing SykWT were found in the spleen, whereas cells expressing SykY/A had an almost complete block in B-cell differentiation similar to that seen in the control (Fig. 7*A*). A similar result was obtained in the bone marrow (BM), where cells expressing SykWT differentiated into IgM$^+$ B cells, whereas cells carrying the mutant SykY/A did not develop further (Fig. 7*B*). In comparison with SykWT cells, a higher amount of SykY/A expressing cells was found in the spleen (Fig. 7*C*, *Left*) whereas a comparable number of GFP$^+$CD19$^+$ cells expressing either GFP (control), SykWT, or SykY/A was present in the BM (Fig. 7*C*, *Right*). This finding indicates that enhancing the Syk serine kinase activity dramatically impairs B-cell development. Furthermore, in contrast to B-cell differentiation, the elevated number of GFP$^+$CD19$^+$ SykY/A cells found in the spleen suggests that pre-B cells expressing mutant Syk could have an enhanced ability to either proliferate or survive.

Discussion

We have shown here that Syk is a dual-specificity kinase with opposing signaling activities on the BCR. As a PTK, it initiates and amplifies signal transduction from the BCR. As a serine kinase, it inhibits and limits signaling output from this receptor.

In activated B cells, Syk not only phosphorylates the BCR signaling subunit Ig-α but also several other protein substrates including CD19 and SLP-65 (13, 14). At present, it is not clear whether the negative regulatory influence of the serine kinase activity of Syk is only because of S197 phosphorylation or also because of the S/T phosphorylation of other Syk substrates. However, our analysis of the Ig-αA mutant suggests that pS197 plays a major role in the negative regulation of BCR signal transduction. In this respect, our results show that blocking S197 phosphorylation increases, whereas augmenting S197 phosphorylation (via the SykY/A mutation) inhibits the signaling output of the BCR. Furthermore, the balance between tyrosine and serine kinase activity of Syk appears to regulate B-cell dif-

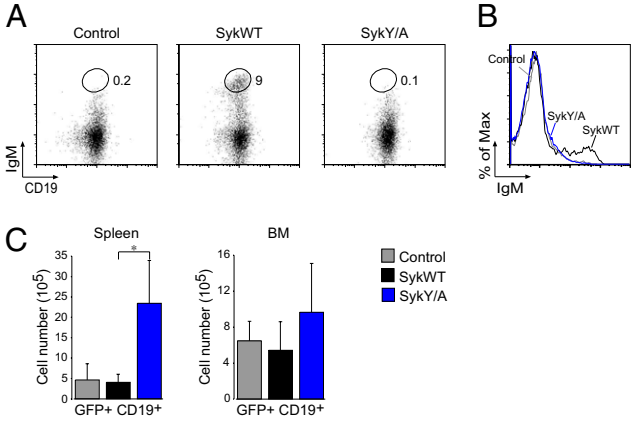

Fig. 7. B-cell development defect of pre-B cells expressing SykY/A. Syk KO pre-B cells transduced with either control vector (GFP), SykWT, or SykY/A were injected i.v. into *RAG-2*$^{-/-}$/$\gamma_c^{-/-}$ mice. Nine days after injection, cells from spleen (*A*) and BM (*B*) were analyzed by flow cytometry. (*A*) B cells are shown in the dot plots with anti-CD19 and anti-IgM antibodies. The cells were gated on the GFP$^+$ population. The number represents the percentage of CD19$^+$IgM$^+$ cells. (*B*) The histogram shows IgM expression of the GFP$^+$CD19$^+$ gated cells. (*C*) Histograms indicate the number of the GFP$^+$CD19$^+$ cells found in the spleen (*Left*) and in the BM (*Right*). Error bars show the means + SEM (each with five mice; *, $P < 0.01$). The cell injection was done two times using three mice per group per experiment. In these two experiments, the cells were independently infected and sorted for GFP expression.

ferentiation. In vivo, pre-B cells expressing the SykY/A mutant fail to develop into IgM^+ B cells.

Syk was found to be a frequently mutated PTK in human tumor samples (37). Several small inhibitors of Syk have been generated in recent years and some of these are now being tested in clinical trials for their role in inhibiting autoimmune or tumor diseases (38). However, with the finding that Syk is a dual-specificity kinase, it may now be possible to screen for more specific drugs, which only inhibit one of the two activities of Syk.

The different time courses of tyrosines and S197 phosphorylations of Ig-α suggest that the phosphorylation of the ITAM tyrosines is crucial to allow the S197 phosphorylation. The finding that Syk cannot phosphorylate the S197 residue of the Ig-αFF mutant lacking the two ITAM tyrosines supports this notion. Thus, the Ig-α ITAM phosphorylation seems to be a prerequisite for S197 phosphorylation.

The dual-specificity of Syk may have evolved to limit the extent of Syk activation at the BCR. Indeed, once Syk has formed the first initiating Syk/BCR complex, it can rapidly phosphorylate neighboring ITAM tyrosines, thus amplifying the BCR signal. The parallel phosphorylation of Ig-α S197 by Syk may limit this signal amplification. The molecular mechanisms of how S197 phosphorylation inhibits BCR signaling are not clear at present. It is feasible that pS197 recruits negative regulatory enzymes such as protein phosphatases, which can efficiently counteract Syk activity at the BCR. Our finding that Syk is a dual-specificity kinase with opposing activities at the BCR shows that the regulation of BCR signaling is more sophisticated than appreciated by the current models.

Materials and Methods

Details on mice, cell lines, cell culture, transfection and retroviral transduction, plasmids, antibodies, Dot Blot assay, in vitro kinase and phosphatase assays, isolation of splenic B cells, immunoprecipitation, measurement of Ca^{2+} release and cell stimulation are provided in *SI Materials and Methods*.

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