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Immunology

Bruton's tyrosine kinase activity is negatively regulated by Sab, the Btk-SH3 domain-binding protein

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ABSTRACT **Bruton's tyrosine kinase (Btk) is a cytoplasmic tyrosine kinase that is crucial for human and murine B cell development, and its deficiency causes human X-linked agammaglobulinemia and murine X-linked immunodeficiency. In this report, we describe the function of the Btk-binding protein Sab (SH3-domain binding protein that preferentially associates with Btk), which we reported previously as a newly identified Src homology 3 domain-binding protein. Sab was shown to inhibit the auto- and transphosphorylation activity of Btk, which prompted us to propose that Sab functions as a transregulator of Btk. Forced overexpression of Sab in B cells led to the reduction of B cell antigen receptor-induced tyrosine phosphorylation of Btk and significantly reduced both early and late B cell antigen receptor-mediated events, including calcium mobilization, inositol 1,4,5-trisphosphate production, and apoptotic cell death, where the involvement of Btk activity has been demonstrated previously. Together, these results indicate the negative regulatory role of Sab in the B cell cytoplasmic tyrosine kinase pathway.**

In contrast to the constitutively active kinase activity of the oncogenic form of tyrosine kinases, the catalytic activities of cytoplasmic tyrosine kinases generally appear to be strictly controlled, which contributes to the homeostatic regulation of cytoplasmic signal transductions. This regulatory process is achieved by posttranslational modifications, such as the tyrosine phosphorylation of residue 527 in Src (1, 2) or, in the case of some kinases, by protein interactions with other molecules called trans-inhibitors (3–7).

Bruton's tyrosine kinase (Btk) is a cytoplasmic tyrosine kinase that is crucial for the maturation of B lineage cells, and its deficiency is involved in the pathogenesis of both human X-linked agammaglobulinemia (8, 9) and murine X-linked immunodeficiency (10, 11). Btk, together with Itk, Tec, Txk, and Bmx, is a member of a recently identified family of cytoplasmic tyrosine kinases (the Btk/Tec family) (12). An ancestral member of this family also can be found in *Drosophila* [Tec29 (13, 14), formerly Dsrc 28C (15)]. The Btk/Tec family kinases share a common feature with the Src and Abl family kinases, namely, the presence of the Src homology 3 (SH3) domain (12). Several studies have demonstrated that deletions or mutations of the SH3 domains in Src or Abl led to oncogenic activation of these kinases (16–18), which suggests a negative regulatory role for the SH3 domain. Also, in the case of Btk and its related kinases, some findings suggest a regulatory role for the SH3 domain in the activation of these kinases (19, 20). With regard to the Src-family kinases, three-dimensional structure analysis has revealed that the interaction between phosphotyrosine 527, which is found in the carboxyl-terminal region, and the SH2 domain enables the interaction between the SH3 domain and the linker region preceding the catalytic domain to take place, which, in turn, results in keeping the catalytic domain inactive (21, 22). Because Btk and Abl family kinases differ significantly from Src family kinases in that they do not contain the carboxyl-terminal tyrosine corresponding to the residue 527 of Src, it is conceivable that the SH3 domains in the Btk and Abl kinases may be involved in the regulation of kinase activity in a manner different from that of Src. To account for this difference, a trans-inhibitor mechanism has been postulated (19, 23, 24).

We previously reported the identification of a 70-kDa Btk-SH3 domain-binding protein termed Sab (SH3 domain-binding protein that preferentially associates with Btk) by using a Far Western cloning strategy (25). Sab was shown to exhibit a higher selectivity for binding to the SH3 domain of Btk than those of other cytoplasmic tyrosine kinases (Lyn, Fyn, Lck, Src) or other cytoplasmic molecules (PLCγ2, PI3K, Grb2, Crk). Though Sab can associate with Btk *in vivo*, tyrosine phosphorylation of Sab by Btk has not been observed (unpublished data). This observation suggested that Sab is not a substrate of Btk and suggested, rather, that Sab may participate in the regulation of the Btk activity. This study represents an attempt to clarify the function of Sab and presents evidences that Sab acts as a trans-inhibitor of Btk.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Expression Constructs. DT40 cells (26, 27) and 293T (28) cells were described previously, as was anti-Btk mAb 43-3B (29). Anti T7-tag antibody was purchased from Novagen. The anti-chicken IgM mAb M4 (30) was provided by Max Cooper (University of Alabama, Birmingham). Human wild-type (8) and WW251LL [SH3-mutated Btk (25); tryptophans 251 and 252 in the Btk-SH3 domain were replaced by leucines] Btk cDNAs inserted into pEF-BOS vector (31) have been described elsewhere as well as T7 epitope-tagged human Sab cDNA inserted into the pRc/CMV vector (DT7-Sab) (25) and T7 epitope-tagged human Btk cDNA inserted into the pApuro vector (32). For transient expressions, these vectors were transfected into 293 T cells with Lipofectamine (GIBCO/BRL). To generate the Sab-overexpressed cell line, the DT7-Sab vector was transfected with the murine stem cell virus puro vector (33) into DT40 cells by electroporation followed by selection with the aid of 0.5 μg/ml of puromycin. The glutathione *S*-transferase (GST)-Sab

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Abbreviations: BCR, B cell antigen receptor; SH3, Src homology 3; GST, glutathione *S*-transferase; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB016835 and AA567603).

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construct was generated by in-frame insertion of human Sab cDNA into pGEX-3X (Pharmacia) (25). The fusion construct of GST and a truncated Sab [the Btk-binding site (Fig. 1*A*) was deleted: Sab(Δ30)] was produced by PCR followed by in-frame ligation into pGEX-2T (Pharmacia).

Btk Kinase Inhibition by Sab. The T7-Btk pApuro vector was transfected into 293T cells. After 48 hr, the cells were lysed with a kinase lysis buffer (1% Triton X-100/10 mM NaH_2PO_4/Na_2HPO_4, pH 7.0/150 mM NaCl/5 mM EDTA/1 mM PMSF/10 μg/ml leupeptin) and the T7 epitope-tagged Btk protein in the lysate was immunopurified with the anti-T7-tag antibody. *In vitro* kinase reactions were assembled by resuspending approximately 10 ng of the immunopurified Btk protein on protein A-Sepharose beads in a reaction buffer (20 mM Pipes, pH 7.0/20 mM $MnCl_2$) with the addition of appropriate amounts of GST proteins and 2 μg of a peptide substrate. The reaction volume was 40 μl. The reaction was initiated by the addition of 20 μCi (1 μCi = 37 kBq) of [γ-^{32}P]ATP (Amersham) and 20 pmol of unlabeled ATP and then allowed to proceed for 10 min at 25°C. The samples were electrophoresed on an SDS/polyacrylamide gel and visualized by autoradiography, after which the phosphorylated peptides were excised from the gel and the radioactivity was quantified by means of scintillation counting.

Detection of Btk Phosphorylation in DT40 Cells. DT40 cells (1×10^8) were stimulated with 15 μg/ml of the anti-chicken IgM antibody M4 for 3 min and then lysed with a lysis buffer containing 10 mM Tris·HCl at pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 20 mM NaF, 10 μg/ml leupeptin, and 1 mM pervanadate (29). Btk in the lysate was immunoprecipitated with 20 μg of the anti-Btk antibody 43-3B and then immunoblotted with the antiphosphotyrosine antibody 4G10.

Analysis of B Cell Antigen Receptor (BCR)-Induced Apoptosis. DT40 cells were stained with 0.25% trypan blue after the cells had been stimulated with the anti-chicken IgM antibody M4 for appropriate time periods, and the percentages of stained cells were calculated. For an alternative assay, incubation of DT40 cells for 36 hr with M4 was followed by staining with annexin V and propidium iodide (R&D Systems) and analysis by flow cytometry (FACSort; Becton Dickinson).

Other Procedures. Mouse Sab cDNA (Fig. 1*A*) was isolated by screening a C57BL/6 spleen cDNA library (Stratagene) with the human Sab cDNA (25) as the probe. To detect the coprecipitation of Btk with T7 epitope-tagged Sab, both proteins were transiently expressed in 293T cells, which then were lysed with 1% digitonin lysis buffer (25). Calcium measurement and phosphoinositide analysis were described previously (26, 27).

RESULTS

Characterization of the *in Vivo* Association of Sab with Btk. By using GST-fusion protein experiments, we demonstrated previously that Btk binds Sab via the structure of the ligand-binding pocket conserved in the SH3 domain despite the absence of a proline-rich region or the consensus PXXP motif (34) in Sab and hypothesized that the SH3 domain-binding site in Sab (Fig. 1*A*) might mimic a polyproline type II helix structure (35) without a polyproline stretch (25). In the present study, we first characterized the *in vivo* association of Sab and Btk. T7 epitope-tagged Sab was transiently coexpressed with wild-type Btk or SH3-mutated Btk (WW251LL) in 293T cells. The cell lysates were immunoprecipitated with the anti-T7 tag antibody, and the coprecipitated Btk was detected by immunoblotting with the anti-Btk antibody 43-3B (Fig. 1*B*). Inconsistent with the previous results obtained with the *in vitro* GST-fusion protein experiments (25), the binding of Sab to the SH3-mutated Btk was diminished greatly when compared with the binding of Sab to the wild-type Btk. In addition, the association of Btk and Sab was reduced markedly when T7 epitope-tagged Sab(Δ30) [Sab lacking the Btk-binding site determined by *in vitro* GST-fusion protein experiments (25) (corresponding to the amino acid sequence 163–193; Fig. 1*A*)] was expressed in place of T7 epitope-tagged Sab (Fig. 1*C*). These results indicated that, also *in vivo*, Btk binds Sab via the conserved structure of the SH3 domain and the stretch of the

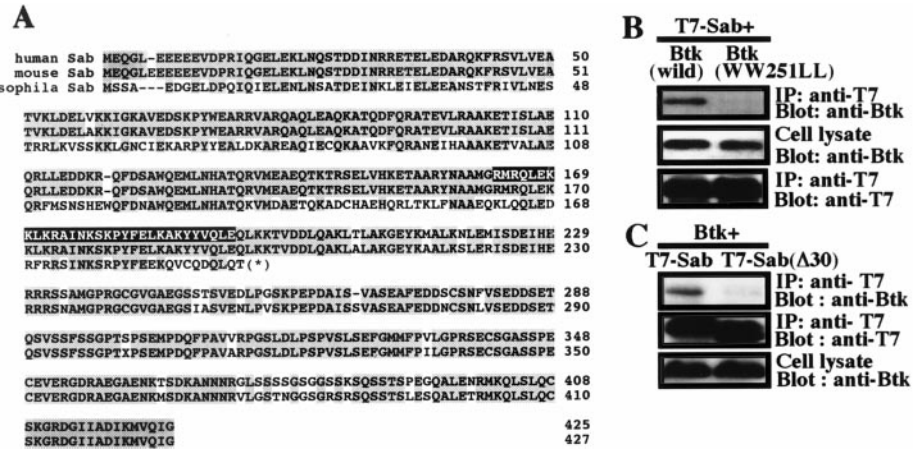

FIG. 1. (*A*) Amino acid sequences of human, mouse, and *Drosophila* Sab and the Btk-binding site. The human Sab sequence was reported previously (25). Mouse Sab cDNA was cloned from the C57BL/6 cDNA library, and its sequence has been deposited in the GenBank database (accession no. AB016835). The *Drosophila* protein sequence was deduced from a 747-bp nucleotide sequence in a Berkeley Drosophila Genome Project/Howard Hughes Medical Institute *Drosophila* expressed sequence tag (accession no. AA567603) and was hypothesized to be a protein homologous with that of human Sab because of its highly conserved amino acid sequence (48% identity with human Sab in the available sequence). *, The C-terminal sequence was not available. The sequences with a gray background indicate identical residues, and those with a black background in the human Sab sequence indicate the minimum region required for the binding to Btk (25). (*B* and *C*) Characterization of the *in vivo* association of Sab and Btk in 293T cells. (*B*) Wild-type or SH3-mutated Btk (WW251LL) was transiently coexpressed with T7 epitope-tagged Sab. The associations between Sab and Btk were evaluated by immunoprecipitation (IP) with the anti-T7-tag antibody followed by immunoblotting (Blot) with the anti-Btk antibody 43-3B (*Top*). (*Middle* and *Bottom*) Confirmation of the expressions of Btk and T7 epitope-tagged Sab proteins, respectively. (*C*) T7 epitope-tagged Sab or Sab(Δ30) was coexpressed with wild-type Btk. The associations were evaluated by immunoprecipitation with the anti T7-tag antibody followed by immunoblotting with the anti-Btk antibody 43-3B (*Top*). (*Middle* and *Bottom*) Confirmation of the expressions of T7 epitope-tagged Sab proteins and Btk, respectively.

30 amino acid residues in Sab is essential for the association of these molecules.

Inhibition of Btk Kinase Activity by Sab. We next tested the possibility that Sab could act as an inhibitor of the Btk kinase activity. T7 epitope-tagged human Btk protein was immunopurified as described in *Materials* and *Methods*. As shown in Fig. 2*A*, silver staining of the immunoprecipitate demonstrated that the purified Btk protein essentially was free of other proteins except for the Ig used for preparation. The kinase activity of the immunopurified Btk protein then was assayed with a peptide substrate with the sequence of CKKVVALY-DYMPMN [corresponding to residues 217–229 (plus cysteine at the amino terminus) of human Btk, in which the tyrosine at residue 223 is the Btk autophosphorylation site]. This peptide has been shown to be a good substrate for Btk (36) and did not contain any serine or threonine residues. As shown in Fig. 2 *B-1* (*Top*) and *B-2*, increasing amounts of the GST-Sab protein added to the kinase reaction resulted in a dose-dependent reduction of the Btk kinase activity as quantitated by the decrease in ^{32}P incorporation into the peptide substrate. In contrast, no reduction of the Btk kinase activity was observed with the addition of the nonfused GST protein or the GST-Sab(Δ30) protein (Fig. 2 *B-1 Middle* and *Bottom* and *B-2*). The time course of Btk-mediated substrate phosphorylation, in the presence or absence of the GST-Sab protein, is shown in Fig. 2*B-3*. Although these results seemed to indicate that the Btk kinase activity was inhibited in the presence of Sab, several other possibilities had to be excluded before a definite conclusion could be reached. First, it was possible that the binding of Sab to Btk might inhibit the access of the peptide substrate to Btk or, alternatively, that the peptide substrate might bind to Sab and, thus, be unable to access Btk, resulting in the apparent reduction of the substrate phosphorylation. However, the results shown in Fig. 2 *B-4* and *B-5* demonstrate that the Btk autophosphorylation also was reduced dose-dependently by the addition of the GST-Sab protein, which indicates that the Btk kinase activity itself was reduced. Second, it was possible that the inhibition of the Btk kinase activity that we observed might represent the dephosphorylation of the substrate and of Btk because of some contaminating phosphatase activity. To test this possibility, the GST-Sab protein was added in the same amounts as shown in Fig. 2*B-2* and incubated for 10 min at 25°C after the *in vitro* kinase reaction without GST protein was terminated. As shown in Fig. 2*C*, the levels of radioactive incorporation did not change as a result of the addition of the GST-Sab protein (solid circles), indicating that no phosphatase was responsible for the reductions in trans- and autophosphorylation seen in Fig. 2*B*. These results demonstrated that Sab actually inhibits the Btk kinase activity *in vitro* via its binding ability to Btk.

Overexpression of Sab in B Cells Interferes with Btk-Related Early-Signaling Events via BCR. The inhibitory effect of Sab on the Btk kinase activity described above led us to examine whether an overexpression of Sab could suppress the Btk-dependent, BCR-coupled cellular events. The roles of cytoplasmic tyrosine kinases in the BCR-signaling pathway have been characterized most effectively in the DT40 chicken B lymphoma cell line because of the facility of *in vitro* genetic dissections of cytoplasmic molecules in this cell line. A dissection of the Btk gene in DT40 cells has been shown to result

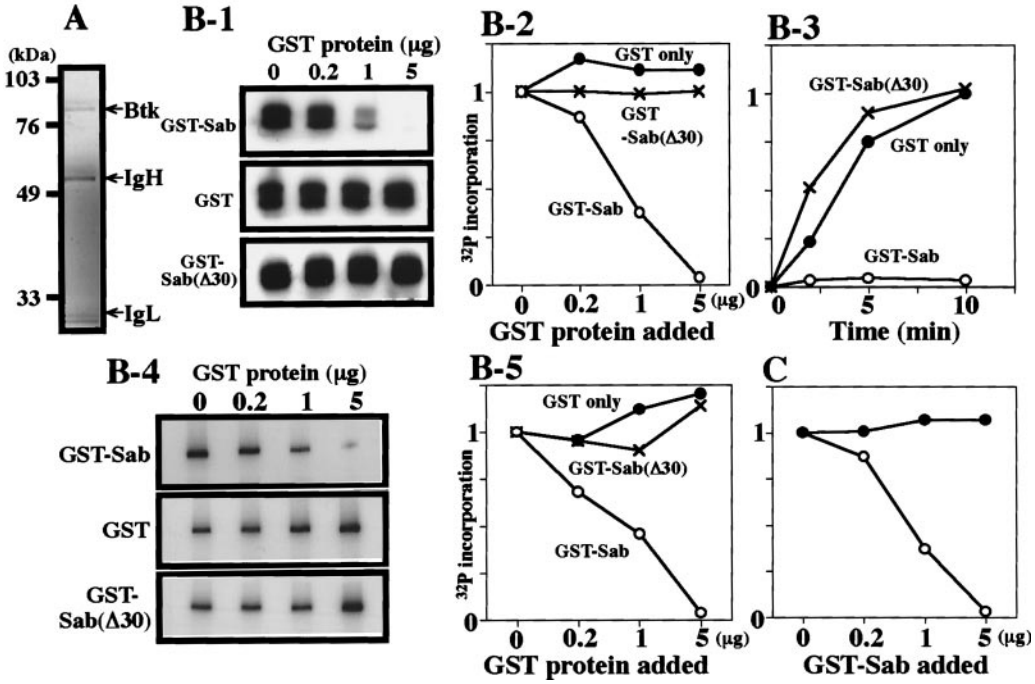

FIG. 2. (*A*) Silver staining of the immunopurified Btk protein used in this experiment. IgH represents the Ig heavy chain, and IgL represents the light chain used for immunopurification of the Btk protein. Molecular mass standards are shown in kDa. (*B-1* and *B-2*) Dose-dependent inhibition of the transphosphorylation activity of Btk by Sab. Approximately 10 ng of the immunopurified Btk protein was mixed with the indicated amounts of GST (●), GST-Sab (○), or GST-Sab (Δ30) (×) protein, and *in vitro* kinase reactions were performed with the addition of the peptide substrate. The relative radioactive incorporation into the peptide substrate is shown. (*B-3*) Time course of radioactive incorporation into the peptide substrate. *In vitro* Btk kinase reaction was performed with the peptide substrate in the presence of 5 μg of GST proteins. (*B-4* and *B-5*) Dose-dependent inhibition of the Btk autophosphorylation by Sab. The conditions for the Btk kinase reaction were the same as those for *B-1* and *B-2*. (*C*) Phosphatase activity is not responsible for the observed reduction of the Btk kinase activity seen in *B-1* and *B-2*. The *in vitro* kinase reaction was performed under the same conditions as those for *B-1* and *B-2* but without the GST protein. After the reaction was terminated, the mixture was incubated with the indicated amounts of the GST-Sab protein for another 10 min at 25°C. Radioactive incorporations were measured by scintillation counting of the phosphorylated peptides (●). For comparison, the open circles show the reduction in the radioactive incorporation into the peptide substrate as a result of the addition of the GST-Sab protein before the *in vitro* kinase reaction (the same dose-dependent reduction as shown in *B-2*).

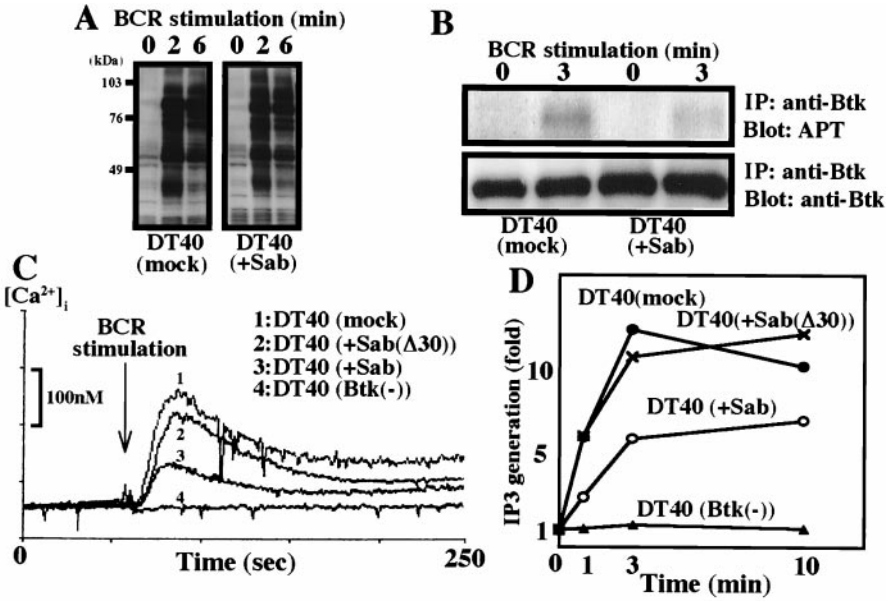

FIG. 3. Overexpression of Sab in DT40 cells interferes with BCR-induced, Btk-related signaling events. (*A*) BCR-induced tyrosine phosphorylation of whole-cell proteins in the mock and the Sab-overexpressed (+Sab) DT40 cells. After DT40 cells were stimulated with M4 for the indicated time periods, the cell lysates were immunoblotted with the anti-phosphotyrosine antibody 4G10. (*B*) Tyrosine phosphorylation of Btk after BCR engagement was reduced in Sab-overexpressed DT40 cells. DT40 cells (mock or Sab-overexpressed) were lysed before and after stimulation with M4 for 3 min. Btk in the lysates then was immunoprecipitated with the anti-Btk antibody 43-3B and immunoblotted with the anti-phosphotyrosine antibody 4G10. (*Lower*) Confirmation of equal amounts of the Btk protein in immunoprecipitates. (*C*) Calcium mobilization after BCR engagement. Intracellular free calcium levels in Fura-2 AM-loaded cells were monitored with a spectrophotometer after stimulation with M4 of the mock, Sab, or Sab(Δ30)-overexpressed and Btk-deficient DT40 cells. (*D*) Ins(1,4,5)P_3 generations were detected by means of extracting soluble inositol from DT40 cells stimulated with M4 for the indicated time periods.

in the complete loss of both calcium mobilization and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] generation after BCR engagement, which indicates that these BCR-coupled cellular events are Btk-dependent (27). Sab cDNA was stably transfected into DT40 cells, and the Sab protein was overexpressed by the cytomegalovirus promoter (25), which resulted in a severalfold increase of the Sab protein in DT40 cells (data not shown). Flow cytometric analysis showed that the mock (rendered puromycin-resistant by transfecting MSCV vector alone) and the Sab-overexpressed DT40 cells expressed comparable levels of surface BCR (data not shown). The mock and the Sab-overexpressed cells were stimulated with the anti-chicken IgM antibody M4, and the cell lysates before and after BCR engagement were immunoblotted with the anti-phosphotyrosine antibody 4G10 (Fig. 3*A*). No significant differences were observed in the global phosphorylation patterns of cellular proteins between the mock and the Sab-overexpressed cells, which seemed consistent with a previous finding that the BCR-induced global phosphorylation patterns were very similar for wild-type and Btk-deficient DT40 cells (27). However, it was found that the BCR-induced tyrosine phosphorylation of the endogenous Btk in the Sab-overexpressed cells was reduced significantly in comparison with that in the mock DT40 cells (Fig. 3*B*). In apparent agreement with this reduction of BCR-induced phosphorylation of Btk, the elevation of intracellular calcium level after BCR engagement was reduced greatly (a 70% reduction compared with the mock cells) in Sab-overexpressed cells (Fig. 3*C*). The BCR-induced Ins(1,4,5)P_3 generation also was reduced significantly (a 40% reduction compared with the mock cells; Fig. 3*D*) in the Sab-overexpressed cells. In contrast, no significant difference was observed between the mock and the Sab(Δ30)-overexpressed cells with regard to the BCR-coupled calcium mobilization and Ins(1,4,5)P_3 generation. These observations indicated that the overexpression of Sab in DT40 cells interfered with the BCR-induced cellular events in which Btk participates.

Overexpression of Sab in DT40 Cells Decelerates the Apoptosis Induced by BCR Engagement. Because the observation described above suggested that Sab suppressed the early-signaling events via BCR, we examined further the possibility that the overexpression of Sab suppressed other Btk-dependent signaling events after BCR engagement. It has been reported that BCR-induced apoptotic cell death observed in the wild-type DT40 cells is delayed significantly by dissecting the Btk gene, suggesting the involvement of Btk in the BCR-induced apoptosis (37). The rate of BCR-induced apoptosis was compared among the mock, Sab-overexpressed, Sab(Δ30)-overexpressed, and Btk-deficient DT40 cells. The percentage of dead cells was calculated by trypan blue staining after the cells had been stimulated for 0, 12, 24, and 36 hr with the anti-chicken IgM antibody M4 (Fig. 4*A*). The Sab-overexpressed DT40 cells died more slowly than the mock cells with a significant difference ($P < 0.05$), whereas the Sab(Δ30)-overexpressed cells died at almost the same rate as the mock cells. After 36 hr of incubation, 73 ± 6% of the mock DT40 cells and 47 ± 4% of the Sab-overexpressed DT40 cells were dead [78 ± 5% of the Sab(Δ30)-overexpressed cells and 26 ± 3% of the Btk-deficient cells were dead at the same time point]. Flow cytometric analysis of the DT40 cells stained with annexin V and propidium iodide (PI) after 36-hr stimulation with the anti-chicken IgM antibody M4 showed that only 48% of the Sab-overexpressed cells were annexin V-positive, whereas 77% of the mock DT40 cells and 61% of the Sab(Δ30)-overexpressed cells were positive (Fig. 4*B*; 25% of the Btk-deficient cells were positive at the same time point). The number of annexin V-positive cells as well as that of trypan blue-stained cells thus was reduced in the Sab-overexpressed DT40 cells in comparison with those in the mock DT40 cells, which indicated that the overexpression of Sab in DT40 cells also suppressed the BCR-induced apoptotic cell death in which the Btk activity previously has been shown to be involved.

DISCUSSION

Clarification of the regulatory mechanism of Btk activity has focused mainly on posttranslational modifications such as

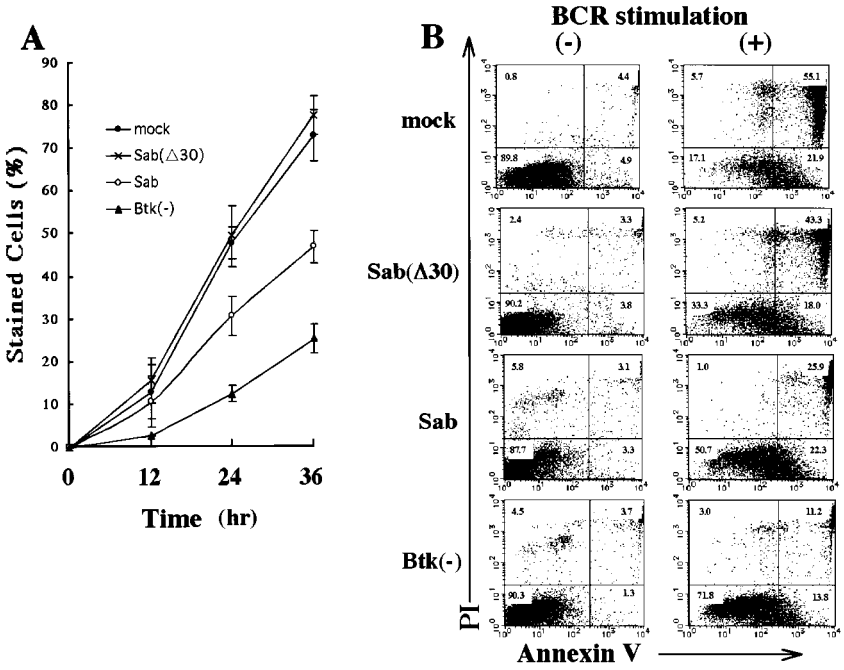

FIG. 4. Overexpression of Sab decelerated the BCR-induced apoptosis of DT40 cells. (*A*) DT40 cells were stained with trypan blue after stimulation with M4 for the indicated time periods. Bars represent ±SE of triplicate experiments. (*B*) DT40 cells were stained with FITC-annexin V and propidium iodide (PI) before or after stimulation with M4 for 36 hr and were analyzed by flow cytometry.

phosphorylation. It has been reported that Btk activity is augmented as a result of the transphosphorylation of the tyrosine 551 residue in its catalytic domain by Src-family kinases such as Lyn (38). On the other hand, Btk activity was reported to be down-regulated by protein kinase C-mediated phosphorylation (39). Recently, the α-subunit of heterotrimeric G protein was found to directly stimulate Btk kinase activity (36, 40), suggesting that the Btk activity also is controlled by the binding of other cytoplasmic molecules. However, no report hitherto has shown the presence of a trans-inhibitor of Btk, although the existence of the trans-inhibitors of Abl (AAP1) (3), Jak (SSI-1/JAB/SOCS-1) (4–6), as well as of Syk (Cbl) (7) has been reported. In this study, we demonstrated that Sab, the Btk-SH3 domain-binding protein, inhibits the auto- and transphosphorylation activity of Btk. The inhibition of the kinase activity was observed with as little as 0.2 μg of GST-Sab for 10 ng of Btk (the molar ratio was approximately 20:1). This stoichiometry in the *in vitro* experiment seemed to be similar to those reported in the inhibition of Abl by AAP1 (3) or in the activation of Btk by Gα protein (36). The observed inhibition of the Btk kinase activity by Sab, together with the evidence that Sab is able to bind selectively to the SH3 domain of Btk (25) and that the Btk kinase activity is not inhibited by the truncated Sab protein, which lacks the Btk-binding site, raises the possibility that Sab is the trans-inhibitor of Btk. This possibility was supported further by the observation that the forced expression of Sab in DT40 cells suppressed the BCR-induced tyrosine phosphorylation of Btk as well as BCR-coupled early signaling events such as calcium mobilization and Ins(1,4,5)P_3 generation, in which Btk activity already has been shown to be involved (27). The observed resistance of the Sab-overexpressed cells to BCR-induced apoptotic cell death also seemed consistent with a previous report that the deficiency of Btk made DT40 cells resistant to BCR-induced apoptosis (37). Together, these findings demonstrate the negative regulatory role of Sab in Btk-related cytoplasmic signaling in B cells.

It is known that some of the reported tyrosine kinase inhibitors (4–6) play an efficient role in the negative feedback for cytoplasmic signaling by means of activation-induced association with corresponding tyrosine kinases as well as of inducible transcription of the genes. However, our preliminary results indicated that the association of Sab with Btk is independent of the activation or phosphorylation of Btk in both reconstituted cells and B cells (data not shown). This finding seems to be similar to that observed in the association of Cbl with Syk in a mast cell line, which was reported to be independent of cell stimulation (41). Although the exact regulatory mechanism of Sab in Btk-dependent cytoplasmic signaling remains to be determined, it is conceivable that the gene expression of Sab also may be controlled in an inducible manner, as that of other trans-inhibitors, and that Sab may have some feedback function in the Btk-signaling pathway. Alternatively, the intracellular localizations of Sab and Btk may change after cell stimulation, and the resulting compartmentation may change the interaction between these molecules.

As reported previously (25), the tissue expression of Sab is quite broad in contrast to the restricted expression of Btk in hematopoietic cells. Although our previous investigation showed that Sab exhibits a high preference for binding to Btk rather than to other cytoplasmic tyrosine kinases (25), the presence of additional targets for Sab such as other members of the Btk/Tec family cannot be ruled out. It also should be remembered that prototypes both of the Btk/Tec family kinase and of Sab (Fig. 1*A*) are present even in *Drosophila* in which no mechanism for antibody production is found. It is conceivable, therefore, that the interaction of Sab and cytoplasmic tyrosine kinases may have been conserved during evolution and is involved in a broad array of cellular events besides antigen receptor-mediated signaling.

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