Phosphorylation- and Activation-independent Association of the Tyrosine Kinase Syk and the Tyrosine Kinase Substrates Cbl and Vav with Tubulin in B-Cells*  

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Aggregation of the B-cell antigen receptor (BCR)1 complex leads to the activation of the 72-kDa Syk protein-tyrosine kinase and the phosphorylation of tubulin on tyrosine. To explore the requirement of Syk catalytic activity for tubulin phosphorylation, tubulin was isolated from cytosolic fractions from anti-IgM-activated B-cells (DT40) that lacked endogenous Syk and immunoblotted with anti-phosphotyrosine antibodies. Tubulin was not tyrosine-phosphorylated in Syk− B-cells. Phosphorylation could be restored by the expression of wild-type, but not catalytically inactive, Syk. However, both catalytically inactive and wild-type Syk were capable of constitutive association with tubulin, indicating that tubulin phosphorylation is not required for this interaction. Anti-phosphotyrosine antibody immunoblotting of proteins adsorbed to colchicine-agarose revealed the presence of three major tubulin-associated phosphoproteins of 110, 90, and 74 kDa, the phosphorylation of which was dependent on Syk expression. The proteins of 110 and 90 kDa were identified as Cbl and Vav, two proto-oncogene products known to become prominently phosphorylated following receptor engagement. Both proteins were shown to be constitutively associated with tubulin.

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1 The abbreviations used are: BCR, B-cell antigen receptor; SH2, Src homology 2; ITAM, immunoreceptor tyrosine-based activation motif; PAGE, polyacrylamide gel electrophoresis; WT, wild-type.

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The recruitment of Syk to the antigen receptor activates and positions the kinase at the plasma membrane, where it can interact with and phosphorylate substrates that are important for receptor-mediated signaling. Tyrosine-phosphorylated and activated Syk is also released from the antigen receptor, which may allow interactions with additional cellular substrates (6). One potential cytosolic substrate with which Syk interacts is α-tubulin (6). To further explore this interaction, we have isolated tubulin-containing cytosolic complexes from B-cells by affinity chromatography and analyzed proteins bound to them. In this report, we provide evidence that Syk associates with tubulin in a manner that is independent of tubulin phosphorylation. In addition to Syk, cytosolic tubulin is constitutively associated with at least three tyrosine kinase substrates, which include the proto-oncogene products Cbl and Vav.

MATERIALS AND METHODS  

Antibodies and Reagents—DT40 and Syk− DT40 chicken B-cells were obtained from Dr. Tomohiro Kurosaki. Syk− DT40 cell lines expressing Syk(WT), Syk(Y130E), and Syk(Y130F) were described previously (7). Syk(K396R) is described below. The 9E10 anti-Myc hybridoma cell line was purchased from the American Type Culture Collection, and the ascites fluid was prepared by the Purdue University Cancer Center Antibody Production Facility. The following antibodies were also used in this report: goat anti-chicken IgM and goat anti-human IgM antibodies (Bethyl Laboratories), goat anti-mouse IgM antibodies (Cappel), anti-phosphotyrosine monoclonal antibody RC20H (Transduction Laboratories), anti-Vav antibody (Upstate Biotechnology), anti-Cbl antibody (Upstate Biotechnology and Santa Cruz Biotechnology), anti-α-tubulin monoclonal antibody (a kind gift of Dr. David Assai, Purdue University) and polyclonal anti-phosphotyrosine and anti-Syk antibodies (prepared as described previously (8, 9)). Colchicine-agarose was prepared as described (10).

Expression of Syk(K396R) in DT40 Cells—Generation of a cDNA for Myc epitope-tagged Syk has been described previously (7). To generate kinase-deficient Myc-tagged Syk (Syk(K396R)) cDNA, site-directed mutagenesis was carried out using the unique site elimination method described by Deng and Nickoloff (11), and the mutation was confirmed by DNA sequencing. Syk− DT40 cells were electroporated with 25 μg of Syk(K396R) cloned into the XhoI site of the pGEM/EPB expression vector (12), along with 2.5 μg p33SS (Stratagene), which contains the hygromycin resistance gene. Cells were selected in RPMI 1640 medium containing 2 mg/ml hygromycin and screened for Syk expression by immunoblotting. The absence of Syk(K396R) catalytic activity was confirmed by an in vitro kinase assay (data not shown).

Cellular Fractionation—Cytosolic fractions from chicken DT40 and murine Bal17 B-cells (5–10 × 10⁶ cells/sample) were obtained as de-
Constitutive Association of Syk, Cbl, and Vav with Tubulin

Syk, Syk−/− DT40 chicken B-cells (13) were transfected with a cDNA encoding wild-type murine Syk (Syk(WT)) (7). These cells express an elevated level of Syk protein that is readily detectable by immunoblotting with anti-Syk antibodies. Cytosolic fractions were prepared from control and anti-IgM-treated cells and adsorbed to colchicine-agarose to isolate tubulin and tubulin-associated proteins. Colchicine-binding proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-Syk antibodies. As shown in Fig. 1B, Syk(WT) could be readily detected in the bound fraction. Receptor cross-linking had no detectable effect on the amount of tubulin-associated Syk(WT). The amount of tubulin bound to the affinity resin, as determined by immunoblotting with anti-tubulin antibodies, also was not detectably altered by receptor engagement (data not shown).

**Syk-Tubulin Association Is Independent of Tubulin Phosphorylation**—To determine whether Syk-catalyzed tubulin phosphorylation was required for Syk association, Syk−/− DT40 cells were prepared that expressed a catalytically inactive form of Syk (Syk(K396R)), in which an essential lysine in the catalytic domain was replaced by arginine. The ability of Syk(K396R) to bind tubulin was assessed by immunoblot analysis of colchicine-agarose-binding proteins prepared from the soluble fractions of the transfected cells. As shown in Fig. 1B, Syk(K396R) still associated with tubulin. Immunoblotting of the affinity-purified tubulin with anti-phosphotyrosine antibodies indicated that tubulin was not phosphorylated on tyrosine in cells expressing only a catalytically inactive form of Syk (Fig. 1C).

**Syk Is Required for the Tyrosine Phosphorylation of Tubulin**—The lack of tubulin phosphorylation in Syk(K396R)-expressing cells suggested that Syk was required for the tubulin phosphorylation that is observed in B-cells following antigen receptor engagement (6). To confirm this, the receptor-stimulated phosphorylation of tubulin was compared in wild-type DT40 cells expressing the normal level of endogenous Syk and Syk−/− DT40 cells. Colchicine-binding proteins isolated from the cytosolic fractions of control and anti-IgM-treated cells were separated by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 2, the phosphorylation of tubulin on tyrosine was completely abrogated in the absence of Syk expression. Immunoblot analysis with anti-tubulin antibodies confirmed the presence of equivalent amounts of tubulin in the samples prepared from the wild-type and Syk−/− cells (data not shown).

Densitometric analyses of anti-tubulin and anti-phosphotyrosine immunoblots were used to determine the extent of phos-
phorylation of cytosolic tubulin by comparing tubulin isolated from cytosolic extracts to purified tubulin phosphorylated in vitro to known stoichiometries using Syk. The results demonstrated that in unstimulated cells, approximately 1.2% of cytosolic tubulin is tyrosine-phosphorylated, whereas in cells stimulated with anti-IgM antibodies, approximately 4.7% of cytosolic tubulin is tyrosine-phosphorylated. These levels of tubulin tyrosine phosphorylation are similar to those observed by Ley et al. (14) in the Jurkat T cell line.

Multiple Tyrosine-phosphorylated Proteins Bind Colchicine-Agarose—Immunoblot analysis with anti-phosphotyrosine antibodies of colchicine-agarose-associated proteins from wild-type DT40 B-cells revealed three phosphotyrosine-containing proteins, in addition to tubulin, the appearance of which was dependent on both receptor cross-linking and the expression of endogenous Syk (Fig. 2). The apparent molecular weights of these proteins, which also appear as a subset of the tyrosine-phosphorylated proteins seen in colchicine-agarose precipitations from Bal17 murine B-cells (see Fig. 4A), were 110,000, 90,000, and 74,000. These data suggested that Syk substrates other than tubulin were associated with the colchicine-agarose affinity resin.

The apparent variation in recovery of phosphoproteins between DT40 and Bal17 B-cells was reproducible and may have resulted from the decreased expression of protein-tyrosine kinases, specifically of the Src family, in the DT40 cell line. In addition to expressing only one Src family kinase (Lyn) (13), we have observed that DT40 B-cells express lower levels of Syk compared with other B-cell lines.

Several experiments were conducted to explore the specificity of the interaction of these tyrosine-phosphorylated proteins with colchicine-agarose. The inclusion of an excess of colchicine blocked the interaction of all of the tyrosine-phosphorylated proteins with colchicine-agarose, indicating that their interaction with the resin was specific for the immobilized ligand (data not shown). In addition, no binding of tyrosine-phosphorylated proteins to either phenyl-agarose or butyl-agarose was observed, indicating that the phosphoproteins were not bound due to nonspecific, hydrophobic interactions (data not shown). To determine whether the binding of these phosphoproteins to colchicine-agarose occurred via their specific interaction with tubulin, immunoprecipitations using a monoclonal anti-tubulin antibody were performed in parallel with adsorption to colchicine-agarose. Bound proteins were separated by SDS-PAGE and immunoblotted with anti-Syk antibodies. As shown in Fig. 3A, both colchicine-agarose and anti-tubulin immune complexes contained a comparable set of phosphotyrosine-containing proteins. The recovery of tubulin-associated proteins is enhanced by the use of colchicine-agarose, which binds a higher fraction of the available soluble tubulin than does the monoclonal antibody. Western blotting analysis of immunoprecipitated tubulin bands in Fig. 3A, lanes 3 and 4, is complicated by their comigration with the heavy chain of the anti-tubulin monoclonal antibody.

To further test this specificity, cells were pretreated with the microtubule-stabilizing agent Taxol to promote the polymerization of soluble tubulin. Cytosolic fractions were prepared from anti-IgM-activated control and Taxol-treated cells and assorbed to colchicine-agarose. As shown in Fig. 3C, Taxol pretreatment essentially removed all detectable tubulin from the cytosolic fraction. This in turn led to a dramatic decrease in the level of colchicine-agarose-associated phosphoproteins that could be recovered (Fig. 3B).

Tubulin Associates with Cbl and Vav—The 120-kDa proto-oncogene product Cbl becomes prominently tyrosine-phosphorylated in response to BCR aggregation and has been shown to associate with Syk in vivo (15, 16). To determine whether the tubulin-associated, 110-kDa phosphoprotein corresponded to Cbl, colchicine-agarose binding proteins prepared from Bal17 B-cells were probed by immunoblotting with anti-Cbl antibodies. As shown in Fig. 4 (lanes 3 and 4), Cbl was constitutively present in the colchicine-agarose-bound fraction. It has been previously reported that the 95-kDa tyrosine kinase substrate Vav associates with both tubulin and ZAP-70 in Jurkat T-cells (17). To determine whether the 90-kDa protein that was present in anti-phosphotyrosine immunoblots of colchicine-agarose adsorbed proteins represented Vav, the immunoblot was reprobed using anti-Vav antibodies. As shown in Fig. 4 (lanes 5 and 6), Vav also was found associated with the colchicine-agarose-bound fraction. Again, as with Cbl, the presence of Vav is constitutive.

To determine the relative stoichiometries of Cbl, Syk, and Vav associated with cytosolic tubulin, immunoblotting of total cytosolic fractions and colchicine-agarose fractions was performed, followed by densitometric analysis. The results demonstrated that approximately 25% of cytosolic Cbl, 22% of cytosolic Syk and 36% of cytosolic Vav associate with cytosolic tubulin in B-cells.

Phosphorylation of Tubulin-associated Proteins, but Not of Tubulin, Requires the Interaction of Syk with the Antigen Receptor—Substitution of Tyr-130 with Glu produces a constitutively active form of Syk with a significantly reduced affinity for the BCR and a reduced ability to support receptor-mediated tyrosine phosphorylation of multiple cellular substrates (7). To determine whether Syk(Y130E) could associate with tubulin, cytosolic fractions were prepared from Syk- DT40 cell lines expressing equivalent levels of Syk(WT) or Syk(Y130E) and adsorbed to colchicine-agarose. Bound proteins were separated by SDS-PAGE and immunoblotted with anti-Syk antibodies.
cross-linking had little effect on the phosphorylation of tubulin—the three tubulin-associated proteins. In contrast, receptor cells, receptor engagement stimulated the phosphorylation of or the Syk(Y130E) mutant (Fig. 6). In Syk(WT)-expressing Bal17 murine B-cells were left unstimulated (−) or stimulated (+) with anti-IgM antibodies. Cytosolic fractions were incubated with colchicine-agarose, and bound proteins were separated by SDS-PAGE. Membranes were probed with anti-phosphotyrosine antibodies (lanes 1 and 2), anti-Cbl antibodies (lanes 3 and 4), or anti-Vav antibodies (lanes 5 and 6).

(Fig. 5A) and then stripped and reprobed with anti-tubulin antibodies (Fig. 5B). These data indicate that the mutation at Tyr-130, which strongly affects Syk-BCR interactions, has little or no effect on the Syk-tubulin interaction.

The phosphorylation of colchicine-agarose-binding proteins was compared in Syk− DT40 B-cells expressing either Syk(WT) or the Syk(Y130E) mutant (Fig. 6). In Syk(WT)-expressing cells, receptor engagement stimulated the phosphorylation of the three tubulin-associated proteins. In contrast, receptor cross-linking had little effect on the phosphorylation of tubulin-associated proteins in the Syk(Y130E)-expressing cells.

DISCUSSION

A small amount of tubulin can be coimmunoprecipitated with Syk in anti-Syk immune complexes prepared from the soluble fractions of anti-IgM activated B-cells (6), indicating that these proteins are able to interact with one another. In the reciprocal experiment, antibodies to tubulin coimmunoprecipitate an endogenous protein-tyrosine kinase activity that catalyzes the tyrosine phosphorylation of tubulin (Fig. 1A). Low levels of a 72-kDa phosphoprotein can be seen in the anti-tubulin phosphorylation assays, but the low levels of Syk in these immune complexes proved difficult to detect by Western blotting with the available anti-Syk antibodies. Tubulin-associated Syk, however, could be detected easily when isolated from a Syk− DT40 B cell line in which murine Syk was expressed at an elevated level (Figs. 1B and 5A). This association permits the in vitro phosphorylation of tubulin by Syk on a single tyrosine that lies near the carboxyl terminus of the α subunit (6).

It should be noted that although tubulin is the predominant phosphoprotein seen in Fig. 1A, longer exposures did reveal the presence of a number of other phosphoproteins, including several between 66 and 205 kDa. The absence of a robust Syk autophosphorylation is not surprising, as we have previously observed significant decreases in Syk autophosphorylation in vitro in the presence of a good substrate (18). This also is likely to explain the absence of other prominently phosphorylated proteins, because it is likely that Syk is the predominant tyrosine kinase in these immune complexes and that tubulin is the best Syk substrate present.

Two additional hematopoietic cell tyrosine kinases, Fyn (19) and ZAP-70 (17), have been reported to bind tubulin. The interaction of Fyn with tubulin is mediated by the Fyn SH2 domain and requires the prior tyrosine-phosphorylation of tubulin (19). However, a similar mechanism of association is unlikely to mediate the Syk-tubulin interaction, because a catalytically inactive form of Syk retains its tubulin binding capacity despite its inability to catalyze tubulin phosphorylation (Fig. 1). Also, the interactions of Syk(WT) with tubulin in Syk(WT)-expressing DT40 cells (Fig. 1B) and of ZAP-70 with tubulin in Jurkat T-cells (17) are independent of receptor engagement. Likewise, a mutant form of Syk (Syk(Y130E)) that exhibits a much reduced affinity for the phosphorylated BCR (7) retains its ability to bind tubulin (Fig. 5A). Thus, tubulin phosphorylation is not a prerequisite for Syk binding. Tubulin, however, is capable of binding to the SH2 domains of Grb2, the p85 subunit of phosphatidylinositol 3-kinase and phospholipase C-γ1 at regions distinct from the phosphotyrosine binding sites (20). Because the amino termini of Syk and ZAP-70 each contain a tandem pair of SH2 domains, an SH2 domain-mediated interaction of these kinases with tubulin in a phosphotyrosine-independent manner remains a possibility.

The nature of the Syk-tubulin interaction is also reflected in the manner in which tubulin becomes phosphorylated on tyrosine. Previous studies implicated Syk in the in vitro phosphorylation of tubulin, because the pretreatment of B-cells with the Syk-selective inhibitor piceatannol abrogated the receptor-stimulated tyrosine phosphorylation of tubulin (6). We have
now extended these findings, using both Syk\(^-\) DT40 cells and Syk\(^-\) cells stably expressing a catalytically inactive form of Syk, to show that Syk catalytic activity is indeed required for tubulin phosphorylation (Figs. 1C and 2). Thus, the phosphorylation state of tubulin on tyrosine in B-cells is an indicator of the presence of tubulin-associated, active Syk kinase. In cells expressing normal levels of endogenous Syk, there is little or no tubulin phosphorylation in the absence of receptor cross-linking (Fig. 2). In cells overexpressing Syk(WT), the basal level of tubulin phosphorylation is somewhat elevated, and this increases with receptor cross-linking (Fig. 1C). In cells overexpressing Syk(Y130E), a form of Syk that is constitutively active and fails to bind to the antigen receptor (7), tubulin is constitutively phosphorylated on tyrosine and this phosphorylation is not enhanced further by receptor cross-linking (Fig. 6).

We observed at least three protein substrates that become phosphorylated on tyrosine following receptor engagement and that co-isolate with soluble tubulin by affinity chromatography on colchicine-agarose (Fig. 2) or by immunopurification with anti-tubulin antibodies (Fig. 3A). These include Cbl, Vav (Fig. 4), and one additional protein of 74,000 Da that remains to be identified. Both Cbl and Vav are known to be phosphorylated by tyrosine kinases activated downstream of immune recognition receptors (15, 16, 21–26). The tyrosine phosphorylation of tubulin is distinguished from the phosphorylation of other proteins, such as Cbl and Vav, which require Syk to be activated by receptor cross-linking. Unlike tubulin, these tubulin-associated proteins are not constitutively phosphorylated in cells expressing Syk(Y130E) (Fig. 6), and this is true in general for the bulk of tyrosine-phosphorylated proteins seen in whole cell lysates (7). If Syk is directly responsible for the phosphorylation of Cbl and Vav, then this difference might be accounted for by differences in the mechanisms by which Syk interacts with its various substrates. Because the recovery of these proteins by affinity chromatography was blocked by free colchicine and required the presence of soluble tubulin dimers in the lysate (Fig. 3B), it appears that some substrates for receptor-activated protein-tubulin phosphatase 2A (43). The frequent reports of tubulin-associating signaling molecules suggests that tubulin or microtubules might function in part to promote the formation of signaling complexes or perhaps aid in the intracellular localization of these signaling molecules.

The physiological relevance of the association of Syk, Vav, and Cbl with tubulin is as yet uncertain. Tubulin can form protein-protein interactions with a wide variety of potential participants of hematopoietic cell signal transduction pathways ranging from receptors (e.g. the Ig-\(\alpha\) and Ig-\(\beta\) components of the BCR in B-cells (34) and CD2 in T-cells (35)) to downstream signaling molecules, such as ZAP-70 (17), Fyn (19), Syk (6), GSK-3 (36), \(\alpha\)-Mos (37), mitogen-activated protein kinase (33), Cdc2 (38), Cdk5 (39), Vav (17, Cbl (this report), phosphoinositide 3-kinase (40), Grb2, phospholipase C-\(\gamma\) (20), neurofibrin (41), several G protein \(\alpha\) subunits (42), and protein phosphatase 2A (43). The frequent reports of tubulin-associating signaling molecules suggests that tubulin or microtubules might function in part to promote the formation of signaling complexes or perhaps aid in the intracellular localization of these signaling molecules.

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