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Regulation of Bruton Tyrosine Kinase by the Peptidylprolyl Isomerase Pin1*

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Bruton tyrosine kinase (Btk) is expressed in B-lymphocytes. Mutations in Btk cause X-linked agammaglobulinemia in humans. However, the mechanism of activation and signaling of this enzyme has not been fully investigated. We have here shown that the peptidylprolyl *cis/trans* isomerase (PPIase) Pin1 is a negative regulator of Btk, controlling its expression level by reducing its half-life, whereas the catalytic activity of Btk was unaffected. The negative regulatory effect of Pin1 was observed both in cell lines and in Pin$^{-/-}$ mice and was found to be dependent on a functionally intact Btk. This may constitute a feedback loop for the regulation of Btk. The target region in Btk was localized to the pleckstrin homology domain suggesting that interphase phosphorylation of serine 115 (Ser-115) in Btk is required, whereas mitosis phosphorylation of serine 21 (Ser-21) is critical. Accordingly, Pin 1 was shown to associate with Btk through binding to Ser-21 and -115, respectively, both of which lie in a classical Pin1-binding pocket. Using a phosphomitotic antibody, it was found that Btk harbors a *bona fide* MPM2 epitope corresponding to a phosphorylated serine or threonine residue followed by a proline. Our results indicate that the peptidylprolyl isomerase Pin1 interacts with Btk in a cell cycle-dependent manner, regulating the Btk expression level.

Bruton tyrosine kinase (Btk)[3] is a nonreceptor tyrosine kinase belonging to the Tec family of protein tyrosine kinases. This family consists of five mammalian members: Btk, Itk, Tec, Bmx, and Txk. In addition, these kinases are also present in other species (1, 2). Btk is expressed in all hematopoietic cells except T-lymphocytes and plasma cells (3). Mutations in the gene coding for Btk result in X-linked agammaglobulinemia in humans (4–6) and X-linked immunodeficiency (*xid*) in mice (7, 8). Btk consists of five domains from the N terminus: the Pleckstrin homology (PH) domain, the Tec homology (TH), which is further subdivided into the Btk motif (BH) and a proline-rich region, the Src homology 3 (SH3) and SH2, and finally the kinase (SH1) domain. The PH domain is important for membrane translocation during B cell receptor signaling (1, 9, 10), whereas the BH Zn^{2+}-binding motif is essential for the stability of the protein (11, 12). The SH2 domain binds to phosphotyrosine-containing peptide motifs (13), whereas the SH3 domain interacts with proline-rich sequences (14).

Previously, two key tyrosine phosphorylation sites (Tyr(P)-551 and Tyr(P)-223) crucial for the enzymatic activation of Btk have been identified (15, 16). Following B cell antigen receptor engagement, Btk translocates to the cell membrane and becomes sequentially phosphorylated on these two tyrosine residues. Notably, Src family kinases (*e.g.* Lyn) phosphorylate first Tyr-551 in the activation loop of the catalytic domain of Btk. This event is followed by autophosphorylation on Tyr-223 in the SH3 domain (15–21). Although, non-tyrosine phosphorylation may also play an important role, as shown by the fact that protein kinase C down-regulates Btk activity (22), further investigation is required to understand the role of serine/threonine phosphorylation in the regulation of Btk.

The peptidylprolyl isomerase Pin1 has emerged as an important regulator of cell proliferation and DNA replication (23–25). Pin1 interacts with a number of phosphoproteins through recognition of phosphorylated serine-proline or threonine-proline motifs (pSer/Thr-Pro) by its N-terminal WW domain (26). The peptidylprolyl bond, which usually exists in two distinct *cis/trans* conformations, is crucial for determining the structure and activity of a protein. Pin1 promotes the isomerization through its C-terminal prolyl isomerase (PPIase) domain (23). Second, Pin1 is pivotal in regulating the function of a diverse array of proteins by modulating protein-protein interaction, kinase/phosphatase activity, gene expression, steady state levels, and subcellular localization of many of its substrates through interaction with specific pSer/Thr-Pro motifs (27–31). Several kinases, including MAPK, cyclin-dependent kinases (Cdk1), and Polo kinases (Plk1) have been shown to phosphorylate serine and threonine residues located in these motifs. Subsequently, phosphorylation of such motifs in target proteins creates epitopes for the mitotic phosphospecific antibody MPM2 (32, 33). Thus, because Pin1 is also a mitotic regulator and binds to pSer/Thr-Pro motifs in target proteins, several of its own substrates are recognized by this antibody. We show here that Pin1 faithfully interacts with Btk and regulates phosphorylation and steady state levels of Btk. To our knowledge, this is the first study of its kind showing the regulation of tyrosine phosphorylation of a non-receptor tyrosine kinase by the peptidylprolyl isomerase Pin1.

EXPERIMENTAL PROCEDURES

Reagents—Anti-Btk antibodies, the protease inhibitor, and phosphatase inhibitors have been described previously (34). The proteasome inhibitor MG132 and the lysosome inhibitor chloroquine were purchased from Sigma. Anti-Pin1 rabbit polyclonal antibody was from Santa Cruz Biotechnology, whereas the anti-Pin1 monoclonal antibody

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[3] The abbreviations used are: Btk, Bruton tyrosine kinase; PH, pleckstrin homology; TH, Tec homology; SH, Src homology; BH, Btk motif; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; GFP, green fluorescent protein; HA, hemagglutinin; BHK, baby hamster kidney.

was kindly provided by Dr. Giannino Del Sal (Laboratorio Nazionale CIB, AREA Science Park, Padriciano Trieste, Italy). Anti-phosphotyrosine monoclonal antibody 4G10 and anti-pSer/Thr-Pro monoclonal antibody MPM2 were from Upstate Biotechnology, Inc., Lake Placid, NY). Redivue Pro-mix L-(^{35}S) *in vitro* cell labeling mix and (γ-^{32}P)ATP were from Amersham Biosciences.

Cell Culture and Transfections—Human embryonic kidney (HEK)293T, NIH3T3, BHK, RBL-2H3 (rat leukemia cell line having mainly mast cell characteristics), and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 1000 units of penicillin-streptomycin (Invitrogen). A20 and Ramos cells were maintained in RPMI 1640 supplemented with 10% (v/v) fetal calf serum and antibiotics. 293T, NIH3T3, BHK, and COS-7 cells were transfected with FuGENE 6 Reagent (Roche Applied Science) according to the manufacturer's instructions. A20 cells were transfected with electroporation in a 0.4-cm gap cuvette at 310 V and 960 microfarads with a Bio-Rad gene pulser.

Small Interfering RNA (siRNA) Preparation and Transfection—A mixture of four synthetic siRNA oligonucleotides targeted against mouse Pin1 was purchased from Dharmacon (Dharmacon Custom SMARTpool). siRNA duplexes (500 pmol) were electroporated into A20 cells (6×10^6 cells/ml).

Plasmid Constructs—Constructs encoding Btk-GFP, E41K-Btk, K430E-Btk, phook-Btk, GFP-Btk, ΔPH-Btk, R28C-Btk, Y223A-Btk, and Y551F-Btk have been described previously (35). The overlap extension PCR technique (36) was used to create Btk mutants; single mutants S21A-Btk, S115A-Btk, and T191A-Btk were expressed using the pSGT vector, whereas double mutant S21A/S115A-Btk was expressed as a fusion protein in the pEGFP-N3 vector. pcDNA3-Pin1 plasmid was kindly provided by Dr. Giannino Del Sal, and the phook-HA-Pin1 plasmid was supplied by Dr. Jim Xiao (Department of Medicine, Boston University School of Medicine, Boston, MA).

Immunoprecipitation and Immunoblotting—Cells were routinely analyzed 48 h post-transfection. Immunoprecipitation and immunoblotting were performed essentially as described previously (34).

Immunofluorescence and Confocal Microscopy—Cytospin preparations containing Ramos cells were fixed with 2% formaldehyde and permeabilized with 0.1% Triton X-100. Confocal microscopy was performed as described previously (34).

In Vitro Kinase Assay—Immunocomplexes containing Btk wild type and its mutants from transfected COS-7 cells were used for kinase assays as described previously (34). Samples were fractionated on 4–20% gradient SDS-PAGE gels. Gels were fixed, vacuum dried, and the amount of incorporated radioactive phosphate in the SH2-SH3 domain was visualized with phosphorimaging device (Bio-Rad Molecular Imager FX PRO PLUS).

Pulse-Chase Assays—Pin1 and Btk co-transfected BHK cells, and Pin1-transfected RBL-2H3 cells were prestarved for methionine and cysteine by replacing the culture medium with Dulbecco's modified Eagle's medium lacking L-methionine and L-cysteine for 30 min. The cells were labeled with 100 μCi ml^{-1} Redivue Pro-mix L-(^{35}S) *in vitro* cell labeling mix (Amersham Biosciences) for 1 h. After labeling, the cells were immediately washed with prewarmed chase medium (Dulbecco's modified Eagle's medium containing 3 mM L-methionine, 1 mM L-cysteine, and 10% fetal calf serum) and then incubated in the chase medium for the indicated chase times. Cells were harvested and lysed in radioimmune precipitation assay buffer, and Btk was immunoprecipitated as described above. The immunoprecipitated Btk was analyzed on 4–20% gradient SDS-polyacrylamide gels, and the gels were fixed in 50% methanol and 10% acetic acid. Labeled Btk was visualized by autoradiography and quantified with a phosphorimaging device.

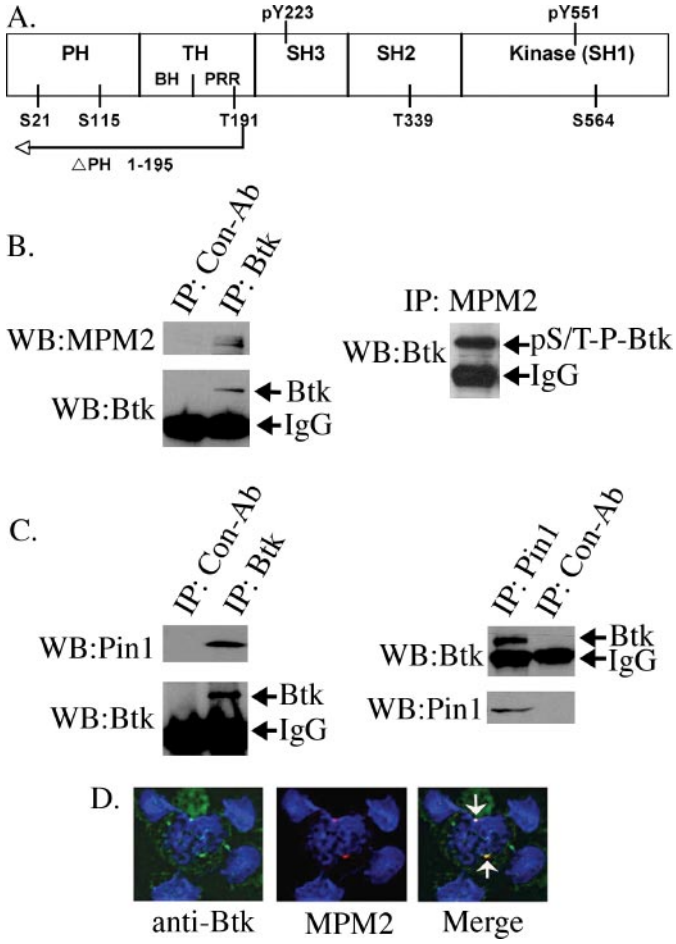

FIGURE 1. **Btk is a substrate of Pin1.** *A*, schematic representation showing the domain organization and tyrosine phosphorylation sites (*top*) and potential proline-directed phosphorylation sites (*bottom*) in Btk. The N-terminal deletion mutant (Δ*PH*) is also indicated. *B*, interaction of Btk with mitotic phosphoproteins. Whole cell lysates from A20 cells were immunoprecipitated (*IP*) with anti-Btk or -MPM2 antibodies and subjected to immunoblotting (*WB*) analysis (*Con-Ab*, control antibody). *C*, reciprocal co-immunoprecipitation analysis shows interaction of Btk and Pin1 in A20 B-lymphocytes. *D*, co-localization of Btk with the mitotic phosphoproteins. Mitotically arrested Ramos B-lymphocytes were fixed and stained with anti-Btk and -MPM2 antibodies, and images were captured by confocal microscopy (*arrows* indicate the centrosome and the colocalization of stainings at this site).

Mice—Pin1-defective mice used in this study were kept at Harvard Medical School and were originally reported to be essentially normal (37) but were later found to display decreased body size, testicular atrophy, and retinal degeneration (27).

RESULTS

In Vivo Interaction of Btk and Pin1—Inspection of the Btk open reading frame identifies five serine and threonine residues followed by a proline, phosphorylation of which could lead to the creation of consensus motif(s) (pSer/Thr-Pro) for binding to Pin1. To assess the possibility that serine/threonine residues in some of these sites are phosphorylated, we decided to use the mitotic phosphospecific monoclonal antibody MPM2, which specifically recognizes phosphorylation events in these motifs (32, 33). As shown in Fig. 1*B*, Btk-interacting proteins were detected in the reciprocal immunoprecipitates from the B-lymphoblastoid cell line A20. Treatment of the clear lysates from the cells with calf intestinal phosphatase prior to immunoprecipitation resulted in the complete disappearance of these proteins, indicating that almost all of

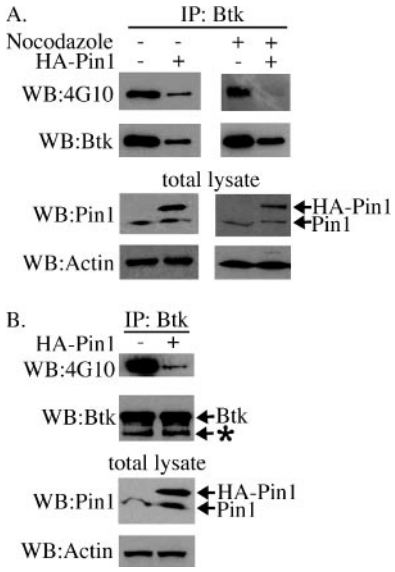

FIGURE 2. **Pin1 negatively regulates Btk.** *A*, COS-7 cells were transiently transfected with Btk with (+) or without (−) HA-Pin1. Cells were lysed, immunoprecipitated (*IP*), and then subjected to Western blot (*WB*) analysis. *B*, A20 cells were transiently transfected with a construct encoding HA-Pin1, and 48 h post-transfection, the lysates were processed for immunoprecipitation and immunoblotting analysis. Nocodazole treatment of cells (50 ng/ml) lasted for 16 h (* indicates a related Btk band of unknown origin).

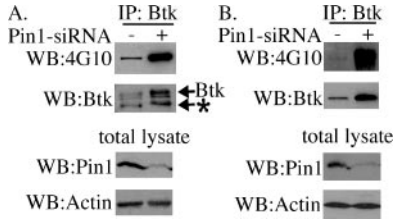

FIGURE 3. **Silencing of Pin1 up-regulates Btk.** *A*, A20 cells were transfected with an siRNA targeted against Pin1. 60 h post-transfection, cells were lysed and immunoprecipitated (*IP*) with anti-Btk antibodies and subjected to Western blot (*WB*) analysis (* indicates a related Btk band of unknown origin). *B*, NIH3T3 cells were co-transfected with Btk and siRNA targeted against Pin1 and thereafter treated as described for *A*.

them were authentically phosphorylated (data not shown). Furthermore, stained Btk colocalized with the MPM2 antibody in mitotically arrested cells (Fig. 1*D*). These results indicate that Btk or a Btk-interacting protein is indeed robustly phosphorylated on a serine or threonine residue in the aforementioned motif. Because Pin1 and MPM2 recognize the same pSer/Thr-Pro motif, these results strongly suggest that Btk could be a potential substrate of Pin1.

Next, we set out to test whether, in native B cells, Btk and Pin1 interact with each other. Reciprocal co-immunoprecipitation analysis in the A20 cell line unequivocally showed that Btk and Pin1 are physically associated with each other (Fig. 1*C*). In addition, the *in vivo* interaction between Btk and Pin1 was replicated in the transfected cell lines COS-7 and BHK (data not shown). These results strongly implicate Btk as a potential substrate of Pin1.

Pin1 Modulates Tyrosine Phosphorylation and Steady State Levels of Btk—Previous studies have shown that Pin1 can modify protein-protein interaction, dephosphorylation, gene expression, steady state levels, and subcellular localization of target proteins (27–31). To probe whether Btk is also a substrate of Pin1, we co-transfected expression vectors encoding Btk and HA-tagged Pin1 into COS-7 cells. The results show that, in cells overexpressing Pin1, Btk was strongly dephosphorylated on tyrosine residues. In addition, steady state levels of Btk were reduced following the ectopic expression of Pin1 (Fig. 2*A* left panel). Similar results were obtained when nocodazole-treated COS-7 cells (arrested at mitosis) were used (Fig. 2*A*, *right panel*), indicating that, in both interphase and mitotic cells, Pin1 down-regulated tyrosine phosphorylation and steady state levels of Btk. To distinguish between the decreased amounts of phosphorylated Btk *versus* the reduced Btk protein level, equal amounts of Btk protein were loaded in the gels. Phosphorylation of Btk in Pin1-transfected cells was reduced as compared with untransfected cells (data not shown). Similar results were obtained in NIH3T3, BHK, and HEK293T cell lines, indicating that it is not a cell type-dependent phenomenon (data not shown). Because Btk and Pin1 were shown to interact with each other also in native cells, we set out to determine whether the increased gene dosage of Pin1 could modulate endogenous Btk. As Fig. 2*B* shows, although steady state levels of Btk were not substantially affected, tyrosine phosphorylation of the protein was severely reduced in ectopically Pin1-expressing cells as compared with the controls.

An important question relates to whether Pin1 causes dephosphorylation of Btk or whether the decreased phosphorylation is secondary to decreased total levels of Btk. In the B cell line A20, which expresses high levels of endogenous Btk, the total amount of this kinase was not detectably reduced following transfection of Pin1, whereas the amount of tyrosine-phosphorylated Btk was attenuated. In contrast, in transfected cell lines, total Btk levels as well as tyrosine-phosphorylated Btk diminished, suggesting the possibility that phosphorylation of a pThr/Ser-Pro motif primarily takes place in pY-phosphorylated Btk and that the loss of this doubly phosphorylated subset is related to the expression level of Btk. This idea is further corroborated by the results obtained from site-directed mutants in which Tyr-551 was replaced (see below).

Tyrosine Phosphorylation of Btk Is Up-regulated in Pin1-depleted B-lymphocytes—The above results clearly indicate that overexpression of Pin1 negatively regulates Btk. Most notably, in cell lines, the degree of expression of Pin1 seems to be critical for regulating the tyrosine kinase activity of Btk. We therefore asked whether down-regulation of endogenous Pin1 in these cells could lead to increased tyrosine phosphorylation and steady state levels of Btk. For this purpose, we employed siRNA technology to generate Pin1-silenced mouse cell lines of B-lymphocyte (A20) and fibroblast origin (NIH3T3). In concurrence with our previous findings, Btk was highly phosphorylated, and moreover, the steady state level of Btk was also up-regulated in A20 cells (Fig. 3*A*). In contrast, although ectopic expression of Pin1 led to a dramatic decrease in the tyrosine phosphorylation of Btk, steady state levels were not significantly affected in Pin1-transfected A20 cells (Fig. 2*B*). Similarly, in Pin1-silenced NIH3T3 cells, tyrosine phosphorylation, as well as steady state levels of Btk, were robustly up-regulated (Fig. 3*B*). In conclusion, the present data suggest that both in Btk-transfected and native cells, Btk is negatively regulated by Pin1 and that the intracellular concentration of Btk may influence this activity.

Btk Half-life Is Affected by the Pin1 Expression Level—Yeh *et al.* (38) recently reported that Pin1 can regulate protein stability by controlling the turnover of c-Myc (38). We performed pulse-chase analysis in Pin1-transfected RBL-2H3 cells (Fig. 4*A*), as well as in Pin1 and Btk co-transfected BHK cells (Fig. 4*B*). Our results clearly show that, in Pin1-transfected cells, the half-life of both exogenous Btk (BHK cells) and endogenous Btk (RBL-2H3 cells) was significantly reduced from 1.7 ± 0.12 to 1 ± 0.10 h and from 3.8 ± 0.14 to 2.7 ± 0.11 h, respectively (data from three experiments). These results indicate that Pin1 facilitates the degradation of the Btk protein.

Inhibition of Proteasomes or Lysosomes Does Not Rescue the Pin1-mediated Degradation of Btk—To determine whether the Pin1-induced degradation of Btk was mediated by the proteasome or lysosome, 293T cells were transiently co-transfected with Btk and an expression vector

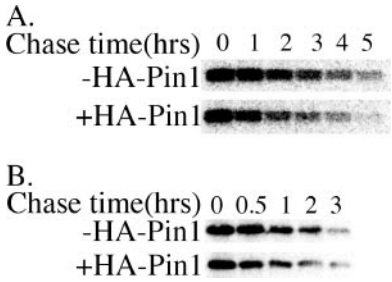

FIGURE 4. **Btk half-life is reduced in Pin1-overexpressing cells.** Pulse-chase radiolabeling assays in Pin1-transfected RBL-2H3 cells (*A*) and Pin1 and Btk co-transfected BHK cells (*B*) were performed 48 h post-transfection.

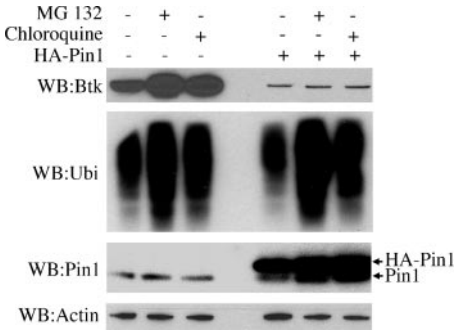

FIGURE 5. **Inhibition of proteasomes or lysosomes fails to rescue Pin1-mediated degradation of Btk.** 293T cells were transfected with Btk in the presence or absence of HA-Pin1. Cells were cultured for 48 h and treated with the proteasome inhibitor MG132 at 10 mM or lysosome inhibitor chloroquine at 100 mM for 16 h. Cells were lysed and subjected to Western blot (*WB*) analysis. *Ubi*, anti-ubiquitin antibody.

encoding Pin1. Cells were treated with proteasome-specific inhibitors MG132 (39, 40) or lysosome-specific inhibitor chloroquine (41). As shown in Fig. 5, both inhibitors augmented protein ubiquitination, whereas neither inhibition of the proteasome nor the lysosome was able to prevent Pin1-mediated loss of Btk. This result suggests that Btk might be degraded through a proteasome- and lysosome-independent process.

The PH Domain, the Tyrosine 551 Phosphorylation, and the Catalytic Activity Are Required for Pin1-mediated Down-regulation of Btk—The PH domain is known to be essential for the lipid binding contributing to its membrane translocation during B cell receptor signaling (35, 42). In addition, the PH domain is crucial for regulating the activity as well as other forms of subcellular localization of Btk (35). Because several of the putative pSer/Thr-Pro motifs in Btk are clustered in the PH-TH domain (Fig. 1*A*), it seemed possible that this module is responsible for the functional interaction with Pin1. To examine this, we employed a PH domain-deleted mutant of Btk (ΔPH-Btk) and transiently transfected it together with an expression construct encoding Pin1 into the 293T cell line. Our results show that Pin1 fails to modulate steady state levels of this deleted form of Btk, strongly suggesting that the PH domain contains the functional motifs required for mediating the interaction of Btk with Pin1 (Fig. 6*A*). These findings were corroborated by results using a naturally occurring (7, 8) PH domain missense mutant (R28C) that impairs tethering of the PH domain to phosphoinositides (9, 34, 42) in the cell membrane (Fig. 6*A*). Conversely, when a constitutively active form of Btk (with enhanced membrane binding (34, 43–46) and carrying another replacement) in the PH domain (E41K) was investigated, this mutant was considerably more sensitive to the Pin1-mediated effects (Fig. 6*B*).

Moreover, because the catalytic activity of Btk is a prerequisite for its phosphorylation and subsequent activity (16, 47), we set out to determine whether Btk activity is also crucial for its regulation by Pin1. Thus,

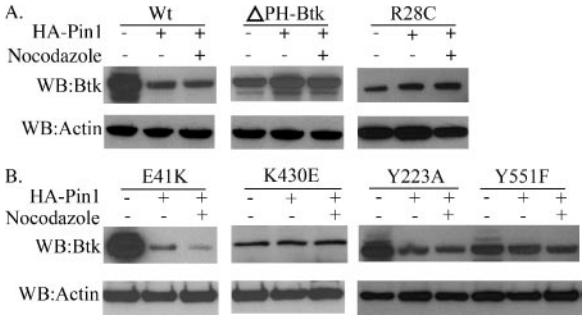

FIGURE 6. **An intact PH domain, the tyrosine phosphorylation state, and the catalytic activity are required for Pin1-mediated down-regulation of Btk.** 293T cells were transfected with wild-type Btk, ΔPH-Btk, and R28C-Btk (*A*) or E41K-Btk, K430E-Btk, Y223A-Btk, and Y551F-Btk (*B*) in the presence (+) or absence (−) of HA-Pin1. Cells were treated with nocodazole (50 ng/ml) for 16 h and subsequently processed for Western blot (*WB*) analysis. *Wt*, wild type.

when Pin1 was co-transfected together with the kinase-defective mutant of Btk (K430E-Btk) into 293T cells, the steady state level of Btk was not affected (Fig. 6*B*).

Accordingly, we investigated whether the Src kinase family target residue Tyr-551 in Btk or the autophosphorylated residue Tyr-223 could be involved. The results show that replacement of Tyr-551 abolished the Pin1 effect, whereas mutation of Tyr-223 did not show any influence (Fig. 6*B*). Taken together, these results indicate that an intact PH-TH domain, as well as an active catalytic site, is necessary for the functional interaction between Btk and Pin1.

Phosphorylation of Serine 21 and Serine 115 Is Required for the Pin1-mediated Dephosphorylation of Btk—The above results indicate that the PH-TH domain mediates interaction of Btk and Pin1. As shown in the schematic representation of Btk (Fig. 1*A*), there are two serine-proline (Ser-21 and Ser-115) and one threonine-proline (Thr-191) motifs that are located in the PH-TH domain. Although under physiological growth conditions, overexpression of Pin1 was shown to modulate the tyrosine phosphorylation status of S21A and T191A mutants, phosphorylation in S115A mutants was virtually unaffected (Fig. 7*A*). In contrast, when cells were arrested at mitosis, Pin1 expression led to the tyrosine dephosphorylation of S115A and T191A mutants, although leaving phosphorylation of S21A mutants intact (Fig. 7*B*). These results indicate that Ser-21 and Ser-115 are important for the functional interaction of Btk and Pin1. This was further corroborated by using the double mutant (S21A/S115A) where there was stable phosphorylation of Btk both in steady state and during mitosis (Fig. 7*C*). Furthermore, co-immunoprecipitation analysis clearly shows that, during interphase, Pin1 binds to Ser-115 of Btk, but during mitosis, Pin1 interacts with Ser-21 of Btk (Fig. 7*D*).

To exclude the possibility that these mutants affect the catalytic activity of Btk, the *in vitro* kinase activity of wild-type Btk and the mutants were measured. The results show that all these mutants have the same kinase activity as wild type, and overexpression of Pin1 does not affect Btk kinase activity (Fig. 8 and data not shown). Altogether, these findings indicate that Btk is differentially phosphorylated on serines in a cell cycle-dependent manner. In steady state, phosphorylation of Ser-115 is responsible for the interaction of Btk and Pin1. In mitosis, however, phosphorylation of Ser-21 is the critical event mediating this interaction.

In Pin1-deficient Mice, Btk Is Strongly Phosphorylated on Ser/Thr-Pro Motif(s), and Btk Expression Is Up-regulated—The above results unequivocally indicate that, at least in cell lines, the intracellular level of Pin1 is a critical component for fine-tuning tyrosine phosphorylation and the stability of Btk. To determine whether similar conditions also prevail *in*

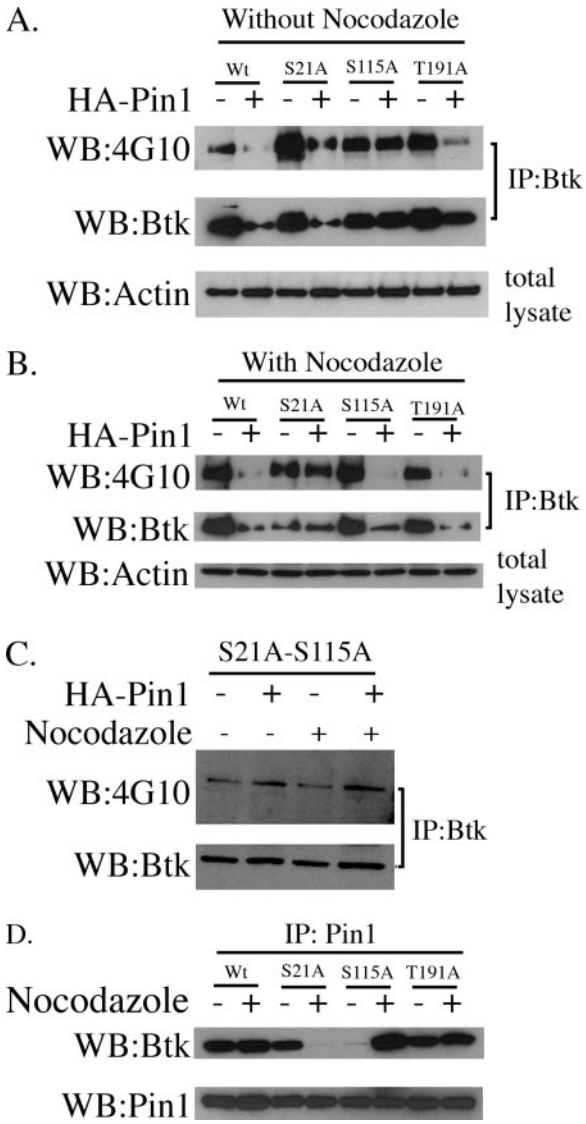

FIGURE 7. **Proline-directed phosphorylation is required for the Pin1-mediated dephosphorylation of Btk.** *A* and *B*, phosphorylation of serine 115 is critical for the negative regulation of Btk by Pin1 in interphase cells, and phosphorylation of serine 21 is important in mitotic cells. Constructs encoding Btk mutants S21A, S115A, T191A, and wild-type Btk were co-transfected with (+) or without (−) HA-Pin1 into COS-7 cells. Cell lysates were subjected to immunoprecipitation and immunoblotting analysis. *C*, the double mutant S21A/S115A-Btk abrogates Pin1-mediated dephosphorylation of Btk. COS-7 cells were transfected with a construct encoding the S21A/S115A double mutant of Btk with or without HA-Pin1. Cells were lysed and processed for immunoprecipitation and immunoblotting analysis. *D*, co-immunoprecipitation analysis shows the interaction of mutated Btk and Pin1 in COS-7 cells. Mutants encoding S21A, S115A, T191A, and wild-type Btk were transfected into COS-7 cells. Total lysates from the cells were subjected to immunoprecipitation and immunoblotting analysis. All experiments were performed in the absence or the presence of nocodazole (50 ng/ml) for 16 h as indicated.

vivo, we examined by immunoprecipitation analysis spleen tissue from wild-type and Pin1-deficient mice for the presence of serine/threonine-phosphorylated Btk. Fig. 9 shows that, in the $Pin1^{-/-}$ mice, but not in control littermates, Btk is strongly phosphorylated on pSer/Thr-Pro motif(s). Moreover, Btk expression level was up-regulated in accordance with data obtained in the tissue culture settings.

DISCUSSION

The major finding in this study is the surprising discovery regarding the functional interaction between the peptidylprolyl isomerase Pin1 and the Tec family kinase member Btk. This interaction results such that active forms of Btk are lost, causing a decreased half-life of this

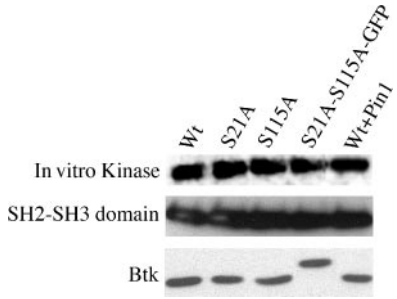

FIGURE 8. **Mutating serine 21, serine 115, or Pin1 expression does not affect the kinase activity of Btk.** Wild-type (*Wt*) Btk and Btk mutants were transfected into COS-7 cells with or without HA-Pin1. 48 h post-transfection, equal amounts of Btk immunocomplexes were subjected to *in vitro* kinase assay. Purified recombinant SH3-SH2 doublet domain of Btk was used as substrate of Btk, and incorporated radioactive phosphate was visualized with a phosphorimaging device.

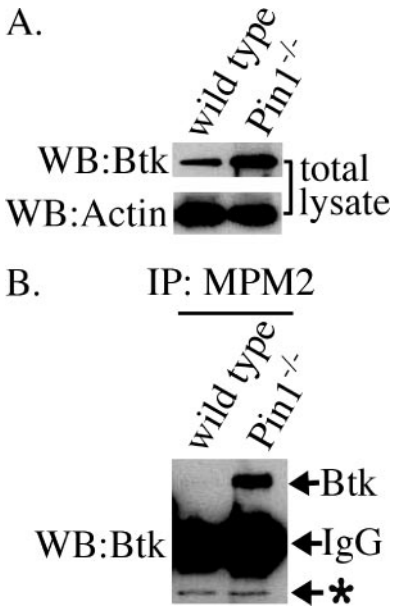

FIGURE 9. **In Pin1-deficient mice, Btk is strongly phosphorylated on serine/threonine-proline motif(s) (pSer/Thr-Pro) and the Btk expression level is up-regulated.** *A*, spleen tissue from wild-type and Pin1-deficient mice was solubilized and subjected to Western blot (*WB*) analysis. *B*, spleen tissue was solubilized with radioimmune precipitation assay buffer, and following immunoprecipitation (*IP*) with an MPM2 antibody, decorated with anti-Btk antibodies (* indicates nonspecific protein, which may serve as an internal control for equal loading).

enzyme. Although many Pin1 substrates have been identified in recent years (48), to our knowledge, this is the first report demonstrating a tyrosine kinase as a substrate of Pin1.

Pin1-catalyzed prolyl isomerization can induce a conformational change in proteins, and previous studies have shown that overexpression of Pin1 is the hallmark in many human cancers (29, 30). In stark contrast, loss of Pin1 function can cause cell cycle progression disorders, neurodegeneration, and alter the primordial germ cell proliferation (23, 49, 50). Recently, it has been reported that cyclophilin A, another member of the peptidylprolyl isomerase family, inhibits the *in vitro* kinase activity of Itk, subsequently leading to the inhibition of T cell signaling (51, 52). Additionally, new data showing overexpression of Pin1 in diffuse large B cell lymphoma have recently been presented, further implicating this enzyme in B cell developmental disorders (53). However, little is known about the molecular mechanism(s) that underlie involvement of this peptidylprolyl isomerase in B-lymphocyte signaling pathways.

Nevertheless, Btk plays critical signaling role(s) in B cell proliferation, differentiation, and activation (54–56). Because Btk harbors five puta-

tive Pin1 binding motifs and three of these motifs are found in the PH-TH domain (Fig. 1*A*) (which was implicated as the critical region), we set out to investigate whether these might function as potential target sites for Pin1. Indeed, in the present work, we have demonstrated that Pin1 faithfully interacts with Btk and regulates its level. Interestingly, the target Ser-21/Pro motif is conserved in all Tec family kinases carrying a PH domain, *i.e.* Btk, Tec, Itk, and Bmx, whereas the Ser-115/Pro motif is found in human, chimpanzee, mouse, rat, and chicken Btk and also in the kinase Tec.

Our results clearly indicate that overexpression of Pin1 results in reduced tyrosine-phosphorylated Btk as well as decreased total levels of Btk, which may have implications for B cell differentiation and development. Tyrosine phosphorylation and activation of Btk occurs following ligand stimulation of a wide variety of receptors, such as the B cell receptor, Fc-receptors, receptor tyrosine kinases, G-protein-coupled receptors, and cytokine receptors (34, 43–45).

Pin1 interacts with its substrates by first recognizing the pSer/Thr-Pro motif (26). Such a motif can sometimes function as an epitope to the mitotic phosphospecific monoclonal antibody MPM2 (32, 33). In steady state, however, this antibody failed to detect ectopically expressed Btk. In contrast, in mitotically arrested cell lines and more importantly in Pin1-deficient mice, phosphorylation of Btk could readily be detected with this antibody, indicating that the presence of the corresponding epitope may be cell cycle-related. As mentioned above, Btk harbors several motifs of that nature, which if phosphorylated, could serve as *bona fide* motifs for Pin1. Residues Ser-21 and Ser-115 are a case in point. Site-directed mutation of each of these amino acids compromises the interaction between Btk and Pin1, strongly suggesting that they may function as PPIase docking sites. Most notably, the tyrosine kinase activity of Btk seems to correlate with the phosphorylation status of these two sites, where Ser-21 is the predominant site in mitosis, whereas Ser-115 is preferred during interphase. Moreover, localization of Thr-191 coincides with a classical consensus MAPK phosphorylation site. However treatment of B cells with MAPK kinase inhibitors (SB203580 and U0126 (57, 58)) did not have any effect on the phosphorylation status of Ser-21 or Ser-115 as detected by an anti-pSer/Thr-Pro monoclonal antibody MPM2 (data not shown). Thus, identification of the elusive kinase(s) responsible for this type of phosphorylation remains to be established.

In the present work, we have also demonstrated that Btk activity is controlled in a cell cycle-dependent manner, which correlates with phosphorylation of Ser-21 and Ser-115 (Fig. 7). Accordingly, during mitosis, but not in interphase, Btk was shown to be strongly tyrosine-phosphorylated (data not shown). We therefore believe that, in steady state, Pin1 binds to the phosphorylated Ser-115 and through this interaction can regulate Btk function. Conversely, in mitotic cells, this interaction seems to be mediated by the phosphorylation of Ser-21. Altogether, these results indicate that Btk may perform distinct functions in different phases of the cell cycle. Although Btk is mainly a cytoplasmic kinase, it shuttles to the nucleus (35) and because endogenous Pin1 is mainly distributed in the nucleus, they may interact in this compartment. However, we noticed that Pin1 is also located in the cytoplasm, making it possible for the interaction to occur outside the nucleus, and results from immunoprecipitations suggest that the interaction may preferentially take place in the cytoplasm.[4] Dougherty *et al.* (59) reported that Pin1 can physically interact with the cytoplasmic signaling molecule Raf-1 and that it regulates the activities of Raf-1. To our knowledge, Raf-1 is not known to shuttle between the cytoplasm and the nucleus, suggesting that this interaction also occurs in the cytoplasm.

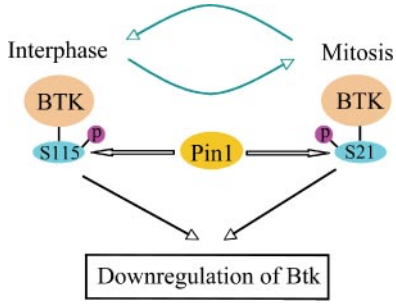

FIGURE 10. **Proposed model for the interaction of Pin1 and Btk.** Pin1 binds to phosphorylated Ser-115 or phosphorylated Ser-21 of Btk during interphase or mitosis, respectively. Through this binding, Pin1 negatively regulates Btk levels by exerting its isomerase activity, causing a conformational change in Btk.

To date, protein kinase C is the only known serine/threonine kinase(s), which can down-regulate Btk kinase activity by phosphorylating the serine 180 in Btk (22). Previous studies have shown that Pin1 can facilitate dephosphorylation of some of its substrates by phosphatase PP2A and that this phosphatase is conformationally specific, dephosphorylating only *trans* pSer/Thr-Pro motifs (31). We used the PP2A-selective inhibitor, okadaic acid, to examine whether PP2A is involved in the process by which Pin1 modulates Btk activities, but we found no evidence to support that PP2A participates in this process (data not shown). Earlier reports suggest that Tec family kinases may have a role in the regulation of the transcription factor nuclear factor of activated T cells, and the phosphatase calcineurin was involved in those processes (60–62). Although we confirm the physical association between Btk and calcineurin, we did not find evidence to show that calcineurin can affect the interaction between Pin1 and Btk (data not shown).

Genetic modification of the ATP-binding site (Lys-430) abolishes the catalytic function of Btk. However, studies from several laboratories have shown that Tec family kinases can partially function as adapter molecules independent of their kinase activity (63, 64). Nevertheless, intact kinase activity is absolutely essential for phosphorylation and activation of Btk (16, 47). In the current study, we show that an intact catalytic site, as well as the regulatory Tyr-551 residue and an intact PH-TH domain, are required for the Pin1-modulated effects on Btk. Accordingly, the constitutively active mutant E41K, which is characterized by enhanced membrane tethering, was particularly sensitive to the Pin1-mediated effects. We have found that Btk is ubiquitinated and degraded by the 26 S proteasome.[5] However, proteasomal degradation does not seem to be the mechanism(s) by which Pin1 affects the Btk level.

We reasoned that it would be crucial if the observed interaction between Pin1 and Btk in the studied cell lines should also have some relevance in an *in vivo* setting. Accordingly, when spleen tissue from Pin1 knock-out mice was analyzed, Btk was found to be strongly phosphorylated on Ser/Thr-Pro motif(s), and its expression levels were elevated (Fig. 9), suggesting an *in vivo* relationship between the two proteins. This finding may be physiologically significant and have implications regarding the involvement of Pin1 in the molecular pathogenesis of X-linked agammaglobulinemia.

The physiological role of Pin1 may be related to the feedback control of Btk activity. Thus, when Btk becomes activated, Pin1 down-regulates its expression, whereas inactive forms of Btk were unaffected. Interest-

[4] L. Yu, A. J. Mohamed, L. Vargas, A. Berglof, and C. I. E. Smith, unpublished data.

[5] L. Vargas, L. Yu, B. Nore, A. J. Mohamed, and C. I. E. Smith, submitted for publication.

ingly, Raf-1, one of the molecules signaling downstream of Btk, is instead activated by Pin1 (59). Whether the regulation of Btk and Raf-1 by Pin1 is in any way correlated remains to be elucidated. There are at least two, not mutually exclusive, scenarios for a feedback loop: Btk could either regulate Pin1 or, alternatively, Btk could regulate Pin1 target sites within the Btk PH domain. We are presently addressing this experimentally. Finally, we are herein proposing the following model (Fig. 10), which may shed light on how the petidylprolyl isomerase Pin1 modulates the tyrosine phosphorylation of Btk. Briefly, recognition of two differentially phosphorylated serine-proline sites by the peptidyl-prolyl isomerase Pin1 in a cell cycle-dependent manner, negatively regulates the cytoplasmic tyrosine kinase Btk.

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