Bruton’s tyrosine kinase (Btk), a nonreceptor cytoplasmic tyrosine kinase belonging to the Tec family of kinases, has been shown to be critical for B cell proliferation, differentiation, and signaling. Loss-of-function mutations in the Btk gene lead to X-linked agammaglobulinemia (XLA), a primary immunodeficiency in humans, and the less severe condition xid in mice. Although Btk is mainly localized in the cytoplasm under steady state conditions, it translocates to the plasma membrane upon growth factor stimulation and cross-linking of the B cell receptor. Nevertheless, in ectopically as well as endogenously Btk-expressing cells, it can also translocate to the nucleus. Deletion of the pleckstrin homology (PH) domain (∆PH1) leads, however, to an even redistribution of Btk within the nucleus and cytoplasm in the majority of transfected cells. In contrast, an SH3-deleted (∆SH3) mutant of Btk has been found to be predominantly nuclear. We also demonstrate that the nuclear accumulation of ∆PH1 is dependent on Src expression. This nucleocytoplasmic shuttling is sensitive to the exportin 1/CRM1-inactivating drug, leptomycin B, indicating that Btk utilizes functional nuclear export signals. In addition, while the ∆PH1 mutant of Btk was found to be active and tyrosine-phosphorylated in vivo, ∆SH3 displayed decreased autokinase activity and was not phosphorylated. Our findings indicate that the nucleocytoplasmic shuttling of Btk has implications regarding potential targets inside the nucleus, which may be critical in gene regulation during B cell development and differentiation.

Bruton’s tyrosine kinase (Btk)1 is a cytoplasmic tyrosine kinase belonging to the Tec family of kinases, a subgroup of the nonreceptor protein-tyrosine kinases (1–3). Btk is the only member of this family in which mutations have been found to be associated with disease. To date, more than 300 unique mutations have been identified in this gene (4). Loss-of-function mutations in Btk lead to X-linked agammaglobulinemia in humans, the hallmark of which is the absence of circulating B lymphocytes. A mutation in the corresponding gene in mice results in X-linked B cell immunodeficiency with a milder phenotype (5, 6). The genetic blueprint of Btk reveals a complex structure shared by many other members of this group of kinases. It is characterized by a pleckstrin homology (PH) domain, a Tec homology (TH) domain, an SH3 domain, an SH2 domain, and finally a kinase domain. The TH domain itself is split into an amino-terminal part, the Btk motif, and a C-terminal domain with a doubly repeated proline-rich stretch (1, 7). On the other hand, the PH domain of Btk has been shown to bind to phosphorylated inositol lipids and may also have other functions, such as protein-protein interactions (8).

Although Btk has been shown to be ubiquitously expressed in nearly all cells of the hematopoietic lineage, except plasma and T cells, it is only the B cells that have been shown to be most vulnerable regarding the functional integrity of Btk. Thus, Btk has been shown to be crucial for B lymphocyte development, differentiation, proliferation, and signaling (9–11). Although the molecular mechanism(s) underlying Btk activation and signaling in B cells are not fully understood, a plethora of Btk-interacting proteins as well as downstream targets/substrates have been described over the last years (7, 12).

Although Btk has been shown to be predominantly cytoplasmic and can translocate to the plasma membrane upon, for example, growth factor stimulation of cells, a small pool of the protein can also translocate to the nucleus under certain physiological growth conditions (13). Most proteins that normally reside in the nucleus contain specific targeting sequences called nuclear localization signals (NLS). The core of the classical NLS utilized by the majority of mammalian proteins consists of a few positively charged residues, mainly arginine and lysine, that assist the interaction with the importin α/β complex before docking to the nuclear pore (14). The cut-off size regarding the transport of macromolecules through the nuclear pore complex is believed to be roughly 40–50 kDa. Thus, smaller proteins can simply diffuse in and out of the nucleus, while proteins that are larger than 60 kDa gain access to the nuclear compartment only if they contain import signals. Proteins that have functions in both the nucleus and cytoplasm contain nuclear export signals (NES) in addition to the NLS. NES are entities specifically recognized by the nuclear export receptor CRM1/exportin 1 for transport of proteins out of the nucleus (15). The prototype NES is a leucine-rich hydrophobic sequence initially identified in Rev, an mRNA-exporting protein from the human immunodeficiency virus type 1 (16). The nuclear export of proteins that contain NES can be inhibited by leptomycin B, a Streptomyces metabolite that binds covalently to CRM1 (17, 18).

In the present work, we have extended our initial investiga-

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1 The abbreviations used are: Btk, Bruton’s tyrosine kinase; PH, pleckstrin homology; SH2 and -3, Src homology 2 and 3, respectively; GFP, green fluorescent protein; NLS, nuclear localization signal(s); NES, nuclear export signal(s); CRM1, chromosome region maintenance; TH, Tec homology.

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Nucleocytoplasmic Shuttling of Bruton’s Tyrosine Kinase*

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tion regarding the intracellular distribution of Btk to include events taking place in the nucleus. Our results show that Btk can translocate to the nucleus of cells. In addition, we provide evidence that Btk utilizes functional CRM1-dependent nuclear export signal(s) to shuttle between the nucleus and the cytoplasm. Also, in Src-deficient fibroblasts, the nucleocytoplasmic shuttling of a deleted PH domain mutant of Btk was found to be dependent on c-Src expression.

MATERIALS AND METHODS

Reagents—The protease inhibitor mixture in the form of a tablet was from Roche Molecular Biochemicals. All phosphatase inhibitors were purchased from Sigma. LMB was a generous gift of Dr. Minoru Yoshida (University of Tokyo, Japan). Anti-Btk rabbit polyclonal antibody has been described previously (13), as has the use of anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY). Rabbit antiserum to Lyn was from Santa Cruz Biotechnology.

Cell Lines and Transfections—COS-7, 293T, NIH 3T3, SYF, and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum and 1000 units of penicillin/streptomycin (Life Technologies, Inc.). The B cell lymphoid cell lines Ramos and Raji and the pre-B cell line Nalm6 were cultured in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. The HMC-1 mast cell line was grown in Iscove’s medium supplemented with 10% fetal calf serum and antibiotics. All cells were cultured at 37 °C in a humidified 5% CO2 incubator. Transfections were carried out by using the Fugene 6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Cell fusion and the interphase heterokaryon assay were performed essentially as described (19).

Plasmid Constructs—The Btk-GFP and K430E-GFP constructs have been described previously (13). For generation of a GFP-Btk chimera protein, the Btk cDNA was cloned in frame into plasmid pEGFP-C1 (CLONTECH) using conventional cloning procedures. The double tyrosine-mutated Btk, Y223A/Y551F (2YF), was generated by site-directed mutagenesis. For the SH3-GFP (SH3) fusion construct, residues 201–253 were deleted from the SH3 domain using overlapping extension polymerase chain reaction. This modified segment was transferred to plasmid Btk-GFP by replacing the corresponding fragment in the wild-type counterpart. All constructs involving manipulations by polymerase chain reaction were sequenced to ensure the absence of mutations. To create a PH-deleted green fluorescent protein (GFP) fusion construct, the Btk cDNA was excised using relevant restriction endonucleases that selectively truncate the PH-TH region, and the resulting fragment was subsequently ligated in frame into the C terminus of plasmid pEGFP-C2 (CLONTECH). These constructs have been referred to as GFP-PH1 (Δ1–195) and GFP-PH2 (Δ1–173), respectively, throughout (or ΔPH1 and ΔPH2). Plasmid pSGT-Src527 was kindly provided by Dr. G. Superti-Furga (EMBL, Heidelberg, Germany).

Subcellular Fractionation—Actively growing cells were harvested by low speed centrifugation at 4 °C and washed twice with ice-cold phosphate-buffered saline. They were resuspended in lysis buffer (10 mM Hepes, 2 mM MgCl2, 15 mM KCl, 0.1 mM EDTA, and 0.15% Nonidet P-40, with protease and phosphatase inhibitors), subjected once to a freeze-and-thaw procedure, and kept on ice for 15 min. The supernatant was centrifuged at 14,000 rpm for 20 min and stored as cytosolic extract. The pellet was washed with lysis buffer, and nuclei were centrifuged at 14,000 rpm for 20 min and stored as nuclear extract. The supernatant was centrifuged at 14,000 rpm for 20 min and stored in aliquots at −80 °C for later use.

Immunoprecipitation, Kinase Assay, Immunoblotting—Cells were routinely analyzed 48 h post-transfection. Immunoprecipitation, in vitro kinase assay, and Western blotting were performed essentially as described (13).

Immunofluorescence—Cells were seeded overnight at 50% confluence onto coverslips in six-well dishes and transfected the following day with the different fusion constructs. Leptomycin B-treated cells were cultured in the presence of 10 ng/ml LMB for 3–16 h. Cells were processed 48 h post-transfection for confocal microscopy analysis as described (13).

RESULTS

We have undertaken a GFP fusion approach to investigate and explore the intracellular localization of Btk and also its trafficking properties. In an earlier work, we extensively described the usefulness of this system for dissecting the dynamics of in vivo redistribution of this protein in response to a wide variety of stimuli, both in native and in transfected cells (13). We have previously described a fusion construct consisting of Btk and GFP, where the Btk cDNA is genetically hooked to the moiety does not affect its trafficking properties and intracellular distribution adversely, we have fused the Btk cDNA to the amino terminus of endogenous Btk. N, nuclear; C, cytosolic; WB, Western blot.

FIG. 1. Expression of Btk-GFP, GFP-Btk, and GFP-ΔPH2 (ΔPH2) in 293T cells. A, immunoblot analysis showing total lysates from cells transfected with the different fusion proteins. Lane 1 corresponds to native Btk from the mast cell line, RBL-2H3. Equal loading of each sample was verified by Ponceau S staining of the filter (not shown). B and C, confocal images showing the subcellular distribution of Btk-GFP in transiently transfected cells, COS-7 and the pre-B cell line Nalm6. The fluorescence intensity changes across the white line were plotted as line intensity histograms in the right panel. D, subcellular distribution of endogenous Btk. N, nuclear; C, cytosolic; WB, Western blot.
indicating that tagging of Btk with GFP does not compromise the function and/or localization of Btk in any way.

**Constitutive Nuclear Translocation of Btk**—To further monitor the trafficking and subcellular localization of Btk, we transfected Btk-GFP and GFP-Btk fusion constructs into COS-7 cells and carefully analyzed their intracellular distribution. Although Btk has been found to be predominantly cytoplasmic, the protein also localized to the nucleus in a small subset of cells, corresponding to less than 10% of the total (Fig. 1B). In addition, Btk translocation to the nucleus was reproducible in several different cell lines irrespective of whether these have been transiently or stably transfected (not shown). Finally, transfection of the pre-B cell line Nalm6 with the same construct gave a similar fluorescence pattern, whereby Btk, again in a few cells, localized to practically all parts of the cell including the nucleus (Fig. 1C).

To exclude the possibility that the observed nuclear localization of Btk in these transfected cells is artifactual due to the consequences of overexpression, we investigated Btk localization in different hematopoietic cells. Since we have not been able to use anti-Btk antibodies successfully in an immunofluorescence setting, we employed biochemical and subcellular fractionation techniques for determining localization in the nucleus. Btk was detected in the nuclear fraction in B cells (Ramos), pre-B cells (Nalm6), and mast cells (HMC-1), indicating that the protein can indeed localize to the nucleus of endogenously expressing cells. Nuclear accumulation of Btk was, however, more robust in Nalm6 (pre-B) and HMC-1 (mast) cells than in mature B cells (Fig. 1D and data not shown).

**Leptomycin B Induces Nuclear Accumulation of Btk**—The presence of Btk in the nuclei of transfected cells as well as hematopoietic cells indicates that there might be a dynamic movement of the protein from the cytoplasm to the nucleus. To obtain further insight into the mechanism of how Btk translates to the nucleus of cells, we treated Btk-GFP-transfected COS-7 cells with the exportin 1 (CRM1)-inactivating antibiotic, leptomycin B. Under steady state conditions, Btk localized exclusively to the cytoplasm in more than 90% of untreated cells (Fig. 2, A and B). Incubation of cells with the drug resulted in the dramatic accumulation and retention of Btk in the nucleus of all cells, indicating that Btk or a Btk-interacting protein might indeed harbor functional nuclear export signal(s) (Fig. 2, C and D). Similarly, LMB treatment induced the nuclear accumulation of endogenous Btk in hematopoietic cells (Fig. 3, A and B).

**ΔPH1 Is Efficiently Translocated to the Nucleus**—Inspection of the amino-terminal region of the PH domain of Btk reveals several short lysine- and arginine-rich regions that could function as potential nuclear localization signals. Furthermore, the PH domain also contains a unique motif with considerable resemblance to the bipartite-basic type of nuclear localization signal carried by nucleoplasmin. To determine whether these basic-rich motifs of the PH domain can play any role in the nucleocytoplasmic shuttling of Btk, a mutant protein that lacks its entire PH-TH domain doublet was generated. Transfection of a plasmid construct encoding ΔPHBtk fused to the C terminus of GFP (GFP-ΔPH1) in COS-7 cells resulted in a diffuse fluorescence pattern strongly reminiscent of that of GFP, with some cells displaying higher intensity in the nucleus (Fig. 4B). Although ΔPH1 did not translocate to the plasma membrane, it distributed equally well in the cytoplasm and the nucleus in the majority (>80%) of cells. Together, these results strongly suggest that while the PH-TH region seems to influence the nuclear export, it is not required or crucial for the translocation of Btk to the nucleus.

**Src Promotes ΔPH1 Nuclear Translocation**—Phosphorylation and activation of Btk in transfected fibroblast cells, such as COS-7 cells, has been shown to be dependent on the concomitant overexpression of most members of the Src family of protein-tyrosine kinases (21). We have shown earlier that, when co-expressed with Src, Btk translocated to the plasma membrane of cells, while a portion of it accumulated intracellularly (13). However, overexpression of active Src in cells normally causes profound morphological changes such as cell rounding and subsequent detachment of cells from the substrate, making interpretation of results difficult. Therefore, we have undertaken another approach to investigate the biochemical and functional roles of Src regarding the subcellular distribution of Btk. We therefore transfected the Src-deficient cell line, SYF, with our fusion constructs. SYF is an immortalized fibroblast cell line derived from a knockout mouse having the major ubiquitously expressed Src family members (Src, Yes, and Fyn) deleted (22). Subcellular localization of the wild-type Btk and the SH3-deleted mutant was indistinguishable from that in COS-7 cells (Fig. 4, compare A and D and compare C and F). In contrast, the PH-deleted mutant was excluded from the nucleus and was entirely cytoplasmic (Fig. 4E). However, LMB treatment induced complete accumulation of this mutant in the nucleus (Fig. 5, A and B). Next, we decided to determine whether reconstitution of c-Src into these Src-deficient cells...
affects subcellular distribution of ΔPH1. Therefore, we cotransfected the SYF cells with c-Src and plasmids encoding the different fusion constructs. While the subcellular localization pattern of Btk and ΔSH3 remained the same in the absence or presence of active Src, ΔPH1 accumulated in the nucleus in all cells (Fig. 5, C and D). These results suggest that the nucleocytoplasmic shuttling of the PH-deleted mutant of Btk is controlled by members of the c-Src family.

**SH3 Deleted Btk Is Predominantly Nuclear**—The SH3 domain of Btk is an important module mediating protein-protein interactions as well as potential intramolecular associations involving the proline-rich motifs (23, 24). In addition, one of the major tyrosine phosphorylation sites of Btk has been shown to reside in the SH3 domain (25). Remarkably, not a single X-linked agammaglobulinemia-causing missense mutation has so far been found in the SH3 domain, indicating that it plays a fundamental role in the regulation of Btk. However, deletion of the SH3 domain of Btk has been shown to produce a protein that had decreased autokinase activity in comparison with the wild type (26). In contrast, a naturally occurring alternatively spliced isoform of Tec having its entire SH3 domain deleted has been shown to be highly active (27). We decided, therefore, to reinvestigate and evaluate the role of the SH3 domain in the regulation of Btk. Along that line, we set out also to explore whether this domain contains information that could be essential for the intracellular trafficking and subcellular localization of the protein. Thus, the large majority of amino acids that contribute to the SH3 domain were deleted, and the resulting mutant was fused to GFP (ΔSH3-GFP). The fusion protein was expressed in COS-7 and SYF cells, respectively. As shown in Fig. 4, C and F, deletion of the SH3 domain renders Btk predominantly nuclear in more than 90% of cells. Similar results were obtained in HeLa cells, indicating that the observed protein distribution was not due to the cell line used (not shown).

Since the ΔSH3 is the prototype nuclear form of Btk, we asked whether it cannot only enter and accumulate in the nucleus but can also be efficiently exported from there. Therefore, we utilized an interspecies heterokaryon analysis, a very sensitive in vivo assay that allows the simultaneous monitoring of the exit of the protein of interest from the donor nucleus to the cytoplasm and import into the surrounding acceptor nuclei. HeLa cells transfected 24 h earlier with a plasmid encoding ΔSH3-GFP were mixed and fused with a large excess of NIH 3T3 fibroblast cells. ΔSH3 could be detected readily in the nuclei of the mouse 3T3 cells (acceptor nucleus) following cell fusion after only 1 h (Fig. 5, E and F). These data strongly suggest that Btk unequivocally shuttles between the cytoplasm and the nucleus.

In addition, the autokinase activity of this mutant was found to be severely diminished, indicating that the SH3 domain could be important for the enzymatic regulation of Btk (Fig. 6B). Finally, the ΔSH3 mutant was not tyrosine-phosphorylated in vivo (Fig. 6C).

**ΔPH1 Is Active and Tyrosine-phosphorylated**—Earlier studies have shown that the PH domain may exert a certain degree of inhibitory effect on the activity of some members of Tec family kinases, protein kinases B and protein kinase D (28–31). Therefore, we decided to characterize this mutant in order to understand the implications of deleting the PH domain of Btk. To test whether the mutant protein has some autokinase activity, we transfected 293T cells with the GFP-ΔPH construct and performed an in vitro kinase complex assay. We found that deletion of the PH domain did not compromise the autokinase activity of Btk (Fig. 6B). Moreover, the ΔPH2 mutant displayed higher activity than ΔPH1 and was comparable with that of the wild-type protein. Next, we sought to determine whether tyrosine phosphorylation of this construct occurs in vivo despite the removal of the PH domain. Total lysate from 293T cells transfected with this construct was subjected to immunoprecipitation followed by immunoblotting with an anti-phosphotyrosine antibody. Fig. 6C shows that the ΔPH mutants can undergo tyrosine phosphorylation similar to the wild-type counterpart.

**Active Btk Is Present in the Nucleus**—We also sought to determine whether the small fraction of Btk that is present in the nucleus is phosphorylated. Accordingly, we used the pre-B cell line Nalm6 as a model cell to investigate the tyrosine

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**Fig. 4.** Confocal microscopy of COS-7 (upper panel) and Src kinase family defective SYF cells (lower panel) transfected with the different fusion constructs. A and D, Btk-GFP; B and E, GFP-ΔPH1; C and F, ΔSH3-GFP.

**Fig. 5.** Src induced nuclear translocation of ΔPH1. SYF cells were transfected with the GFP-ΔPH1 construct and treated overnight with LMB (A and B). C and D, SYF cells were cotransfected with plasmids encoding constitutively active c-Src and GFP-ΔPH1. B and D show fluorescent staining of nuclei by Hoechst. E and F show interspecies heterokaryon analysis between ΔSH3-GFP-transfected HeLa cells and NIH 3T3. Hoechst staining was used to distinguish between human and mouse cell nuclei. H, human; M, mouse. The arrows indicate mouse and human nuclei within the heterokaryon (F). Both cells are also positive for GFP fluorescence (E).
phosphorylation of Btk. The main reasons for choosing this cell line are 1) this is the developmental stage at which the phenotype in X-linked agammaglobulinemia is first manifested, 2) expression of Btk is relatively high compared with mature B cells, making biochemical detection easier, 3) tyrosine phosphorylation of Btk is constitutive, obviating the need for prior stimulation of cells (32), and 4) we found in preliminary studies that, under normal physiological growth conditions, a substantial amount of Btk is present in the nucleus (this study). To that end, cytoplasmic and nuclear fractions have been extracted from actively growing Nalm6 cells, and lysates from these were subjected to immunoprecipitation using anti-Btk antibodies followed by Western blotting with the anti-phosphotyrosine antibody, 4G10. Our results show that Btk can be readily tyrosine-phosphorylated in these cells and that this active Btk exists in both the cytosolic and nuclear fractions (Fig. 7, left). The amount of tyrosine-phosphorylated Btk in the nucleus, however, is far less than that in the cytosolic fraction. Instead, a shorter significantly phosphorylated Btk-specific band can be detected in the nuclear fraction of these cells. Moreover, under the experimental conditions employed, the anti-Btk antibody precipitated several additional tyrosine-phosphorylated proteins ranging between 56 and 160 kDa (Fig. 7, short arrows).

FIG. 6. A, schematic representation showing the different Btk mutants used in this study. The solid lines correspond to the deleted region(s) in the Btk cDNA. The arrows indicate the mutagenized amino acid. B, in vitro kinase complex assay in 293T cells transfected with the fusion constructs. C, tyrosine phosphorylation of the fusion constructs. Lysates from 293T cells transfected with the fusion proteins were subjected to immunoprecipitation (IP) and immunoblotting (WB) analysis.

DISCUSSION

The major finding in this study has been the discovery of the remarkable ability of Btk to shuttle between the cytoplasm and the nucleus. The majority of proteins that do so, however, perform duties in both compartments. Btk translocates to the nucleus but seems to be exported rapidly. As far as we know, Btk phosphorylates its major substrates primarily in the cytoplasm or in the inner surface of the plasma membrane. Whether corresponding nuclear targets of Btk also exist remains to be established. There are, however, examples of other nonreceptor tyrosine kinases that translocate to the nucleus by virtue of their distinct nuclear localization signals (33). In addition, these kinases utilize specific NES in order to exit from the nucleus. We do not, at the present time, understand the molecular mechanism(s) underlying the nucleocytoplasmic shuttling of Btk. Two lines of evidence suggest, however, that this shuttling is independent of the tyrosine phosphorylation status of the protein. This is clearly evident in circumstances whereby in vivo tyrosine phosphorylation of Btk is not feasible. Transfected fibroblasts and unstimulated B cells are a case in point. Yet, in both cases, Btk translocates to the nucleus. Second, Btk mutants that lack catalytic activity or have their major tyrosine-phosphorylated residues mutated can translocate to the nucleus (data not shown). These findings provide further evidence that neither tyrosine phosphorylation nor the catalytic activity is required for the nuclear translocation of Btk. Nonetheless, the machinery used by Btk to get into the nucleus is enigmatic, and whether it is mediated by specific NLS remains to be established.

There is, however, a short basic region containing a cluster of positively charged residues in the PH domain that may, at first glance, correspond to an NLS region. Our analysis, however, indicates that these sequences are neither necessary nor required for the nuclear translocation of Btk, since a PH-deleted mutant of Btk has been found to be equally distributed between the nucleus and cytoplasm. The PH domain, on the other hand, functions as a protein-protein interacting module that mediates the association of Btk with other ligands. Therefore, disruption of such interaction(s) as a result of deleting the PH domain may compromise the cytoplasmic retention or sequestering of Btk.

Although both ΔPH1 and ΔSH3 mutants efficiently translocate to the nucleus, they show significant biochemical and subcellular localization differences. First, ΔPH1 displays substantial autokinase activity. In addition, we found that the ΔPH1 mutant is also tyrosine-phosphorylated. This observation is in striking contrast to recent data reported by others where in vivo tyrosine phosphorylation of a similar construct involving Btk failed to materialize unless it was delivered to the plasma membrane (28). The most plausible explanation regarding these discrepancies is differences in the nature of the constructs as well as the expression systems employed. We used a PH-TH deletion construct, while the deletion in those constructs was confined either to the PH domain or included part of the TH. Moreover, the host cells that we used for
expressing the constructs were the human embryonic kidney cell line 293T, while these investigators used COS-7 and Jurkat cells, respectively (28, 29). Finally, Itk may be different with regard to its phosphorylation and activation mechanisms. The tyrosine phosphorylation of a PH domain-lacking molecule is intriguing, since the PH domain has itself been suggested to be a prerequisite for the plasma membrane targeting and subsequent activation of Btk (34). On the other hand, the ΔSH3 mutant, which had substantially low autokinase activity, compared with ΔPH1 and wild-type Btk, showed virtually no evidence of in vivo tyrosine phosphorylation (Fig. 6C).

While the PH domain-deleted mutant is excluded from the nucleus in the Src-deficient cell line SYF and may also show cell line-dependent localization, the ΔSH3 is predominantly nuclear (Fig. 4, C and F). Furthermore, the nucleocytoplasmic shuttling of ΔPH1 is found to be dependent on Src expression, indicating that a Btk-interacting protein may translocate to the nucleus upon tyrosine phosphorylation by Src. Alternatively, another Src-dependent protein that sequesters Btk in the cytoplasm may release it when tyrosine-phosphorylated, leading to the nuclear translocation of Btk. Finally, interaction of Btk with other Src substrates may expose masked NLS.

As described above, deletion of the SH3 domain renders Btk predominantly nuclear, suggesting that this domain contains nuclear export signals (and/or) enables Btk to localize to the cytoplasm through its protein interacting capacity. Careful inspection of the PH domain reveals a unique leucine-rich sequence motif located between residues 22 and 32 (PLN-KKRLPLL) that mimics classical NES. Leptomycin B also induced nuclear accumulation of the ΔPH1 mutant in SYF cells, suggesting that other candidate NES sequences, in addition to this motif, might exist in Btk. This argument has support, since the increased accumulation of ΔSH3 in the nucleus suggests that the leucine-rich motif in the PH domain is not sufficient for mediating nuclear export of Btk. Alternatively, the nuclear translocation of ΔSH3 could be dominant over its nuclear export, since, in an interspecies heterokaryon analysis, this mutant has been shown to be exported efficiently from the donor nucleus (producer cell) and accumulated in that of a recipient cell shortly afterward.

In conclusion, in the current study we have demonstrated that active Btk, albeit in a much lower amount, is present in the nucleus of hematopoietic cells. It is intriguing to learn that Btk can, under certain conditions, move to the nucleus perhaps to regulate gene expression. In fact, Btk and Tec have been shown to regulate gene expression. In fact, Btk and Tec have been shown to modulate transcription of cytokine genes in mast and hematopoietic cells, suggesting that other candidate NES sequences, in addition to this motif, might exist in Btk. This argument has support, since the increased accumulation of ΔSH3 in the nucleus suggests that the leucine-rich motif in the PH domain is not sufficient for mediating nuclear export of Btk. Alternatively, the nuclear translocation of ΔSH3 could be dominant over its nuclear export, since, in an interspecies heterokaryon analysis, this mutant has been shown to be exported efficiently from the donor nucleus (producer cell) and accumulated in that of a recipient cell shortly afterward.

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