eIF4B phosphorylation by Pim kinases plays a critical role in cellular transformation by Abl oncogenes

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Abstract
Alterations in translation occur in cancer cells but the precise pathogenic processes and mechanistic underpinnings are not well understood. In this study, we report that interactions between Pim family kinases and the translation initiation factor eIF4B are critical for Abl oncogenicity. Pim kinases Pim-1 and Pim-2 both directly phosphorylated eIF4B on Ser406 and Ser422. Phosphorylation of eIF4B on Ser422 was highly sensitive to pharmacological or RNAi-mediated inhibition of Pim kinases. Expression and phosphorylation of eIF4B relied upon Abl kinase activity in both v-Abl- and Bcr-Abl-expressing leukemic cells, based on their blockade by the Abl kinase inhibitor imatinib. Ectopic expression of phosphomimetic mutants of eIF4B conferred resistance to apoptosis by the Pim kinase inhibitor SMI-4a in Abl-transformed cells. In contrast, silencing eIF4B sensitized Abl transformed cells to imatinib-induced apoptosis and also inhibited their growth as engrafted tumors in nude mice. Extending these observations, we found that primary bone marrow cells derived from eIF4B-knockdown transgenic mice were less susceptible to Abl transformation, relative to cells from wild-type mice. Taken together, our results identify eIF4B as a critical substrate of Pim kinases in mediating the activity of Abl oncogenes, and they highlight eIF4B as a candidate therapeutic target for treatment of Abl-induced cancers.
Introduction

Expression of Abl proteins is associated with a variety of hematopoietic malignancies (1-2). In mice, the \textit{v-Abl} oncogene of Abelson murine leukemia virus (A-MuLV) induces pre-B-cell transformation through constitutively activated JAK-STAT signaling (2-3). In humans, chromosomal translocations result in the formation of \textit{Bcr-Abl} hybrid gene that mediates the pathogenesis of chronic myelogenous leukemia (CML) (4). Despite extensive studies, the precise mechanisms by which Abl oncoproteins cause cancer are not fully understood.

Growing evidence suggests that the full transforming activity of Abl involves the activation of STAT/Pim pathway (2, 5-7). For example, previous studies have demonstrated that \textit{v-Abl} cannot efficiently transform bone marrow cells derived from Pim-1/Pim-2 double-deficient mice (Pim-1^{-/-}/Pim-2^{-/-}) (6), indicating that Pim-1 and Pim-2 are required for \textit{v-Abl}-mediated tumorigenesis. A report from the other group also showed that the signal transmitted through Pim kinases is important for survival of hematopoietic cells transformed by Bcr-Abl (8). In addition, it has been revealed that Pim-1 expression was markedly up-regulated following Bcr-Abl-dependent activation of STAT5 (9-10). Together, these observations demonstrate that Pim pathway contributes to cellular transformation by \textit{v-Abl} and Bcr-Abl. However, the precise roles of Pim kinases in Abl-mediated transformation remain elusive.

The Pim kinases are a family of three ubiquitously expressed serine/threonine (Ser/Thr) kinases (Pim-1, Pim-2 and Pim-3). Pim-1 was first identified as a common
integration site in Moloney murine leukemia virus-induced T cell lymphoma (11). Subsequent studies have shown that Pim kinases contribute to both cell proliferation and survival and have thus been implicated in the control of tumor formation (12). For example, Pim-1 and Pim-2 have been demonstrated to be transcriptionally up-regulated in various hematopoietic malignancies (13). In addition, a high frequency of Pim-1 somatic mutation has been found in diffuse large B-cell lymphoma cases (14). Recent studies have also revealed that enhanced Pim expression is associated with other tumors including human prostate, pancreatic, colon and gastric cancers (11, 15-16). These findings suggest that up-regulation of Pim kinases or Pim mutation may provide a selective advantage during cellular transformation. On the other hand, an increasing number of substrates have been identified that are phosphorylated by Pim kinases. These substrates include transcriptional regulators, cell cycle regulators, signaling intermediates, and apoptosis mediators (17-23). Interestingly, eukaryotic translation initiation factor 4B (eIF4B) has recently been identified as a novel Pim-2 substrate (17). Pim-2 kinase has also been shown to modulate protein translation regulator eIF4E-binding protein 1 (4E-BP1) (24-27).

The control of mRNA translation is preferentially exerted at the initiation phase, which is crucial for the specific expression of genes important for development, cell growth, proliferation, and survival. eIF4B is part of the protein complex involved in the ribosome recruitment to the 5′ ends of eukaryotic mRNAs and is thought to stimulate eIF4F activity by potentiating the eIF4A RNA helicase activity in unwinding secondary structures in the 5′ untranslated region (5′UTR) of the mRNA (28). Therefore, eIF4B is a
key component in the regulation of eukaryotic translation initiation. eIF4B is a phosphorylated protein, and its phosphorylation is responsive to extracellular stimuli including serum, insulin and phorbol esters (29-30). Additionally, it has been revealed that eIF4B is regulated by several proto-oncogenic signaling pathways including MAPK, PI3K/mTOR and Akt (30-32). Moreover, eIF4B has been shown to regulate translation of proliferative and pro-survival mRNAs with structured 5’UTR and thus control cell proliferation and apoptosis (24, 33). For example, recent studies have demonstrated that eIF4B is involved in regulating the expression of Cdc25C, c-Myc, Bcl-2, and XIAP. These observations suggest that eIF4B may contribute to cellular transformation.

In this study, we investigated the role of Pim kinases leading to eIF4B phosphorylation downstream of Abl signaling. Our data revealed that expression and phosphorylation of eIF4B are Abl kinase dependent in Abl transformants. Furthermore, we demonstrated that Pim kinase-phosphorylated eIF4B, especially on Ser422, is required for efficient cellular transformation by Abl oncogenes. These results provide novel insights into complex mechanisms by which Abl oncoproteins cause hematopoietic malignancies. In addition, our experiments establish a key role for eIF4B phosphorylation and thus, eIF4B may be a potential target for treatment of Abl-induced cancers.
Materials and Methods

Cell lines and cell culture

Cancer cell lines K562, 293T, Jurkat, A549, and HeLa were purchased from American Type Culture Collection. Hepatocellular carcinoma cell line SMMC-7721 and breast carcinoma cell line MCF-7 were purchased from National Platform of Experimental Cell Resources for Sci-Tech (http://cellresource.cn, Beijing, China). Cells were grown in DMEM or RPMI1640 supplemented with 10% FBS (Gibco) and antibiotics (penicillin and streptomycin) (Invitrogen) as previously described (7). v-Abl transformed mouse cell lines named as NS2 and W44 in this study were generated and cultured as described previously (6, 34). These cell lines were characterized in our laboratory as CD10⁺/CD19⁺ pre-B cells. The 32D myeloid cells stably expressing wild-type Bcr-Abl or Bcr-Abl-T315I were kind gifts from Dr. Guangbiao Zhou (Institute of Zoology, Chinese Academy of Science, Beijing, China). Pim- or eIF4B-overexpressing Abl transformants (K562, NS2 and W44) were generated by stably infecting the cells with retroviruses or lentiviruses encoding Pim or eIF4B using viral vectors pMSCV-GFP or pNL-GFP (Addgene) as previously described (35). Short hairpin RNA (shRNA)- expressing stable Abl transformants were generated by infection of the cells with lentiviruses expressing specific shRNA in pSIH-H1-GFP vector (System Biosciences) as described previously (36). The shRNA sequences are described in Supplementary Materials and Methods.

Antibodies and Reagents
The following antibodies were used in this study: anti-FLAG (Sigma F1804 M2); anti-Pim-1 (Santa Cruz sc-13513 12H8); anti-c-Myc (Santa Cruz sc-40 9E10); anti-Bcr (Santa Cruz sc-885 N-20); anti-Bcl-xL (Cell Signaling 2764 54H6); anti-eIF4B (Cell Signaling 3592); anti-Phospho-eIF4B (Ser406) (Cell Signaling 5399); anti-Phospho-eIF4B (Ser422) (Cell Signaling 3591); anti-Bcl-2 (Cell Signaling 2870 50E3); anti-c-Abl (Merck Millipore OP19 19-110); anti-p70 S6K and anti-Phospho-p70S6K (Thr389) (Cell Signaling 9202 and 9205). All other antibodies were obtained and used as described previously (7). Pim-1/Pim-2 kinase inhibitor SMI-4a was purchased from Calbiochem (San Diego, CA).

**GST pull-down experiment, and in vitro kinase assay**

GST fusion proteins were expressed and purified as previously described (6, 35). Pull-down assays were performed by incubating aliquots of purified GST fusion protein beads with cell lysates at 4°C overnight. Beads were washed and examined by Western blotting. For *in vitro* kinase assay, purified GST fusion proteins were mixed with each other in the kinase buffer incubated at 30°C for 30 min (37). Samples were separated by electrophoresis and probed with the indicated antibodies.

**Immunoprecipitation, immunoblotting, and apoptosis assay**

Preparation of cell extracts, immunoprecipitation and immunoblotting were performed as previously described (6, 36). Where indicated, immunoblot signals were quantified by densitometry. For apoptosis assay, cells were treated with 10 µM imatinib.
or 50 µM SMI-4a. Cells were then stained with propidium iodide/Annexin V-FITC, and analyzed by fluorescence-activated cell sorter (BD Bioscience, San Jose, CA).

**Examination of tumorigenicity using xenograft model in nude mice**

Nude-mouse injection was carried out as described previously (35). Tumor growth was monitored and measured in volume (length × height × width) at the indicated time points after inoculation. Bioluminescent imaging was used to detect tumor growth from GFP-expressing cells. Images were quantified as photons/s using the indigo software (Berthold Technologies, Germany).

**Generation of eIF4B-knockdown transgenic mice**

eIF4B-knockdown transgenic mice were generated by the microinjection method as previously described (38). Western blotting using anti-eIF4B antibody was performed to determine the interference efficiency. The transgenic founders with high interference efficiency were selected and maintained on a C57BL/6J genetic background.

**Primary murine bone marrow transformation assay**

Bone marrow cells were freshly isolated from 5-6 week old mice. v-Abl- and Bcr-Abl-mediated bone marrow transformation was performed as previously described (6, 35). Transformation efficiency was scored by counting the number of the wells containing the transformed cell clones showing cytokine-independent growth 3 weeks after infection.
DNA construction and mass spectrometry (MS)

DNA construction and mass spectrometry analysis are described in the Supplementary Materials and Methods.
Results

eIF4B was found to be a potential target of Pim-1 kinase in v-Abl transformants

Substrates of Pim kinases in Abl transformants are poorly characterized. To identify novel Pim targets in Abl transformants, we employed v-Abl-transformed pre-B cell lines derived from mice (6). The v-Abl transformants were metabolically labeled with $H_3^{32}$PO$_4$. Radiolabeled Pim-1 was then isolated by immunoprecipitation, and phosphorylation was examined by autoradiography. Several phosphorylated proteins were detected in the precipitated protein complex (Fig. 1A). The identities of Pim-1 and SOCS-1 were confirmed by Western blotting. As expected, when expressed in cells, Pim-1 became constitutively auto-phosphorylated. The observed phosphorylation of SOCS-1 is consistent with the previous studies (6, 39). Interestingly, we observed a highly phosphorylated protein at ~80 kDa. Subsequent analysis of tryptic fragments by mass spectrometry led to the identification of this protein as eIF4B (Fig. 1A and Supplementary data 1).

To confirm the interaction between Pim-1 and eIF4B in the v-Abl transformants, we performed the GST pull-down experiments. Association of eIF4B with Pim-1 was observed when either GST-eIF4B was used to pull down Pim-1 (Fig. 1B) or GST-Pim-1 was used to pull down eIF4B (Fig. 1C). Furthermore, we found that eIF4B wild-type and its Ser406Ala (S406A) and Ser422Ala (S422A) mutants had similar capacity to bind Pim-1. Strikingly, however, kinase-dead (KD) mutant of Pim-1 (Pim-1 K67M) lost the ability to interact with eIF4B. These results suggest that interaction between Pim-1 and eIF4B may require Pim kinase activity or the residue lysine 67 on...
Pim-1 may be critical for the physical interaction between these proteins.

**Pim-1 and Pim-2 can directly phosphorylate eIF4B on Ser406 and Ser422**

Previous studies have demonstrated that eIF4B is serine-phosphorylated on Ser406 and Ser422 by several kinases and phosphorylation of these residues is essential for optimal translational activity of eIF4B (17, 30-31). Also, previous experiments have revealed the substrate consensus sequence of Pim kinases (R-X-R-R/H-X-S/T) (17). Using an in silico search, we found that Ser406 and Ser422 in the arginine-rich motif region of eIF4B are potential Pim phosphorylation sites. To test this possibility, 293T cells were co-transfected with eIF4B and Pim kinase, and eIF4B phosphorylation status was then determined by Western blotting with the specific antibodies. We found that expression of Pim-1 or Pim-2 resulted in marked increase in Ser422 phosphorylation and slight increase in Ser406 phosphorylation of eIF4B (Fig. 2A-D). Importantly, expression of Pim-1-KD or Pim-2-KD mutant abolished the phosphorylation, indicating that phosphorylation of eIF4B is Pim kinase-dependent in the cells (Fig. 2A-D). However, expression of Pim-3 had little effect on eIF4B phosphorylation (Supplementary Fig. S1). We further performed in vitro kinase assay by incubating the purified recombinant GST-Pim kinases with eIF4B protein. Indeed, Pim-1 or Pim-2 was able to phosphorylate eIF4B at Ser406 and Ser422, and mutation of these sites to Ala abolished the phosphorylation (Fig. 2E-H). In addition, KD mutation of Pim kinases failed to phosphorylate eIF4B at Ser406 and Ser422. These experiments demonstrate that Pim-1 and Pim-2 directly phosphorylate eIF4B on Ser406 and Ser422.
eIF4B is highly expressed and phosphorylated in v-Abl and Bcr-Abl transformants in an Abl kinase-dependent manner

It is believed that deregulated translational control plays an important role in oncogenic transformation (40). To explore the contribution of eIF4B in Abl-induced cellular transformation, we investigated the eIF4B expression and phosphorylation status in Abl transformants as compared with other cell lines and normal controls. Interestingly, we observed that eIF4B was overexpressed in all three Abl-transformed cell lines analyzed (v-Abl-transformed cell lines W44 and NS2, and Bcr-Abl-expressing K562 leukemic cell) (Fig. 3A and Supplementary Fig. S2A). Strikingly, eIF4B was also highly phosphorylated in Abl-transformed cells, especially on Ser422 residue (Fig. 3A).

To examine whether eIF4B is expressed and phosphorylated downstream of Abl signaling, Abl transformants were treated in a time course with imatinib (Fig. 3B-D and Supplementary Fig. S2B-E). The results showed that the endogenous protein levels of eIF4B decreased after treatment with imatinib, suggesting that expression of eIF4B is Abl kinase dependent. Similar to the situation seen with eIF4B expression, the phosphorylation of eIF4B on Ser406 displayed a similar decrease after imatinib treatment. However, the eIF4B phosphorylation on Ser422 was more sensitive to imatinib treatment as indicated by rapidly reduced phosphorylation levels after exposure to the compound (Fig. 3C and Supplementary Fig. S2B-C). To confirm that expression and phosphorylation of eIF4B is Abl kinase dependent, we employed
Bcr-Abl T315I mutant to test the effect of imatinib. As expected, imatinib treatment had little effect on eIF4B expression and phosphorylation in cells expressing the imatinib-resistant mutant (Fig. 3D). These results reveal that eIF4B is expressed and activated by Abl signaling and Ser422 is a critical residue in eIF4B that can be regulated by this signaling.

**Pim-1 and Pim-2 regulate eIF4B phosphorylation in v-Abl and Bcr-Abl transformants**

To determine whether Pim kinases are able to regulate eIF4B phosphorylation in Abl-transformed cells, SMI-4a, a novel small molecule inhibitor of Pim-1 and Pim-2 kinases (41), was employed. We found that eIF4B protein level and phosphorylation on Ser406 slightly decreased in Abl transformants treated in a time course with SMI-4a (Fig. 4A and B). Interestingly, treatment with SMI-4a led to a more rapid reduction in the phosphorylation of eIF4B on Ser422 (Fig. 4A, B and Supplementary Fig. S3A and B), similar to the pattern observed in imatinib treatment (Fig. 3B-C). To determine the responsible kinases that phosphorylate eIF4B in Abl transformants, we generated shRNA-based Pim-1, Pim-2, or S6K knockdown cells (Fig. 4C-E and Supplementary Fig. S3C-E). Phosphorylation of eIF4B on Ser422 was greatly suppressed by silencing the Pim-1 and clearly decreased after Pim-2 depletion (Fig. 4C and data not shown). In contrast, eIF4B is still phosphorylated in Abl transformants upon silencing S6K (Fig. 4D and E). Similar results were obtained from experiments using rapamycin (Supplementary Fig. S3F and G). These data suggest that the ability of Abl
oncoproteins to regulate eIF4B phosphorylation, especially on Ser422 residue, is mainly mediated by their downstream effectors Pim kinases.

To further test this possibility, we generated v-Abl- and Bcr-Abl-transformed cell lines stably expressing either Pim WT, Pim KD mutants or GFP control using bicistronic retroviruses (Supplementary Fig. S3H). Although the endogenous Pim expression was inhibited by imatinib treatment, the ectopic expression of Pim-1 and Pim-2 caused a marked increase in eIF4B phosphorylation on Ser422 compared to the GFP control (Fig. 4F, G and Supplementary Fig. S3I). As expected, the effect of Pim kinases was dependent on the catalytic activity, since the cells expressing Pim KD mutants recapitulated that of the control. These results confirm that phosphorylation of eIF4B on Ser422 is regulated by Pim-1 and Pim-2 kinases in Abl transformants.

**eIF4B plays a critical role in regulating survival of Abl transformants and functions downstream of Pim signaling in these cells**

Since eIF4B was found to be regulated by Abl kinase-dependent oncogenic Pim signaling and eIF4B is involved in control of pro-survival mRNA translation, we hypothesized that eIF4B might play an important role in regulating survival of Abl transformants. To examine this possibility, we generated stable v-Abl- and Bcr-Abl-transformed cell lines expressing eIF4B-specific shRNAs (Fig. 5A, 5B and Supplementary Fig. S4A). v-Abl transformants initiated apoptosis following imatinib treatment, and approximately 51% of wild-type cells remained viable after incubation with this inhibitor for 15 hours under our culture condition. By contrast, only
approximately 28% eIF4B silencing cells were viable under the same conditions (Fig. 5C). Similarly, approximately 48% of Bcr-Abl positive K562 cells expressing control shRNA were viable after treatment with imatinib for 32 hours. Strikingly, only 27% of K562 cells expressing eIF4B-specific shRNA exhibited viable following the same treatment (Fig. 5D). These experiments demonstrate that silencing eIF4B sensitizes Abl transformants to undergo apoptosis induced by imatinib, although there is no significant difference in the survival of untreated cells expressing control or eIF4B shRNA (Supplementary Fig. S4B and C).

Further, we asked whether eIF4B functions downstream of Pim signaling in Abl transformants. To address this issue, we generated Abl transformants stably expressing either GFP control, eIF4B WT, or its phosphomimetic mutants (Supplementary Fig. S4D). These cells were treated in a time course with Pim inhibitor SMI-4a and analyzed for cell survival. Inhibition of Pim kinases with SMI-4a for 20 hours reduced cell viability to approximately 25% in the control v-Abl cells (pNL), to approximately 40% in the cells expressing eIF4B WT or eIF4B S406E, and to 52-54% in the cells expressing eIF4B S422E or eIF4B S406E/S422E (Fig. 5E). Similar results were observed in K562 cells (Fig. 5F). These data indicate that expression of eIF4B WT and its phosphomimetic mutants conferred various resistance to apoptosis induced by the Pim inhibitor. It appeared that the cells expressing S422E, or S406E/S422E were more resistant to SMI-4a-induced apoptosis than those expressing WT or S406E. Since it has been suggested that Jak2 and Stat5 are key upstream factors that regulate Pim expression, we further tested the effects of silencing these factors on eIF4B
phosphorylation. Indeed, Pim-1 expression and phosphorylation of eIF4B on Ser422 were markedly reduced upon depletion of Jak2 and Stat5 (Supplementary Fig. S4E and F). Together, these results reveal that Pim kinase-dependent eIF4B phosphorylation is important for the viability of Abl-transformed cells and Ser422 in eIF4B is a critical phosphorylation site that is regulated by Abl-Jak-Stat-Pim signaling.

In an attempt to provide insights into the mechanism by which eIF4B regulates survival of Abl transformants, we evaluated the expression of Pim kinase target, c-Myc, and several Bcl-2 family members that are the critical regulators of apoptosis. Our results indicate that silencing eIF4B did not alter protein expression of Bcl-XL, but obviously reduced protein expression of Bcl-2 and c-Myc (Fig. 5G).

Silencing eIF4B significantly inhibits tumor formation induced by K562 leukemic cells in xenograft mouse model

In an attempt to gain a better understanding of the role of eIF4B in Abl-mediated tumorigenesis, we investigated the effect of silencing eIF4B on the tumor formation of Abl-transformed cells using xenograft model in nude mice. Each mouse was inoculated subcutaneously with K562 cells stably expressing shRNA targeting either eIF4B or luciferase control. Remarkably, we found that the tumors formed by cells expressing luciferase-specific shRNA grew much faster than those formed by cells expressing eIF4B-specific shRNA (Fig. 6A). These experiments were repeated at least three times to ensure the reliability of the results and consistency of data. By statistical analysis, we found that silencing eIF4B expression in K562 cells significantly inhibited the tumor.
growth (Fig. 6B and C). These observations were further confirmed by bioluminescent imaging performed to detect tumors (Fig. 6D). Additionally, Western blot analysis of tumor extracts demonstrated the eIF4B depletion in the tumor cells expressing eIF4B-specific shRNA (Fig. 6E). Together, these results suggest that eIF4B is required for Abl-dependent tumor growth.

Primary bone marrow transformation assay using transgenic mice demonstrates a requirement for eIF4B in efficient cellular transformation by Abl oncogenes

To further define the role of eIF4B in Abl-mediated tumorigenesis, we wished to establish a more physiological model system for analysis of eIF4B involvement in the Abl transformation. For this, we generated eIF4B-knockdown transgenic mice as previously described (Supplementary Fig. S5A and B) (38). The transgenic founders with high interference efficiency were selected (Fig. 7A). Bone marrow cells derived from eIF4B-knockdown mice or their wild-type littermates were infected with equal titer of Abelson murine leukemia virus (A-MuLV). The capacity of the virus to transform bone marrow cells was measured as previously described (6, 35). eIF4B wild-type cells infected with the A-MuLV displayed v-Abl transformation with average results of 14 wells showing cytokine-independent growth of cell clones per 96-well plate (Fig. 7B). However, silencing eIF4B significantly decreased v-Abl transformation efficiency. This study demonstrates that disrupting the eIF4B expression has profound effects on the v-Abl-mediated bone marrow transformation.

To confirm this observation, we generated bicistronic retroviruses encoding the
v-Abl or Bcr-Abl and either eIF4B, eIF4B-S422A or GFP control (Supplementary Fig. S5C and D). Bone marrow cells derived from eIF4B-knockdown or wild-type mice were infected with these retroviruses. As expected, GFP and eIF4B-S422A failed to complement eIF4B deficiency for v-Abl- or Bcr-Abl-mediated transformation (Fig. 7C and D). In contrast, significantly increased transformants were found in cells infected with the retroviruses encoding Abl and eIF4B wild-type proteins. To demonstrate the involvement of Pim kinase in the cellular transformation, we generated bicistronic retroviruses encoding the v-Abl or Bcr-Abl and either Pim-1, Pim-1-KD mutant or GFP control (Supplementary Fig. S5E and F) and performed the bone marrow transformation. We observed that overexpression of Pim-1 resulted in significantly increased Abl transformation efficiency in wild-type mice but not in eIF4B-knockdown mice (Fig. 7E and F), suggesting that Pim promotes Abl transformation through phosphorylating eIF4B. Together, these results suggest that v-Abl- and Bcr-Abl-mediated cellular transformation critically requires activated eIF4B that is regulated by Pim-dependent phosphorylation mainly on Ser422.
Discussion

Abl-induced tumorigenesis is a complicated process, involving the activation of signaling pathways that regulate cell survival and proliferation (2, 42). It has been demonstrated that constitutive activation of STAT signaling endows the Abl transformed cells with cytokine-independent growth (43-45). Importantly, activation of STAT signaling strongly induces the expression of Pim kinases in response to Abl oncogenic signaling. Pim-1 and Pim-2 have been shown to be critically required for malignant transformation of hematopoietic cells by Abl oncogenes (6, 8). Despite these progresses, the mechanisms underlying Pim action in Abl transformation and substrates of Pim kinases in Abl transformants are poorly characterized. Here, we, for the first time, demonstrate that Pim-1 and Pim-2 phosphorylate eIF4B mainly on Ser422 and to a lesser extent, on Ser406 in Abl-transformed cells, revealing a novel Pim kinase-dependent eIF4B activation that plays a crucial role in Abl-mediated cellular transformation (Supplementary Fig. S5G). However, Pim-3 has little effect on eIF4B phosphorylation. This is consistent with the previous observation that Pim-3 is not required for v-Abl transformation and it might have different function in these cells (6).

Pim kinases are able to phosphorylate multiple targets and have therefore been implicated in diverse biological processes. For example, there is compelling evidence demonstrating that Pim kinases are of great importance in cellular transformation due to their oncogenic activities that exert influence in regulation of cell survival signaling by phosphorylating BAD and regulation of cell cycle progression through phosphorylation
of p21^{CIP1}, p27^{kip1}, Cdc25A and Cdc25C, as well as regulation of c-Myc activity (25). In addition, Pim-1 and Pim-2 kinases are involved in regulating phosphorylation of SOCS-1, and thereby alter the inhibitory effects of SOCS-1 (34, 39). Based on their oncogenic potential, the Pim kinase family is emerging as an important new target for drug discovery (46). However, malignant transformation of lymphoid and myeloid cells involves the dysregulation or mutation of genes including PI3K/Akt pathway and Pim family (14, 47). The Akt and Pim kinases produce parallel oncogenic signals and share many molecular targets normally involved in regulating the cell proliferation and survival. Therefore, resistance to targeted Pim or PI3K/Akt therapeutics may become a major clinical problem because the redundancy of oncogenic signaling pathways provides back-up mechanisms that allow cancer cells to escape (26, 48).

In this study, our results represent the first report of Pim kinases-mediated phosphorylation of eIF4B in Abl transformants, suggesting that Pim kinases regulate cap-dependant protein translation in these cells. We found that expression of eIF4B S422E phosphomimetic mutant was able to complement inhibition of Pim kinases for Abl transformant survival, implying that phosphorylation of this site plays a key role in eIF4B activity. These data indicate that eIF4B Ser422 phosphorylation is critically required for efficient cellular transformation by Abl oncogenes. In addition, the results reveal that eIF4B mediates some of the effects of the Pim kinases on hematopoietic cell transformation. Previous studies from other group have shown that activity of eIF4B can be regulated through phosphorylation by Akt (30). These experiments reveal that
ability of Akt to phosphorylate eIF4B is potentially critical for the transforming capacity of this kinase. Our results presented in this study provide strong evidence that eIF4B can also be phosphorylated by Pim kinases. Together, these data suggest that the function of eIF4B in regulating cell survival and proliferation is mediated by several proto-oncogenic signaling pathways. These findings indicate that eIF4B represents one of the focal points whereby Akt, Pim and other oncogenic signaling converge to exert their effects on malignant transformation. Therefore, targeting the convergence of oncogenic survival signals on eIF4B is an attractive alternative for development of anti-cancer therapies.

Our study has also begun to address the mechanism by which eIF4B may affect Abl transformation. We observed that depletion of eIF4B led to a significant reduction in the expression of Bcl-2 and c-Myc. This is consistent with the previous experiments showing that eIF4B regulates translation initiation of mRNAs harboring strong to moderate secondary structures in their 5’UTRs such as Bcl-2 and c-Myc (40). It has long been suggested that resistance to apoptosis in Abl-transformed cells is associated with increased expression of anti-apoptotic proteins including Bcl-2 (49-50). Importantly, our observation indicates that Abl kinase-dependent expression and phosphorylation of eIF4B are responsible to control of the anti-apoptotic protein expression. Further research is required to address whether this regulation occurs in human leukemia patients and has any prognostic significance in the leukemia.
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References


Figure Legends

Figure 1. Pim-1 interacts with eIF4B in v-Abl-transformed cells and 293T cells. A, phosphoamino acid(s) in v-Abl-transformed cells was metabolically labeled by adding 500 µCi (18.5 MBq) of H$_3^{32}$PO$_4$ for 2 hours. $^{32}$P-labeled Pim-1 protein was isolated by immunoprecipitation, and phosphorylation was detected by autoradiography ($^{32}$P). Pim-1 and SOCS-1 were examined by Western blotting (WB) and Commassie Blue Staining (CBS). eIF4B was identified by mass spectrometry. B, 293T cells were transfected with Myc-Pim-1 or Myc-Pim-1-KD (Pim-1K67M). Lysates were incubated with purified GST, GST-eIF4B, GST-eIF4BS406A or GST-eIF4BS422A coupling beads. The bound proteins were identified by WB using anti-Myc antibody. C, 293T cells were transfected with Flag-eIF4B, Flag-eIF4BS406A or Flag-eIF4BS422A. Pull-down assay using purified GST, GST-Pim-1, or GST-Pim-1-KD coupling beads was performed as described in (B).

Figure 2. Pim-1 and Pim-2 kinases directly phosphorylate eIF4B. A, 293T cells were co-transfected with Pim-1 or Pim-1-KD and eIF4B or eIF4BS422A mutant. Cell lysates were examined by Western blotting using indicated antibodies to determine the phosphorylation of eIF4B Ser422 mediated by Pim-1. B, experiments were performed to test phosphorylation of eIF4B Ser422 mediated by Pim-2 as described in (A). C and D, experiments were performed as described in (A) to examine the effect of Pim-1 (C) and Pim-2 (D) on the phosphorylation of eIF4B Ser406. E-H, in vitro kinase assay: E, purified recombinant GST-eIF4B or GST-eIF4BS422A protein was incubated with
purified GST, GST-Pim-1 or GST-Pim-1-KD. Phosphorylation of eIF4B on Ser422 was examined by immunoblotting and GST-fusion proteins were stained with Commassie Blue. F, experiment was performed as described in (E) to test the effect of Pim-2 on the phosphorylation of eIF4B Ser422. G and H, experiments were carried out as described in (E) to determine whether Pim-1 (G) or Pim-2 (H) directly phosphorylates eIF4B Ser406.

Figure 3. Expression and phosphorylation of eIF4B exhibit an Abl kinase-dependent manner. A, eIF4B expression and phosphorylation were examined by Western blotting in Abl-transformed cells (K562, W44, NS2), indicated human cell lines, human peripheral blood mononuclear cells (Normal) and mouse bone marrow cells (BMC). B, v-Abl-transformed cells were treated in a time course with imatinib (10 μM). Cell lysates were analyzed by immunoblotting to examine the eIF4B expression and phosphorylation with indicated antibodies. C, eIF4B expression and phosphorylation levels in panel B were quantitated by densitometry and normalized to actin expression levels. The levels of eIF4B expression and phosphorylation are 100% at 0 hour. Plotted are results from three independent experiments. Error bars represent SEM, n=3. D, Bcr-Abl transformed cells (32D-Bcr-Abl and 32D-Bcr-Abl -T315I) were treated in a time course with imatinib (20 μM). Cell lysates were analyzed by immunoblotting with indicated antibodies.
Figure 4. Pim-1 and Pim-2 kinases regulate eIF4B phosphorylation in Abl transformants. A, v-Abl-transformed cells were treated in a time course with SMI-4a, an inhibitor of Pim-1 and Pim-2 kinases. Cell lysates were analyzed by immunoblotting to examine the eIF4B expression and phosphorylation levels as indicated. B, K562 cells were treated with SMI-4a and analyzed by immunoblotting as described in (A). C, K562 cells expressing luciferase-specific shRNA or Pim-1-specific shRNAs were analyzed by immunoblotting with indicated antibodies. D and E, v-Abl transformed cells (D) or K562 cells (E) expressing luciferase-specific shRNA or S6K-specific shRNAs were analyzed by immunoblotting with indicated antibodies. F, v-Abl transformants ectopically expressing either empty vector (EV), Pim-2-WT, or Pim-2-KD were treated with imatinib and analyzed by immunoblotting as indicated. G, experiment using K562 cells expressing EV, Pim-2-WT, or Pim-2-KD was performed as described in (F).

Figure 5. eIF4B plays a critical role in regulating survival of Abl transformants. A and B, eIF4B expression in v-Abl-transformed cells (A) and K562 cells (B) expressing luciferase shRNA (sh-luciferase) or eIF4B-specific shRNAs (sh-eIF4B) targeting different regions of eIF4B sequences were analyzed by immunoblotting. C and D, survival of v-Abl-transformed cells (C) and K562 cells (D) expressing luciferase shRNA or eIF4B-specific shRNAs upon treatment of imatinib was analyzed by flow cytometry after propidium iodide staining. Plotted are the results from three independent experiments. Error bars represent SEM. E and F, shown is cell survival.
analysis of v-Abl-transformed cells (E) and K562 cells (F) ectopically expressing either empty vector (pNL), eIF4B-WT or its phosphomimetic mutants treated with SMI-4a. G, shown is an immunoblot of K562 cells expressing sh-luciferase or sh-eIF4B probed as indicated.

**Figure 6. eIF4B deficiency attenuates tumor formation caused by K562 cells.** A, nude mice were subcutaneously injected with K562 cells stably expressing sh-luciferase or sh-eIF4B. The tumor volumes were measured at indicated time points. Plotted are results from three independent experiments. Error bars, SEM; n=9. B, tumors were excised from mice. Shown are representative images from five independent experiments with similar results. C, shown is relative volume of tumors excised from nude mice injected with K562 cells expressing sh-luciferase or sh-eIF4B. The average volume of tumors caused by K562 cells expressing sh-luciferase is 100%. Error bars, SEM; n=9, *, P < 0.05. Differences between variables were tested for significance using the Student's t test. D, over a 14-day period after inoculation, tumors stably expressing sh-luciferase or sh-eIF4B were measured by bioluminescent imaging. Shown are representative images from at least three independent experiments with similar results. E, eIF4B expression in representative tumors expressing sh-luciferase or sh-eIF4B was examined by immunoblotting.

**Figure 7. eIF4B is required for efficient bone marrow transformation by Abl oncogenes.** A, eIF4B expression in representative tissues (spleen) from
eIF4B-knockdown transgenic mice (TG) and wild-type littermates (WT) was examined by immunoblotting. B, bone marrow cells from wild-type and eIF4B-knockdown transgenic mice were infected with A-MuLV viruses. Transformation efficiency was scored as described in Materials and Methods. Plotted are results from three independent experiments. Error bars, SEM; n = 3. **, P < 0.01. C and D, experiments were performed as described in (B). Bone marrow cells from wild-type and eIF4B-knockdown transgenic mice were infected with the bicistronic retroviruses expressing v-Abl (C) or Bcr-Abl (D) and either GFP, eIF4B-WT or eIF4B-S422A as indicated. Plotted are results from three independent experiments. Error bars, SEM; n = 3. *, P<0.05, **, P<0.01. E and F, bone marrow cells from wild-type and eIF4B-knockdown transgenic mice were infected with the bicistronic retroviruses expressing v-Abl (E) or lentiviruses expressing Bcr-Abl (F) and either GFP, Pim-1-WT or Pim-1-KD as described in C and D. Plotted are results from three independent experiments. Error bars, SEM; n = 3. *, P<0.05, **, P<0.01.
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Figure 2

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Bcr-Abl
v-Abl
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Change from baseline (%)

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E

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<td>Imatinib (hr)</td>
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G

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<td>Myc-Pim-2</td>
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<td>actin</td>
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Figure 5

A

v-Abl transformed cell

sh-luciferase

sh-eIF4B-1

sh-eIF4B-2

sh-eIF4B-3

sh-eIF4B-4

eIF4B

actin

B

Bcr-Abl transformed cell

sh-luciferase

sh-eIF4B-1

sh-eIF4B-2

eIF4B

actin

C

Percent viability

0hr 5hr 10hr 15hr

Imatinib treatment (v-Abl)

D

Percent viability

0hr 8hr 16hr 24hr 32hr

Imatinib treatment (Bcr-Abl)

E

Percent viability

0hr 5hr 10hr 15hr 20hr

SMI-4a treatment (v-Abl)

F

Percent viability

0hr 12h 24h 36h 48h

SMI-4a treatment (Bcr-Abl)

G

eIF4B

c-Myc

Pim-1

Bcl-2

Bcl-XL

actin

sh-luciferase

sh-eIF4B

sh-luciferase

sh-eIF4B

sh-luciferase

sh-eIF4B
Figure 6

A

B

C

D

E

sh-luciferase
sh-eIF4B

Experiments
1 2 3 4 5

Tumor volume (mm³)

5d 10d 15d 20d

Change from baseline (%)

sh-luciferase
sh-eIF4B

Actin

Experiment 1

Experiment 2
Figure 7

A

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<tr>
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eIF4B

actin

B

WT  TG

Wells of survivors/96 wells

C

** **

Wells of survivors/96 wells

D

** *

Wells of survivors/96 wells

E

* **

Wells of survivors/96 wells

F

* **

Wells of survivors/96 wells
eIF4B phosphorylation by Pim kinases plays a critical role in cellular transformation by Abl oncogenes


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