Cytokines direct the regulation of Bim and $p27^{kip1}$ mRNA stability by Heat shock cognate protein 70

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Abstract

Previous gene-targeting studies indicated that Bim, a BH3-only death activator, and p27\(^{\text{KIP1}}\), a cyclin-dependent kinase inhibitor, regulate total cell number in the body. Cytokines contribute to this process primarily by negatively regulating the steady-state levels of Bim and p27 mRNAs. Here we present a novel mechanism for cytokine-mediated post-transcriptional regulation of Bim and p27 mRNA levels via the activity of Heat shock cognate protein 70 (Hsc70), which enhances the stability of specific mRNAs by binding to AU-rich elements (AREs) in their 3'-untranslated regions. The RNA-binding potential of Hsc70 is regulated by co-chaperones, including Bag-4 (also SODD), CHIP, Hip and Hsp40. Cytokines that down-regulate Bim and p27 operate via Ras-activated signaling pathways, which in turn control the expression or function of these co-chaperones. Thus, exposure of cells to cytokines ultimately leads to the destabilization of Bim and p27 mRNAs and the promotion of cell division and survival. This unanticipated role for a chaperone/co-chaperone complex in the control of mRNA stability appears to be critical for hematopoiesis and leukemogenesis.
Introduction

Cytokines promote cell division and the survival of hematopoietic progenitors by negatively regulating the activity of two key factors: p27KIP1 (Toyoshima and Hunter 1994), a cyclin dependent kinase inhibitor, and Bim (O'Connor et al, 1998; Hsu. et al, 1998), a BH3-only cell death activator in the Bcl-2 superfamily (reviewed in Inaba, 2004). Recent reports indicate that neurotrophic factors also promote the survival of neuronal cells by negatively regulating Bim (Putcha et al, 2001; Whitfield et al, 2001; Biswas and Greene 2002). Disruption of the murine p27 gene caused hyperplasia of all organs, including hematopoietic progenitors in the spleen and T cells in the thymus (Fero et al, 1996). The importance of Bim in hematopoiesis was demonstrated in Bim-deficient mice, which manifested both hyperplasia of hematopoietic progenitors in the bone marrow and increased white blood cell count in peripheral blood (Bouillet et al, 1999). In addition, T-cell development was perturbed, and most older mice accumulated plasma cells and succumbed to autoimmune kidney disease. Thus, the regulation of p27 and Bim by cytokines is critical for homeostasis of cell number within the body, especially in the hematopoietic and immune systems.

Upstream signaling pathways through which cytokines down-regulate Bim differ between cell lineages. In hematopoietic progenitors, a role of Ras and/or phosphatidylinositol-3 kinase (PI3-K) pathways has been demonstrated (reviewed in Miyajima and Kinoshita, 1999), however, discrepancies between experimental systems have been observed in the subsequent activation of Raf/mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR) or Akt (reviewed in Inaba, 2004). In neuronal cells, jun-N-terminal kinase (JNK) and/or e-Jun are implicated in neurotrophic

The molecular mechanisms by which cytokines negatively regulate Bim function also differ between cell types. At least three different mechanisms have been reported. First, in the presence of interleukin (IL)-3, the steady-state level of Bim mRNA decreases in the IL-3-dependent murine hematopoietic progenitor cell lines Baf-3 and FL5.12 (Shinjyo et al, 2001; Dijkers et al, 2000). Nerve growth factor (NGF) functions similarly to negatively regulate Bim in NGF-dependent neuronal cells, including primary cultures of rat sympathetic neurons or neuronally-differentiated PC-12 cells (Biswas and Greene, 2002; Putcha et al, 2001; Whitfield et al, 2001). Second, Bim protein levels are regulated by proteasome-dependent degradation in serum-deprived fibroblasts and M-CSF-dependent osteoclasts. The phosphorylation of Bim, which is induced by the presence of serum or cytokines, enhances the efficiency of its ubiquitination (Ley et al, 2003; Akiyama et al, 2003; Ley et al, 2004; Luciano et al, 2003). Third, in the IL-3-dependent mouse cell line FDC-P1, the presence of IL-3 has no effect on steady-state levels of Bim mRNA or protein, but instead affects the subcellular localization of Bim (Puthalakath et al, 1999). In this cell line, Bim binds to dynein motor complexes on microtubules in an IL-3-dependent fashion. Withdrawal of IL-3 results in dissociation of Bim from microtubules followed by Bim binding to and inhibiting the function of anti-apoptotic Bcl-2 family members.

The diversity of these regulatory mechanisms suggests that regulation of Bim function is multi-faceted, allowing flexibility in response to different signals or in different cell types (Yamaguchi et al, 2003). Among these mechanisms, our observations suggest that regulation of steady-state Bim mRNA levels is a primary contributor to
homeostasis of hematopoiesis. We observed that in the Sca-1<sup>+</sup>-c-Kit<sup>+</sup>Lin<sup>−</sup> cell fraction of primary murine bone marrow cultures, which contain hematopoietic stem cells and early undifferentiated hematopoietic progenitors exclusively, Bim mRNA levels increase in cells deprived of stem cell factor (SCF) and thrombopoietin (Kuribara et al., 2004). Recent studies indicate that cytokines also contribute to the regulation of steady-state levels of p27 mRNA (Dijkers et al., 2000; Parada et al., 2001; Stahl et al., 2002), although p27 protein levels has been considered to be regulated predominantly by cyclin E-<em>cdk2</em>-mediated phosphorylation, which targets p27 for proteasome-dependent degradation (Pagano et al., 1995; Hengst and Reed, 1996; Dijkers et al., 2000). These results suggest that by promoting increases in the levels of Bim and p27 mRNAs, cytokine deprivation can decrease cell number in the body.

Negative regulation of Bim by NGF in neuronally-differentiated PC12 cells was demonstrated to depend on sequence-specific binding sites for the FOXO3a (also FKHR-L1) transcription factor located within the Bim gene enhancer (Gilley et al., 2003). The efficiency of p27 gene transcription in Baf-3 cells has also been reported to be regulated by FOXO3a (Dijkers et al., 2000). Since FOXO3a is inactivated by phosphorylation events mediated by both Ras and PI3-K signaling pathways (Guo et al., 1999; Rena et al., 1999; Tang et al., 1999), we hypothesized that FOXO3a was a primary regulator of cytokine-dependent Bim transcription in hematopoietic cells.

To test this hypothesis, we identified <em>cis</em>-acting regulatory elements of the Bim gene in Baf-3 cells, but found that none were IL-3 dependent (Matsui et al., 2005). Furthermore, FOXO3a binding sites within the Bim gene that function as NGF-dependent enhancers in PC12 cells do not affect Bim transcriptional efficiency in Baf-3 cells. Moreover, nuclear run-off assays demonstrated nearly the same degree of depletion of Bim transcriptional activity in both cell types.
*novo* transcription in Baf-3 cells in the presence or absence of IL-3. These data led to the alternative hypothesis that IL-3-dependent decreases in Bim mRNA levels could occur post-transcriptionally.

Here, we report that the stabilities of Bim and p27 mRNAs are regulated by cytokines in Baf-3 cells. Heat shock cognate protein 70 (Hsc70) specifically binds to and stabilizes Bim and p27 mRNAs. Co-chaperones, which associate with Hsc70 in a cytokine-dependent fashion, regulate the RNA-binding potential of Hsc70. We suggest that this mechanism plays a critical role in the regulation of hematopoietic cell number in the body.
Results

Destabilization of Bim and p27 mRNA by IL-3 in Baf-3 cells

Within the 5.1 kb human Bim messenger RNA, the 3'UTR is long (approximately 4.2 Kb) relative to the coding region (0.6 Kb) (Figure 1A). The 3'UTR contains a number of AUUUA pentamer repeats, which are key cis-acting sequences within the AU-rich sequence elements (AREs) that play a critical role in the regulation of mRNA stability. Between the eight pentamers in human mRNA and the six pentamers in mouse, four are conserved. In a comparison between the 3'UTRs of human and mouse Bim messages, the overall nucleotide sequence homology is low, with the exception of a centrally located 1 kb region of high sequence conservation (76%) that includes three conserved AUUUA pentamers.

To test the effect of IL-3 on the stability of Bim mRNA, we transfected IL-3-dependent Baf-3 cells with plasmids expressing a hybrid mRNA with the Bim 3'UTR terminating the β-globin coding sequence. As previously demonstrated (Xu et al, 1998), full-length β-globin mRNA expressed in Baf-3 cells was stable in the presence or absence of IL-3 (Figure 1B). In contrast, replacement of the native β-globin 3'UTR with the entire Bim 3'UTR reduced the stability of the fusion mRNA in cells cultured in the presence of IL-3, but not in its absence.

We measured the half-lives of Bim mRNAs synthesized and radiolabeled in vitro in cytosol (S100 extract) preparations. The half-life of a Bim 3'UTR mRNA with a 5' cap and a poly(A) tail of 100 residues [cap⁺/poly(A)⁺] was less than 10 minutes in cytosol preparations from IL-3-treated Baf-3 cells (Figure 1C and D). In contrast, the half-life of cap⁺/poly(A)⁺ Bim mRNA increased to approximately 30 minutes in cytosol from Baf-3
cells cultured without IL-3. In extracts prepared from control FDC-P1 cells (another IL-3-dependent cell line in which Bim expression is not affected by IL-3), the half life of cap+/poly(A)+ Bim mRNA was approximately 30 minutes either in the presence or absence of IL-3. We tested the effects of a cap and poly(A) tail on Bim mRNA stability in Baf-3 cell lysates in the absence of IL-3. The half-life of cap+/poly(A)+ or cap+/poly(A)+ Bim mRNA was less than 10 minutes (Figure 1E). The half-life of cap+/poly(A)+ Bim mRNA increased to more than 10 minutes, however it was still less stable than cap+/poly(A)+ Bim mRNA. In cytosol extracts from IL-3-treated Baf-3 cells, Bim mRNAs were unstable regardless of a cap or poly(A) tail (Figure 1F). These data suggest that in Baf-3 cells, IL-3 destabilizes Bim mRNA in a cap- and poly(A)-dependent fashion.

We also tested the stabilities of cap+/poly(A)+ p27 and c-fos mRNAs in Baf-3 cell extract. The half-life of p27 mRNA was lengthened by IL-3 starvation similar to Bim mRNA, while that of c-fos mRNA was unchanged (Figure 1G).

**Hsc70 binds to and stabilizes Bim mRNA**

To identify RNA-binding proteins that may function as IL-3-dependent stabilizing or destabilizing factors, we looked at cytosolic proteins that bind to the 3'UTR of Bim mRNA using RNA-affinity chromatography (Skalweit et al, 2003). Extracts of Baf-3 or FL5.12 cytosol were incubated on RNA-affinity columns prepared with cap+/poly(A)+ RNAs encoding the 3'UTRs of Bim or p27. Bound proteins from both extracts resolved by SDS-PAGE and stained with silver showed similar banding patterns, and both included one band at approximately 75 kDa which was enhanced in extracts prepared from IL-3-starved cells (Figure 2A, lanes 1 to 4, 7 and 8). In contrast, the patterns of silver staining resulting from Baf-3 extract proteins bound to a c-fos 3'UTR mRNA
column (lanes 5 and 6), or FDC-P1 extract proteins bound to Bim mRNA (lanes 9 and 10) were substantially different from the previous experiment, with less signal from the 75 kDa protein. Protein from the 75 kDa band was isolated and identified as Hsc70 by mass spectrometry analysis (Figure 2B). Immunoblot analysis of bound proteins from Baf-3 and FDC-P1 cell extracts to Bim mRNA using Hsc70-specific antibodies confirmed this result (Figure 2C). To test whether Hsp70, a protein closely related to Hsc70, could also be involved in the control of mRNA stability in Baf-3 cells, we tested for the presence of Hsp70 by immunoblot analysis with an Hsp70-specific antibody. Hsp70 was undetectable in Baf-3 cells cultured at 37°C in the presence or absence of IL-3 (data not shown).

Hsc70 has been reported to bind directly to AREs (Henics et al, 1999; Zimmer et al, 2001; Wilson et al, 2001), although the biological significance of these interactions has not been determined. To test whether Hsc70 stabilizes Bim mRNA, Hsc70 was immunodepleted from cytosolic extracts prepared from Baf-3 cells cultured without IL-3 using Hsc70-specific antibodies. The half-life of cap⁺/poly(A)⁺ Bim mRNA in Hsc70-depleted cytosol, which contains virtually no Hsc70 (Figure 3A, lane 2, lower middle panel), was less than 5 minutes (Figure 1B, indicated as 't-Id').

The RNA-binding and -stabilizing function of Hsc70 was further analyzed using cytosol extracts prepared from IL-3-treated Baf-3 cells supplemented with purified Hsc70 protein. We observed more Hsc70 protein bound to the Bim mRNA affinity column, but neither the cap nor the poly(A) tail affected Hsc70's RNA-binding ability (Figure 2D). The stability of Bim mRNA was evaluated after 30 minutes incubation (Figure 2E). Hsc70 stabilized cap⁺/poly(A)⁺ Bim mRNA, but did not stabilize those lacking either the cap or poly(A) tail.
Co-chaperones control mRNA affinity of Hsc70 in an IL-3-dependent manner

Because IL-3 starvation did not increase the concentration of Hsc70 in Baf-3 cells (Figure 3A, lanes 1 and 2, top panel), we hypothesized that IL-3 negatively regulates the RNA-binding potential of Hsc70. To test this hypothesis, we compared the ability of Hsc70 protein complexes in Baf-3 cells cultured with or without IL-3 to bind Bim mRNA. Although approximately equal amounts of Hsc70 protein were immunoprecipitated from IL-3-treated and -untreated cells (Figure 3A, lanes 1 and 2, upper middle panel), the Hsc70 complexes formed in the presence of IL-3 bound cap’/poly(A)’ Bim mRNA less efficiently than those formed in the absence of IL-3 (Figure 3B). In contrast, no differences were observed in the binding abilities of the two extracts for c-fos mRNA.

Hsc70 can associate with ATP as well as ADP, although only Hsc70-ATP can function as an ATPase (reviewed in Mayer et al, 2001). Using thin-layer chromatography, we observed that Hsc70 protein complexes isolated from IL-3-treated Baf-3 cells have high ATPase activity, whereas the activity of the complexes isolated from untreated cells does not exceed background levels (Figure 3C). This result suggests that the binding potential of Hsc70 to Bim mRNA may be related to its association with either ATP or ADP.

The binding of co-chaperones to Hsc70 have been shown to regulate its ATPase activity (reviewed in Mayer et al, 2001). Hsc70 association with members of the Bag protein family or CHIP induces the formation of Hsc70-ATP (Takayama et al, 1999; Ballinger et al, 1999), whereas association with Hsp40 induces the formation of Hsc70-ADP (Minami et al, 1996) and is stabilized by Hip (Hohfeld et al, 1995). Of the three members of the Bag-family detected in immunoblots of Baf-3 cell extracts (Bag-1,
-2, and -4; Figure 3A, top panel), only the level of Bag-4 increased in IL-3-treated cells. Similarly, the abundance of Hsc70-Bag-4 complexes also increased with IL-3 (upper middle panel). Although the expression levels of CHIP, Hip and Hsp40 were not altered by the presence of IL-3, the relative abundance of Hsc-70-CHIP complexes increased, while those with Hip and Hsp40 decreased in extracts of IL-3-treated Baf-3 cells. To test the role of Ras pathways in IL-3-mediated regulation of Hsc-70-co-chaperone complex formation, we undertook a similar analysis in extracts from Baf-3 cells that express a constitutively-active Ras mutant (RasG12V). In these cells, there was a reversal in the relative abundances of Hsc-70-co-chaperone complexes seen in IL-3-starved Baf-3 cells (bottom panel), suggesting that Ras pathways mediate the IL-3-dependence of co-chaperone associations with Hsc70.

**Co-chaperones regulate RNA-binding potential of Hsc70**

A cytosol-free system was used to test whether CHIP, Hip or Hsp40 affects the RNA-binding potential of Hsc70. In immunoblot assays of proteins bound to Bim mRNA-coated beads, the affinity of purified Hsc70 protein with Bim mRNA was low (Figure 4A, lane 2), but was enhanced with the addition of Hsp40 or Hip (lanes 3 and 4). In contrast, CHIP did not enhance the affinity of Hsc70 with RNA (lane 5), but reversed the stabilization of Hsc70-Bim mRNA complexes by Hsp40 or Hip (lanes 6 and 7). RNA gel retardation assays using a probe containing two conserved AUUUA pentamers in the 3'UTR of Bim mRNA were used to test the binding of the Hsc70/Hsp40/Hip complex to the ARE. In the presence of increasing amounts of an equimolar mixture of recombinant Hsc70, Hsp40 and Hip, increasing amounts of radiolabeled RNA probe migrated in a slower mobility complex (Figure 4B, lanes 2 to 5), which was further shifted by addition
of an Hsc70 antibody (lane 6).

To test the effects of Bag-4 on the mRNA binding and stabilizing potential of Hsc70, we used recombinant Bag-4 and its mutant Bag-4ΔC protein, which lacks the Bag domain necessary for binding to Hsc70 (Takayama et al., 1999). When Bag-4 or Bag-4ΔC was incubated with cytosol extracts from Baf-3 cells cultured without IL-3, association of Hsc70 with Bim mRNA was inhibited by Bag-4 but not by Bag-4ΔC (Figure 4C). Consequently, the degradation of Bim mRNA was accelerated by Bag-4, but not by Bag-4ΔC (Figure 4D).

Hsc70 binds to PABP and AUF1

Since both a cap and poly(A) tail are required for stabilization of mRNA by Hsc70 (Figure 2E), it is possible that Hsc70, like other ARE-binding proteins such as AUF1 (Laroia et al., 1999; Grosset et al., 2000), stabilizes mRNA via interactions with the eIF4G/eIF4E ternary complex (translation initiation factors which bind to the cap) and poly(A) binding protein (PABP). To investigate this possibility, we used co-immunoprecipitation to detect interactions between Hsc70 and eIF4G, PABP or AUF1. AUF1 was readily detected in immunoblots of Hsc70 co-immunoprecipitates from Baf-3 cells, confirming a previously published result that Hsc70 physically interacts with AUF1 (Figure 5A) (Laroia et al., 1999). eIF4G and PABP also co-immunoprecipitated with Hsc70, and the recoveries of AUF1, eIF4G and PABP were similar in extracts prepared from cells cultured with or without IL-3. Regulation of the interaction between AUF1 and Bim 3’UTR mRNA by IL-3 was investigated in a pull-down experiment with Bim mRNA-coated beads and cytosol extracts prepared from Baf-3 cells cultured in the presence or absence of IL-3. Immunoblots showed that among the proteins that bound to
Bim mRNA in vitro, virtually equal amounts of AUF1 were detected in cytosols from Baf-3 cells cultured in the presence or absence of IL-3 (Figure 5B), indicating that, unlike Hsc70, the affinity of AUF1 with Bim mRNA is not affected by IL-3.

**Hsc70 and Bag-4 affect apoptosis of Baf-3 cells induced by IL-3 starvation**

To obtain insights into the biological significance of this mRNA-stabilizing mechanism, we initially tried to knock Hsc70 gene expression down using a small interference (si) RNA-expression plasmid vector, piGENE-mU6 (Miyagishi et al, 2004). By transfection with the piGENE-mU6-2sm or -3sm vector, each of which contains a fragment for a target sequence of Hsc70 mRNA (see Materials and Methods), a greater-than-60% reduction of Hsc70 protein was achieved when whole cells were analyzed (Figure 6A). Cells undergoing apoptosis by IL-3 starvation which are positively stained by FITC-VAD-FMK were decreased by transfection with piGENE mU6-2sm or -3sm (Figure 6B), suggesting that Hsc70 facilitates apoptosis induced by IL-3 starvation. We next generated Bag-4 over-expression lines from Baf-3 cells to investigate the effect of Bag-4 on Bim and p27 protein levels (Figure 6C). In these lines, increases in Bim and p27 protein induced by IL-3 starvation were suppressed after 8 hours, suggesting that Bag-4 contributes to the down-regulation of Bim and p27. Consistent with the decrease in Bim and p27 mRNA levels, Bag-4-overexpressing cells continued to divide and survived longer than Baf-3 parent cells in IL-3-free medium (Figure 6D). To test the role of Bag-4 in normal hematopoietic progenitors, we measured the levels of Bag-4 mRNA by real-time RT-PCR using template RNA isolated from murine early undifferentiated hematopoietic progenitors (Sca-1\(^+\)-Kit\(^+\)Lin\(^-\)), which proliferate in a SCF- and thrombopoietin-dependent manner. Bag-4 mRNA levels declined when these cells were
deprived of cytokines (Figure 6E), suggesting that Hsc70-mediated regulation of mRNA stability occurs in native hematopoietic progenitors.


**Discussion**

We and others have previously demonstrated that by regulating the steady-state levels of Bim and p27 mRNAs, cytokines contribute to homeostasis of cell number in the body. Here we show that cytokines lead to a decrease in the half-lives of Bim and p27 mRNAs by changing the cytosolic environment to one that does not favor complex formation between Hsc70 (an mRNA stabilization factor) and the 3'UTR sequences of these genes. In Baf-3 cells cultured in the presence of IL-3, Hsc70 associated more readily with Bag-4 and CHIP, which induce the ATP-bound form of Hsc70 that binds less stably to mRNA. In the absence of IL-3, Hsc70 associated more readily with Hip and Hsp40, which promote ADP-bound Hsc70 that binds with high stability to mRNA.

We demonstrated previously that activation of Ras by IL-3 is a critical step for decreasing Bim mRNA in Baf-3 cells (Kuribara *et al*, 1999; Shinjyo *et al*, 2001). Consistent with these data, Ras$^{G12V}$ substituted for IL-3 as a regulator of co-chaperones (Figure 3A), which ultimately controls Bim mRNA levels. IL-3 promotes increases in the intracellular concentration of Bag-4, which is low relative to the concentration of Hsc70. In Baf-3 cells, both in the presence and absence of IL-3, the majority of Bag-4 protein associated with Hsc70. Thus, reduction of Bag-4 by IL-3 starvation should directly reduce the amount of Hsc70-bound Bag-4. In contrast, CHIP, Hip and Hsp40 were more abundant in Baf-3 cells, and only a fraction of the total of these proteins associated with Hsc70. Hence, we propose that the primary mechanism by which IL-3 regulates Bim and p27 is via Ras-mediated changes in the availability of co-chaperones that associate with Hsc70, and corresponding changes in the association of Hsc70 with ATP or ADP. Our preliminary results (not shown here) suggest that IL-3 signaling does not change the
phosphorylation state of either Hsc70 or these three co-chaperones.

Hsc70 and Hsp70 have been reported to bind directly to AREs (Henics et al, 1999; Zimmer et al, 2001; Wilson et al, 2001). Our cytosol-free system demonstrated that complexes of Hsc70 with Hsp40 and/or Hip bind to ARE sequences within a Bim mRNA probe (Figure 4A and B). Consistent with these data, Hsc70 complexes formed in Baf-3 cells in the absence of IL-3 associated strongly with Bim and p27 3'UTR, however, they did not bind well to the c-fos 3'UTR (Figure 2A), which also contains typical AREs. Future experiments comparing these 3'UTR sequences may provide clues for elucidating the binding specificity of Hsc70.

Classification of AREs, either by experimental criteria or comparative analysis of AUUUA pentamer repeats, has been attempted by several groups (Xu et al, 1997; Bakheet et al, 2001). The AREs of Bim, p27 and c-fos, which are widely distributed within their respective 3'UTR regions, are all classified as ‘WAUUUAUW and a U-rich region’ motif (W stands for A or U) (Wilusz et al, 2001). It should be noted that although AREs are binding sites for Hsc70, non-ARE flanking sequences might also function as cis-acting regulatory elements which ultimately could specify the binding potential of Hsc70 to AREs. Little is known about contribution of non-ARE sequences to the regulation of mRNA decay, but a recent report of CA repeats mediating the constitutive degradation of Bcl-2 mRNA (Lee et al, 2004) suggests the possible involvement of non-ARE sequences in the control of mRNA stability.

Hsc70 interacts with a protein complex composed of eIF4G, PABP and AUFl that binds to the Bim 3'UTR (Figure 5A and B). This complex binds to the cap through eIF4E/eIF4G, to a poly(A) tail through PABP, and to AREs through AUFl, which contributes to the rapid turnover of ARE-containing mRNAs through its degradation by
ubiquitin-protease pathways (Laroia et al, 1999). In view of these data, a simple model for IL-3-mediated regulation of Bim is that AUF1 is the primary occupant of the AREs of Bim, p27 and c-fos mRNAs in the presence of IL-3. In the absence of IL-3, RNA-binding ADP-bound Hsc70 replaces AUF1 on the AREs of Bim and p27 (but not c-fos) mRNAs, forms a stable complex with eIF4G/eIF4E and PABP, and ultimately enhances the stability of Bim and p27 mRNAs. Alternatively, by joining the existing AUF1/eIF4G/eIF4E/PABP complex, Hsc70 could stabilize it, rather than replacing AUF1. In this scenario, interactions between Hsc70 and AUF1 (Figure 5A) may contribute to the stabilization of the RNA-protein complex with AUF1. However because c-fos mRNA, which does not associate with Hsc70 (Figure 3B), was not stable in Baf-3 cells, the key factor determining the stability of these ARE-containing mRNAs should involve Hsc70 binding to mRNA.

It has been clearly demonstrated that Bim plays a critical role in the regulation of hematopoietic cell number in the body (Bouillet et al, 1999). Therefore, altered regulation of Bim could be involved in leukemogenesis. Indeed, we recently reported that negative regulation of Bim by the Bcr-Abl chimeric kinase contributes to an increase in white blood cell and hematopoietic progenitor counts in the chronic phase of chronic myelogenous leukemia (CML) (Kuribara et al, 2004). In addition, we have preliminary data suggesting that Bim is one of the major molecular targets of constitutively active mutants of the Flt-3 receptor tyrosine kinase, which are detected in approximately 30% of patients with acute myeloid leukemia. Finally, anti-leukemic drugs such as imatinib mesylate, which target these leukemogenic molecules, at least in part inhibit proliferation of leukemic blasts by increasing Bim protein (Kuribara et al, 2004). Because negative regulation of Bim by Bcr-Abl and constitutively active Flt-3 mutants may operative via
the Hsc70 protein complex, our laboratory endeavors to further understand the formation and regulation of this complex as a means to identify new molecular targets for anti-leukemic drugs.
Materials and methods

Cells and cell culture
IL-3-dependent cells were cultured with and deprived of IL-3 as described previously (Shinjyo et al, 2001). Baf-3 cells expressing Ras^{G12V} (Kinoshita et al, 1995) were a kind gift from Dr. Atsushi Miyajima. Bag-4 over-expression lines were established according to standard procedures as described previously (Shinjyo et al, 2001). Murine undifferentiated early hematopoietic progenitors (Sca-1^+c-Kit^Lin^-) were isolated and cultured as previously described in serum-free medium containing stem cell factor and thrombopoietin (Kuribara et al, 2004).

Measurements of mRNA stability
mRNA stability in Baf-3 cells was measured as previously described (Xu et al, 1998). Briefly, cells maintaining a plasmid (pTet-off, Clontech, Palo Alto, CA) that encodes a tetracycline (Tet)-regulated transactivator protein were transfected by electroporation with an expression plasmid encoding the Tet-responsive element (TRE) upstream of either the full length human β-globin gene (pTRE-βG) or a Bim/β-globin gene hybrid in which the 3'UTR of the β-globin gene is replaced by the entire 3'UTR of Bim (pTRE-βG/Bim). Following a 12-hr recovery period that included tetracycline (2 μg/ml), antibiotic was removed from the culture medium for 3 hours, and the cells were then cultured in the presence of Tet with or without IL-3 for various times. Levels of mRNAs were measured in quantitative real-time reverse transcribed polymerase chain reactions (RT-PCR) with the following pair of oligonucleotide primers: 5'-GGGATCTGTCCACTCCTGATGCTG -3' in exon 2, and 5'-
ATGGGCCAGCAGCACAGACCAGCAC -3’ in exon 3.

The half-lives of \textit{in vitro}-synthesized mRNAs incubated with preparations of cultured cell cytosolic (S100) extracts were analyzed as described previously (Ford and Wiluss, 1999). Target mRNAs approximately 1 kb in length included either the highly conserved region between human and mouse in the Bim 3'UTR, or the entire 3'UTR sequence of p27 or c-fos mRNAs. Radiolabeled mRNAs synthesized \textit{in vitro} with or without the cap analog ($^{7}$MeGpppG) and/or a poly(A) tail of 100 bp were incubated with S100 extracts at 37°C for increasing amounts of time. Following each time point, the signals from full-length mRNAs resolved on 5% acrylamide gels containing 7 M urea were quantified using a Bas3000 phosphoimager (Fuji film, Tokyo, Japan).

\textit{Enrichment and identification of RNA-binding proteins}

RNA-affinity chromatography was performed as described (Skalweit \textit{et al}, 2003). Briefly, biotinylated mRNAs encoding the full-length 3'UTRs of Bim, p27 or c-fos were synthesized \textit{in vitro} without the cap analog or poly(A) tail. The mRNAs loaded onto streptavidin-coated agarose beads were exposed to cytosol S100 extracts for 45 minutes at 4°C and then 15 minutes at room temperature. After gentle washing of the protein-bound beads in washing buffer (150 mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris pH 7.5, 0.5 mM DTT), bound proteins were released from the beads by boiling in SDS sample buffer, resolved by SDS-PAGE and visualized by silver staining or immunoblot analysis. Protein bands of interest were recovered and analyzed using a matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF/TOF) mass spectrometer (Brucker, Bremen, Germany).
**Functional analysis of Hsc70 protein complexes**

Hsc70 protein complexes were immunoprecipitated using protein G beads and a monoclonal antibody that specifically recognizes Hsc70, but not Hsp70 (1B5, Stressgen, Victoria, Canada). The RNA-binding ability of the Hsc70 protein complexes were analyzed by incubating *in vitro* synthesized radiolabeled mRNA with the resulting immunoprecipitates in RNA binding buffer (10 mM MOPS pH 7.2, 50 mM KCl, 3 mM MgCl₂, 0.5 mM ATP, 2 mM DTT) containing RNase inhibitor (RNase OUT, Invitrogen) at 37°C for 30 minutes. After washing the beads, the amount of RNA bound to the beads was determined using a scintillation counter. ATPase activity was assayed as previously described (Takayama *et al*, 1999).

**In vitro RNA-binding assay**

The ability of Bag-4 to destabilize Hsc70-mRNA complex was analyzed by incubating biotinylated Bim mRNA and glutathione beads bound with either glutathione-S-transferase (GST), GST-Bag-4 (the entire protein), or GST-Bag-4AC (aa 1 to 378) in cytosol from Baf-3 cells cultured without IL-3. Following a 15-minute incubation, the beads were pelleted by centrifugation and mRNA was recovered from the supernatant with streptavidin-coated agarose beads. Hsc70 bound to either GST- or streptavidin-coated beads was detected by immunoblot analysis. For cytosol-free RNA-binding assays, biotinylated Bim mRNA synthesized *in vitro* (10 μg) and bound to streptavidin-coated agarose beads was incubated with Hsc70 (500 ng) with or without equimolar concentrations of Hsp40, Hip or CHIP protein in RNA binding buffer at 4°C for 45 minutes and then at room temperature for 15 minutes. After washing the beads, bound proteins were released by boiling in SDS sample buffer, resolved by SDS-PAGE,
and analyzed by Western immunoblots using Hsc70 antibody.

RNA gel retardation assays were performed as described previously with minor modifications (Mahtani et al. 2001). Briefly, increasing amounts of equimolar mixture of recombinant Hsc70, Hsp40 and Hip were incubated with a radiolabeled RNA probe (5'-UCUGUGUGAUGUGUCCACUGUUUCAUAUAAUGCUGUACGUAGAAA UAUUGUAUAUUUAUUUCUGCUUAUUUAUGUCUUAUUUCUGAAA-3'; a region containing two conserved AUUUA pentamers in the 3'UTR of human Bim mRNA) in a total volume of 20 µl RNA-binding buffer containing 20 mM HEPES (pH 7.6), 3 mM MgCl₂, 40 mM KCl, 2 mM DTT and 5% glycerol. To test for the presence of Hsc70, the binding reaction was pre-incubated with 1 µl of rabbit polyclonal antiserum against Hsc70 (SPA-816, Stressgen) for 30 min on ice before adding the probe. The reaction mixture containing probe was incubated on ice for 20 min, and then RNase T1 and heparin sulfate were added to final concentrations of 50 U/ml and 5 mg/ml, respectively, prior to incubation for a further 20 min on ice. The resulting binding reactions were resolved by electrophoresis on a nondenaturing 4% polyacrylamide gel run at 150 V for 3 h at 4°C.

**RNA interference experiments**

For expressing siRNA, a piGENE-mU6 vector (Miyagishi et al. 2004), which expresses mouse U6 promoter-driven hairpin type dsRNA was used. DNA fragments (5'-GAATTCGCTGGAGTTATGCgtgtgctgtcc GCATAGGACTCCAGTGAGTTC -3' for 2sm; 5'-GAGTTGAACGAGCATTAGCgtgtgctgtcGTTAATGCTTTGTCAGCTC -3' for 3sm), which contain sense target sequences (capital) with mutations (underlined), an
optimized loop sequence (small letter), and antisense target sequences (bold), were
inserted at the BspM1 site downstream the U6 promoter. Either of piGENE-mU6-2sm,
-3sm, or the empty vector (25 µg) was cotransfected to Baf-3 cells (2 x 10^7) with
pDsRed2-N1 vector (25 µg) (Clontech), an indicator of transfection efficiency, by
electroporation at 290 V and 950 µF. After culturing for 16 hours in the presence of IL-3,
IL-3 was removed and cells were continued to culture for 12 hours. Apoptotic cells were
detected by flow cytometry using CaspACE FITC-VAD-FMK Kit (Promega, Madison,
WI), according to the manufacturer's direction.

**Other experimental procedures and reagents**

Real-time quantitative RT-PCR for Bag-4 in early hematopoietic progenitors was
performed with the primer pair 5'-GCAGGTTGCGATGGCTACTACCCC-3' and
5'-CCATTTCATAAGAATTCAGGCTC-3'. The number of cycles required to produce a
detectable product (Ct) was used to calculate fold-differences in the starting mRNA level,
using 28S ribosomal RNA as an internal control (Kuribara et al, 2004). Immunoprecipitation, immunodepletion and immunoblot analyses were performed as
previously described (Shinjyo et al, 2001). Antibodies used were as follows: actin (1378
996) from Roche Biochemicals (Mannheim, Germany); AUF1 (07-260) and SODD
(Bag-4, 07-092) from Upstate Cell Signaling Solutions (Lake Placid, NY, USA); Bag-1
(FL-274), Hsc70 (B-6) and p27 (C-19) from Santa Cruz Biotechnology (Santa Cruz, CA);
Bag-2 from Oxford Biotechnology (Oxfordshire, UK); Chip (Ab-1) from Oncogene
Research Products (San Diego, CA); eIF4G1 (BL896) from Bethyl laboratories
(Montgomery, TX), Hip (SPA-766), Hsp40 (SPA-400) and Hsp70 (C92F3A-5) from
Stressgen. A rabbit polyclonal anti-PABP was raised against GST-mouse PABP (aa.
213-422) fusion protein, and an anti-Bim was described elsewhere (Shinjyo et al, 2001).

Purified Hsc70 and Hsp40 proteins were purchased from Stressgen. Hexameric
His-tagged mouse Hip protein was expressed in E. coli using the pQE-80L vector (Qiagen,
Hilden, Germany) and purified using Ni-NTA beads. Mouse CHIP protein was
synthesized as a GST fusion protein, and then cleaved and purified from the GST
polypeptide by thrombin according to the standard procedure.

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Thermodynamics and kinetics of Hsp70 association with A + U-rich

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Figure legends

Figure 1  RNA stability of Bim mRNA. (A) A schematic representation of human and mouse Bim mRNAs. Black bars represent the coding regions, open bars untranslated regions, and gray bars regions of strong homology between human and mouse Bim 3'UTR. Black squares above bars indicate AUUUA pentamers conserved between the human and mouse 3'UTRs, open squares are those present in either human or mouse. (B) Stability of the human β-globin mRNA or β-globin (coding region)/Bim (the entire 3'UTR) fusion mRNA in Baf-3 cells cultured in the presence or absence of IL-3. Relative levels of mRNAs in cells cultured for the indicated number of hours after suppression of the tet-regulated transactivator are shown. Message levels were measured by real-time RT-PCR. The mean and standard deviation (SD) of three independent experiments are shown. (C-G) RNA stability assay in vitro. Cytosol preparations from Baf-3 or FDC-P1 cells cultured in the presence or absence of IL-3 were incubated with radiolabeled mRNA as indicated on the right. 'Id' indicates Hsc70-immunodepleted cytosol. 'pA' indicates a poly(A) tail (C). Values for each band were measured and plotted as a percent of the starting point. Results of one representative experiment are shown. Similar results were obtained in three independent experiments (D-G).

Figure 2  Hsc70 binds and stabilizes Bim mRNA. (A) Enrichment of proteins bound to the 3'UTRs of Bim, p27 or c-fos mRNAs in cells indicated above by RNA-affinity chromatography. Proteins were visualized by silver staining. An arrow indicates bands analyzed by MALDI-TOF/TOF. (B) A representative result from MALDI-TOF/TOF analysis. The molecular weight of each peak and the corresponding amino acid residues
of Hsc70 are shown. (C) Immunoblot analysis of proteins binding to the Bim mRNA 3'UTR in Baf-3 and FDPC-1 cells using an Hsc70-specific antibody. Numbers below bands indicate the intensity of each band relative to that of Baf-3 cells in the presence of IL-3. (D and E) Stabilization of mRNA by Hsc70. Increasing amounts of purified bovine Hsc70 (lane 2, 0.8 µg; lane 3, 1.6 µg; lane 4, 3.2 µg) was added to cytosol prepared from 10^7 Baf-3 cells cultured with IL-3. Hsc70 protein bound to a Bim mRNA-affinity column was quantified by immunoblot analysis (D). RNA stability assays in vitro were performed by incubating radiolabeled Bim 3'UTR mRNA with cytosolic extracts for 0 minutes (lane C) or 30 minutes (lanes 1 to 4) (E). Numbers below bands indicate the intensity of each band relative to control experiments (lane 1 for panel D, lane C for panel E).

Figure 3 Analysis of Hsc70 protein complexes formed in Baf-3 cells. (A) Samples prepared from parental Baf-3 cells (top three panels) or cells expressing Ras^G12V (bottom) cultured in the presence or absence of IL-3 were analyzed by immunoblot analysis using antibodies indicated above. Immunoblots showing total protein levels in Baf-3 cells (top panels). Total cell lysates were subjected to Hsc70-antibody immunoprecipitation, and then proteins binding to protein G beads were analyzed by immunoblot analysis ('Hsc70-bound', upper middle and bottom panels). Total cell lysates were subjected to Hsc70-antibody immunoprecipitation, and proteins that did not bind to protein G beads were used in immunoblots ('Hsc70-unbound', lower middle panels). Equivalent cell numbers were analyzed (upper and lower middle panels). (B) RNA-binding potential of Hsc70 protein complexes. Immunoprecipitation was performed using either Hsc70-specific antibodies or isotype-specific control immunoglobulins and protein G
beads. After incubation with radiolabeled Bim (left) or c-fos (right) mRNAs, protein G beads were washed and the amount of RNA bound to the beads was determined using a scintillation counter. The mean ± SD of three independent experiments is shown. (C) ATPase activity of Hsc70 complex was assayed by thin layer chromatography. Ratios of ADP:ATP radioactive signals are shown below.

**Figure 4** Contribution of CHIP, Hip, Hsp40 and Bag-4 to the Hsc70-mediated mRNA stabilization (A) Streptavidin beads (SA) alone (lane 1) or those incubated with biotinylated Bim mRNA (SA-Bim mRNA; lanes 2 to 7) were mixed with purified Hsc70 protein and the co-chaperones indicated above. Hsc70 protein bound to the beads was detected by immunoblot analysis. The intensity of each band relative to that of Hsc70 protein alone bound to SA-Bim mRNA (lane 2) is given below. (B) RNA gel retardation assay. A radiolabeled RNA probe containing two conserved AUUUA pentamers in the context of the 3'UTR of human Bim mRNA (see Materials and Methods) were mixed without (lane 1) or with an equimolar mixture of recombinant Hsc70, Hsp40 and Hip (lane 2: 0.1 μg, lane 3: 0.5 μg, lane 4: 1 μg, lanes 5 and 6: 2 μg; total weight). 1 μl of Hsc70 antiserum was present in lane 6. The positions of free RNA probe, RNA/protein complexes and antibody-supershifted RNA/protein complexes are indicated on the right. (C) Cytosol from Baf-3 cells cultured without IL-3 was incubated with biotinylated Bim mRNA and glutathione beads bound with either GST (lane 1), GST-Bag-4 (lane 2), or GST-Bag-4ΔC (lane 3). The glutathione beads were pelleted and mRNA was recovered from the supernatant with streptavidin-coated agarose beads. Hsc70 protein bound to glutathione beads (upper panel) or biotinylated mRNA recovered by streptavidin beads (lower panel) were detected by immunoblot analysis. (D) Glutathione beads bound with
GST (lane 1), GST-Bag4 (lane 2) or GST-Bag4ΔC (lane 3) and radiolabeled Bim mRNA were incubated with cytosol preparations from Baf-3 cells cultured without IL-3 for 0 (lane C) or 30 (lanes 1 to 3) minutes; numbers below indicate the radioactive signal from each band relative to signal in lane C.

Figure 5 Hsc70 forms a complex with eIF4G, PABP and AUF1. (A) Cytosol extracts of Baf-3 cells cultured with or without IL-3 were used in Hsc70 antibody immunoprecipitations. The precipitants were then analyzed by immunoblot analysis using antibodies against eIF4G, PABP, or AUF1. (B) Streptavidin beads (SA) alone (lane 1) or those incubated with biotinylated Bim mRNA (SA-Bim mRNA; lanes 2 and 3) were mixed with cytosol from Baf-3 cells cultured with or without IL-3. Proteins bound to the beads were separated by SDS-PAGE, followed by immunoblot analysis using an AUF1 antibody.

Figure 6 (A and B) Baf-3 cells were transfected with either the empty piGENE-mU6 vector, piGENE-mU6-2sm, or -3sm vector and cultured in the presence of IL-3 for 16 hours. Immunoblot analysis using Hsc70 antibody (A). Cells transfected with the empty vector were cultured for 16 hours in the presence of IL-3 (upper left); cell transfected with the empty vector (upper right), piGENE-mU6-2sm (lower left), or piGENE-mU6-3sm (lower right) were cultured in the presence of IL-3 for 16 hours and then continued to culture in the absence of IL-3 for 12 hours. In each transfection, pDsRed2-N1 vector, which expresses DsRed protein emanating red fluorescence was transfected as an indicator of transfection efficiency. Cells were stained with FITC-VAD-FMK and analyzed by flow cytometry (B). (C and D) Parental Baf-3 cells and cells over-expressing
Bag-4 (Clone #17) were cultured without IL-3 for the indicated periods. Immunoblot analyses for Bag-4, Bim, p27 and β-actin (C), and cell number (left) and viability (right) (D) are shown. Results of a representative clone (#17) are shown; similar results were obtained with three independent clones. (E) Relative levels of Bag-4 mRNA measured by real-time RT-PCR in murine early hematopoietic progenitors (Sca1'cKit'Lin') cultured in cytokine-free (stem cell factor and thrombopoietin-free) medium for the indicated hours are shown. The mean and SD of three independent experiments are shown.
human BimEL

mouse BimEL

(Fig. 1A)
Bim mRNA, Cap+/pA+

- **Baf-3 IL-3(+)**
- **Baf-3 IL-3(-)**
- **FDC-P1 IL-3(+)**
- **FDC-P1 IL-3(-)**

**Relative signal intensification vs. Incubation time**

(Fig. 1D)
Relative signal intensification

Bim mRNA
Baf-3, IL-3(-)

- Cap+/pA+
- Cap+/pA-
- Cap-/pA+
- Cap-/pA-

Incubation time (Min)
0 5 10 15 20 25 30

(Fig. 1E)
Bim mRNA
Baf-3, IL-3(+) 

Cap+/pA+ 

Cap+/pA- 

Cap-/pA+ 

Cap-/pA- 

0.05 (Min) 

0 5 10 15 20 25 30 

Incubation time 

Relative signal intensification 

(Fig. IF)
Baf-3, Cap+/pA+ mRNA

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(kDa)

200  116  97  66  55  36  31

1  2  3  4  5  6  7  8  9  10

(Fig. 2A)
(Fig. 2C)
Lane: 1 2 3 4

(Fig. 2D)
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(Fig. 3A)
(Fig. 3B)
Baf-3 lysate

IP

no

IL3+ IL3-

isotype control

anti-Hsc70

(Fig. 3C)
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Lane: 1 2 3 4 5 6 7

(Fig. 4A)
Hsc70 Ab

Hsc70

supershifted complex

RNA/protein complex

Free probe

1 2 3 4 5 6

(Fig. 4B)
Glutathione beads-bound Hsc70
Bim mRNA-bound Hsc70

GST

GST Bag4

GST Bag4ΔC

1 2 3
(Fig. 4D)
IL3+  IL3-
elF4G  PABP  AUF1
(Fig. 5A)
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(Fig. 5B)
(Figure 6A)
Apoptotic cells
Intact cells

DsRED (Transfection efficiency)

(Fig. 68)
Hours after IL-3 starvation

(Fig. 6D)
Figure 6E: Relative expression level over time. The graph shows a significant increase in expression level at 0 hours, followed by a decrease at 3 hours and a slight increase at 6 hours. (Fig. 6E)