Pim-1 Kinase Stability Is Regulated by Heat Shock Proteins and the Ubiquitin-Proteasome Pathway

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Abstract

Elevated expression of the serine/threonine kinase Pim-1 increases the incidence of lymphomas in Pim-1 transgenic mice and has also been found to occur in some human cancers. Pim-1 acts as a cell survival factor and may prevent apoptosis in malignant cells. It was therefore of interest to understand to what extent maintenance and degradation of Pim-1 protein is affected by heat shock proteins (Hsp) and the ubiquitin-proteasome pathway in K562 and BV173 human leukemic cells. The half-life of Pim-1 protein in these cells was found to increase from 1.7 to 3.1 hours when induced by heat shock or by treating the cells with the proteasome inhibitor PS-341 (bortezomib). The Hsp90 inhibitor geldanamycin prevented the stabilization of Pim-1 by heat shock. Using immunoprecipitation, it was determined that Pim-1 is targeted for degradation by ubiquitin and that Hsp70 is associated with Pim-1 under these circumstances. Conversely, Hsp90 was found to protect Pim-1 from proteasomal degradation. A luminescence-based kinase assay showed that Pim-1 kinase bound to Hsp70 or Hsp90 remains active, emphasizing the importance of its overall cellular levels. This study shows how Pim-1 levels can be modulated in cells through degradation and stabilization. (Mol Cancer Res 2005;3(3):170–81)

Introduction

The proto-oncogene Pim-1, which is highly conserved in mammalian cells, encodes a serine/threonine kinase that is found at high levels in some leukemias, lymphomas, and carcinomas (1, 2). Pim-1 has been shown to phosphorylate several proteins, including $p21^{cip1/waf1}$ (3), CDC25A (4), PTP-U2 (5), NuMA (6), and PAP-1 (7). Of particular relevance to its role in cancer, Pim-1 has been shown to prevent the normal process of apoptosis, acting as a cell survival factor. Although important in normal cellular processes, survival factors may contribute to malignancy by interfering with proapoptotic signals, preventing programmed cell death and allowing the next round of mitosis to take place. As an example, Lilly et al. (8) found that factor-dependent 32D cells deprived of interleukin-3 survived longer in the presence of overexpressed wild-type Pim-1, whereas dominant-negative Pim-1 caused apoptosis. Pim-1 has also been implicated in antiapoptosis through the phosphorylation of $p21^{cip1/waf1}$ (3), which is thought to mediate survival by binding to and preventing the activation of pro-caspase-3 (9). Most recently, Pim-1 kinase has been found to promote inactivation of the proapoptotic Bad protein by phosphorylating it on the Ser^{112} gatekeeper site (10).

Lymphomas in mice caused by infection with the Moloney murine leukemia virus overexpress Pim-1 because the virus preferentially infects T cells and integrates into the 3′ untranslated region of the gene. This results in the insertion of a premature stop codon in front of the destabilizing A/U-rich element in the 3′ untranslated region that makes *pim-1* transcripts inherently short lived. The loss of this element results in longer-lived *pim-1* mRNAs, which allow for more translation of Pim-1 protein. It has also been shown that Pim-1 protein has a short half-life of ~5 to 10 minutes in primary cells (11, 12). In this study, we have found the Pim-1 protein half-life in tumor cells to be at least 100 minutes. The half-life of Pim-1 has been found to shorten in response to the heat shock protein (Hsp) 90 inhibitor geldanamycin (13), providing evidence that Hsp90 may be a regulator of Pim-1 levels in cells. Therefore, an increase in half-life, rather than just an increase in transcription rate, may be a major factor in the increased levels of Pim-1 observed in tumor cells. This is of particular importance because the crystal structure of Pim-1 reveals that it is a constitutively active kinase (14). Thus, it was of interest to determine the extent to which Pim-1 levels are regulated post-translationally.

When proteins are no longer needed in a cell at the level currently expressed, several different degradation or cleavage events may be invoked to lower the protein levels. Perhaps the most commonly observed mode of degradation is the ubiquitin-proteasome pathway in which the target proteins are tagged for destruction with small chains of ubiquitin and are then enzymatically disassembled by the large multiunit 26S proteasome complex. Cellular proteins have varying half-lives, ranging from minutes to days. Therefore, a population of proteins being prepared for degradation by the ubiquitin-proteasome pathway continually exists. Well-known candidates for degradation by the ubiquitin-proteasome pathway are numerous, including c-Jun (15), IκB (16), Mos (17), STAT1 (18), cyclin E (19), p27 (20), c-Myb (21), pRb (22), and cyclin D1 (23).

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Hsp chaperones have been shown to be involved both in protection of cellular proteins and in their degradation by the 26S proteasome. Hsp90 and Hsp70 can bind to several adaptor proteins that are responsible for either stabilizing the chaperone-substrate complex or using the association to target the substrates to the proteasomes (24). In the latter case, adaptor proteins act as E3 ubiquitin ligases, facilitating the addition of chains of ubiquitin to the client proteins. Hsp90 is often found overexpressed in leukemic cells and cell lines (25). Cells expressing high levels of Hsps can become resistant to chemotherapy (26), suggesting that Hsp90 and other chaperones may protect proteins that are closely involved in tumor cell survival (27).

This study investigated the post-translational regulation of Pim-1 protein in Bcr-Abl tumor cells (K562 and BV173) that occurs by chaperone binding and by degradation. We hypothesized that Hsp chaperone binding and ubiquitin-mediated degradation provide mechanisms for precisely controlling the levels of Pim-1 available for survival activity. We show that a major route of degradation for Pim-1 protein is via the ubiquitin-proteasome pathway and that Pim-1 binds to Hsp90, an association that can be disrupted by the use of a Hsp90-specific inhibitor, geldanamycin. The ubiquitin-mediated degradation of Pim-1 occurs after its dissociation from Hsp90, providing evidence that the chaperone has a protective effect on Pim-1. The association between Pim-1 and Hsp70 exists when Pim-1 is conjugated to ubiquitin. It was also shown that Pim-1 bound to Hsp70 and Hsp90 in these cells remains kinase active. These findings suggest that the protein level of Pim-1 in tumor cells is mediated at least in part through the ubiquitin-proteasome pathway and Hsp70 and Hsp90 binding. Furthermore, concerning cell survival, expression of a kinase-dead Pim-1 caused an increase in cell death that was not augmented by treatment with geldanamycin. Cells expressing wild-type Pim-1 remained susceptible to a reduction in cell survival after treatment with geldanamycin. As an antiapoptotic factor, Pim-1 seems to aid in the survival of tumor cells, whereas a decrease in its expression or activity would shift the balance toward apoptosis.

Results

Inhibition of the 26S Proteasome Prevents Pim-1 Degradation

K562 and BV173, the two cell lines used in this study, express the highest constitutive levels of Pim-1 protein that we have found to date. Both are derived from human chronic myelogenous leukemia, are Philadelphia chromosome positive, and express the Bcr-Abl fusion protein. Data from K562 cells are shown, with consistently similar data being obtained for the BV173 cell line. We have observed that Pim-1 protein has a longer half-life in these and other tumor cell lines than the 10-minute half-life reported for the 34-kDa Pim-1 in primary cells (11). For this reason, we were interested to determine the mechanism responsible for controlling the long half-life of Pim-1 in tumor cells. Furthermore, because the ubiquitin-proteasome pathway has been well established as a major means of protein degradation, we wanted to evaluate the contribution of this pathway to Pim-1 degradation.

PS-341 (bortezomib) is an inhibitor of the ubiquitin-specific 26S proteasome (28). Adding a proteasome inhibitor to cell culture medium does not interfere with ubiquitin tagging but does prevent the 26S proteasome from degrading the marked proteins. As a control, chloroquine and ammonium chloride were added to cells to inhibit lysosomal cathepsins (29). These proteases function in the low pH environment of the lysosome, and the combination of chloroquine and ammonium chloride is often used experimentally to inactivate the cathepsins by increasing lysosomal pH. Lysosomes provide a major route for the degradation of proteins and other cellular components but are not the chief destination for ubiquitin-tagged proteins. We also tested the effects of the Hsp90 inhibitor geldanamycin, which changes the physical association of Hsp90 with its substrates by acting as an ATP analogue that specifically binds Hsp90 in its ATP binding site; thus, the client protein is not protected (30). To show that Pim-1 is a target for degradation by the 26S proteasome, K562 cells were treated with PS-341 or chloroquine/ammonium chloride for up to 16 hours. The resulting Pim-1 Western blot shows the effects of proteasome inhibition on preventing Pim-1 protein turnover (Fig. 1A). The inhibitors of lysosomal degradation, which inactivate cathepsins by raising the pH inside lysosomes (normally pH 5.5), did not cause any significant accumulation of Pim-1 protein. Cells treated with geldanamycin experienced a dramatic decrease in Pim-1, suggesting that maintenance of normal Pim-1 levels is aided by Hsp90. One-hour heat shock at 43°C was seen to increase Pim-1 levels. The pretreatment of cells with PS-341 or heat shock before geldanamycin provides some preservation of Pim-1, although it cannot prevent the action of geldanamycin. When geldanamycin was included in the treatments, there was a significant ($P < 0.05$) decrease in Pim-1 protein compared with heat shock and PS-341 (Fig. 2). These results show that the 26S proteasome is a contributor to Pim-1 degradation and that Hsp90 plays a role in Pim-1 stability in these cells.

The Half-life of Pim-1 Increases with Proteasomal Inhibition

To confirm that inhibition of the 26S proteasome prevents Pim-1 degradation, a half-life study was done. K562 cells were treated with cycloheximide to halt protein synthesis (31, 32) and with PS-341 or DMSO vehicle. Samples were taken at the indicated times (Fig. 1B). A graph of the half-life of Pim-1 (Fig. 1C) confirms that in PS-341-treated cells Pim-1 is significantly more stable than in untreated cells. For consistency, Pim-1 half-life studies were also done with geldanamycin and heat shock in the same experiment and will be discussed later. The normal half-life of Pim-1 in K562 vehicle-treated cells (control) is ~1.7 hours. The half-life of Pim-1 in the presence of PS-341, when proteasomal degradation is inhibited, is ~2.65 hours. This result shows that the 26S proteasome is involved, at least to some degree, in Pim-1 degradation.

Pim-1 Can Be Conjugated to Ubiquitin In vitro

To test whether Pim-1 may be tagged with ubiquitin, we did an *in vitro* ubiquitylation assay using cell lysate and glutathione *S*-transferase (GST)-Pim-1 (59 kDa), AMP-PNP, and/or ubiquitin in a cell-free system. AMP-PNP is a β,γ-nonhydrolyzable ATP analogue. It was used to uncouple ubiquitylation from degradation by allowing the addition of ubiquitin molecules in an increasing chain to the GST-Pim-1

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A

0 4 8 16 h treated

Chlq +NH_4Cl

PS341

GA

GA + PS341

HS

GA + HS

WB: Pim-1 (top)

WB: Actin (bottom)

B

0 1.5 3 6 h post-CHX

Control

PS341

HS

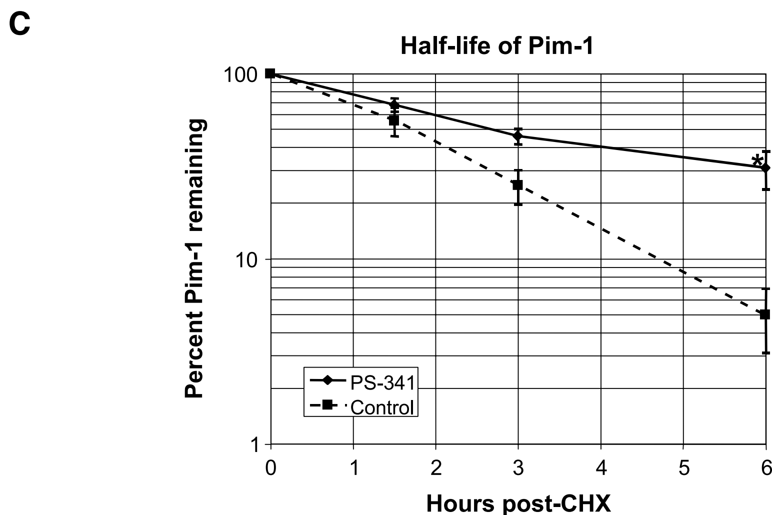
GA

GA+HS

WB: Pim-1 (top)

WB: Actin (bottom)

C



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Treatment	P
PS341	
	P = 0.0056
PS341 + GA	
	P = 0.0012
GA	
	P = 0.4430
Heat Shock + GA	
	P = 0.0001
Heat Shock	

FIGURE 2. Difference (from untreated, set at 100%) in Pim-1 levels after 16-hour treatment.

while preventing their removal by proteasomal degradation, which uses β,γ cleavage of ATP (33). The differing ATP cleavage requirements of these two processes make AMP-PNP a useful analogue for this experiment. As seen in Fig. 3A, GST-Pim-1 is conjugated to ubiquitin when it is present, and this is an ATP-dependent process. In Fig. 3A (*top*), probed with anti-ubiquitin antibodies, high molecular weight forms of ubiquitin-conjugated GST-Pim-1 are present when all components are available, indicating that Pim-1 may become extensively ubiquitylated. In Fig. 3A (*bottom*), highly ubiquitylated forms of Pim-1 were not recognized by our antibody to Pim-1 probably due to epitope masking. Extensive ubiquitylation of a protein is thought to mask the epitope that is recognized by an antibody and thus inhibits the detection of heavily ubiquitylated proteins (34). Both blots show a form of GST-Pim-1 with slightly altered mobility, consistent with a monoubiquitylated protein (35), which may be caused by residual ATP and ubiquitin in the cell lysate. Because of the large amount of GST-Pim-1 added to the lysate, greatly ubiquitylated forms of the protein would not be expected without the addition of exogenous sources of energy and ubiquitin. Thus, only the first lane exhibits a streak of polyubiquitylated GST-Pim-1. The number of molecules added to Pim-1 in this case may be greater than expected *in vivo* due to the prevention of deubiquitylation by a nonhydrolyzable ATP analogue.

Proteasomal Inhibition Results in Ubiquitylated Forms of Endogenous Pim-1 In vivo

To confirm the presence of Pim-1 in the population of cellular ubiquitin-conjugated proteins, total ubiquitin was immunoprecipitated from K562 cells treated with geldanamycin, PS-341, or a combination of the two drugs. Western blotting was done for Pim-1. Figure 3B shows that Pim-1 bound to ubiquitin is detected primarily in cells that have been treated with geldanamycin and PS-341 to prevent immediate degradation by the 26S proteasome. In these samples, Pim-1 association with Hsp90 was disrupted or prevented, allowing Pim-1 to be conjugated to multi-ubiquitin chains, but is best visualized in the lane where PS-341 is present to prevent rapid degradation by the proteasome. Cells treated with PS-341 alone show minimal conjugation of Pim-1 to ubiquitin, because although the proteasome is inhibited Pim-1 may still be protected by Hsp90. These results suggest that Pim-1 experiences less ubiquitin tagging when its binding to Hsp90 is left intact and that, when released from the protection of Hsp90, Pim-1 may be targeted for proteasomal degradation. As a control, a reprobe for Hsp70 shows that this chaperone, often associated with ubiquitin, is present in the total ubiquitin-conjugated fraction in similar amounts. In Fig. 3B (*bottom*), cells treated with geldanamycin and/or PS-341 were immunoprecipitated for Pim-1, and the presence of Pim-1 was confirmed by Western blotting, to show that Pim-1 is present under these circumstances. These results indicate that the Pim-1 protein is attached to endogenous chains of ubiquitin *in vivo*. Taken together with the increase in Pim-1 when the 26S proteasome is inhibited, it suggests that the ubiquitin-proteasome pathway is a major route of degradation for Pim-1.

The Half-life of Pim-1 Increases with Heat Shock but Decreases When Hsp90 Binding Is Inhibited

We have made the observation that Pim-1 protein expression is increased by heat shock (Fig. 1A). This prompted us to investigate whether this effect could be due to a decrease in the turnover of Pim-1 protein. Heat shock has been shown to induce a protective effect on cells in that a 1-hour heat treatment allows them to resist further injury (36), a phenomenon that requires prosurvival activity. In our experiments, K562 cells were heat shocked at 43°C for 1 hour before the addition of cycloheximide. The Western blot is shown in Fig. 1B. The half-life of Pim-1 in heat shocked cells is ~3.1 hours, and there is significantly more Pim-1 left after 6 hours than in untreated cells (Fig. 4A).

Although it was clear that Pim-1 can be degraded by the ubiquitin-proteasome pathway, the circumstances that regulate its availability for degradation were not known at this point. The increase in Pim-1 half-life with heat shock suggested that it might be sequestered by Hsp chaperones, a class of proteins that are either ubiquitously expressed or induced by heat shock and/or other cellular stresses. A report of Pim-1 binding to the chaperone Hsp90 (13) led us to investigate its possible contribution to Pim-1 stability concerning the ubiquitin-proteasome pathway. We employed the inhibitor geldanamycin

FIGURE 1. Pim-1 protein levels and half-life increase with proteasomal inhibition or heat shock. **A.** K562 cells were treated with 1 μmol/L PS-341 or 100 μmol/L chloroquine (*Chlq*) + 2.5 mmol/L ammonium chloride (NH_4Cl) for 4, 8, or 16 hours. K562 cells were treated with 2 μmol/L geldanamycin (*GA*) or 1 μmol/L PS-341, pretreated with 1-hour heat shock (*HS*) at 43°C, or a combination of these for 4, 8, or 16 hours. Cell lysates (30 μg) were run on 11% SDS-PAGE and Western blotting was done with polyclonal Pim-1 antibodies. Actin was used as a loading control. Blots representative of four independent experiments. **B.** For the half-life studies, K562 cells were given 30 μmol/L cycloheximide (*CHX*) to halt translation. Treatments included 1 μmol/L PS-341, 2 μmol/L geldanamycin, or an equivalent volume of DMSO vehicle (*Control*). Heat shock was done by pretreating cells for 1 hour at 43°C and adding DMSO vehicle. All results are compared with the untreated control. Cells were harvested at the times indicated and lysates (30 μg) were separated by 11% SDS-PAGE. Western blotting was done with anti-Pim-1 polyclonal antibodies. Actin was used as a loading control. Representative of three experiments with similar results. **C.** Densitometric analyses of the PS-341 and control blots as in **B** were done with the Quantity-One program. A graph was constructed based on these values. A *t* test done on the values at 6-hour cycloheximide confirms a significant difference between PS-341 and control (*, $P = 0.0037$).

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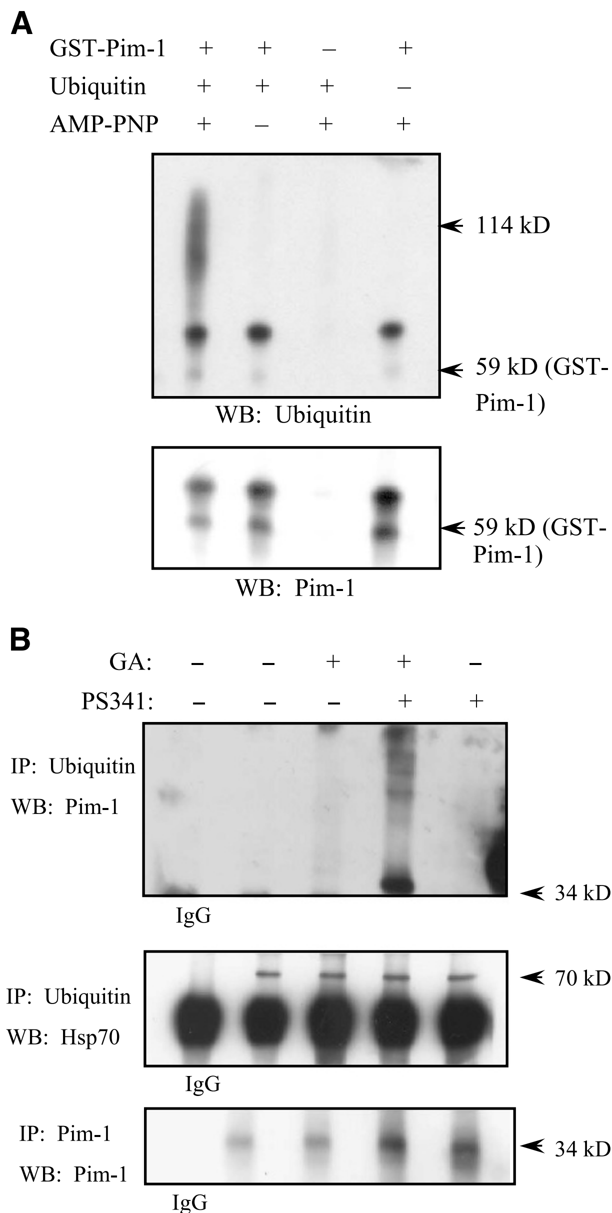

FIGURE 3. Pim-1 is ubiquitylated *in vitro* and *in vivo*. **A.** Using cell lysates as a source of ubiquitylation enzymes, an *in vitro* assay was done with exogenous GST-Pim-1, ubiquitin, and/or AMP-PNP. Reactions were incubated at 37°C for 30 minutes and the reaction was stopped with the addition of cold radioimmunoprecipitation assay buffer. GST-Pim-1 (59 kDa) was isolated on glutathione-Sepharose 4B. Samples were run on 7% SDS-PAGE and Western blotting was done with anti-ubiquitin and anti-Pim-1 polyclonal antibodies. Two additional experiments yielded similar results. **B.** K562 cells were treated for 2 hours with geldanamycin, PS-341, both, or DMSO vehicle. Total ubiquitin was immunoprecipitated from protein lysate (400 μg) and Western blotting was done with monoclonal anti-Pim-1. The blot was reprobed with anti-Hsp70. *Bottom,* an immunoprecipitation of Pim-1 with Western blotting for Pim-1. Preimmune IgG was used as a negative control. Similar results were found in two additional experiments.

to prevent Hsp90 from binding to Pim-1. K562 cells were treated with geldanamycin or DMSO vehicle at the same time that cycloheximide was added to prevent new protein synthesis. The Western blot is shown in Fig. 1B. The normal half-life of Pim-1 without geldanamycin is 1.7 hours in these cells (Fig. 4B). When geldanamycin is present, the half-life of Pim-1 decreases to ~0.7 hour, less than half of normal. This suggests that Pim-1 may be protected from degradation by Hsp90.

The overall increase in levels of Pim-1 protein in these tumor cells when they are subjected to 1-hour heat shock at 43°C seems to be caused at least in part by an increase in half-life. The addition of cycloheximide for this half-life study shows that existing Pim-1 protein is stabilized during heat shock. We suspected that the mechanism behind longer half-life of Pim-1 following heat shock may be the protection provided by Hsp90. A graph of the half-life experiment in which K562 cells were pretreated with geldanamycin before heat shock (Western blot in Fig. 1B) shows that heat shock no longer prolongs Pim-1 stability when Hsp90 is unable to bind it (Fig. 4C). The half-life of Pim-1 when treated with geldanamycin before heat shock is only 1 hour, slightly longer than with geldanamycin treatment alone but much shorter than the 3.1-hour half-life of Pim-1 in cells treated only with heat shock. These results confirm that Pim-1 from heat shocked cells is stabilized by Hsp90, because the presence of geldanamycin prevents the heat shock–induced prolongation of Pim-1 half-life.

Pim-1 Binds to Hsp90 and Hsp70 In vivo

To confirm that Pim-1 does indeed bind to Hsp90, a coimmunoprecipitation was done with the endogenous proteins. Cells were pretreated with geldanamycin or DMSO vehicle. Lysates were immunoprecipitated with mouse monoclonal Pim-1 antibodies or rat monoclonal Hsp90 antibodies. Western blots were done with antibodies to the coimmunoprecipitating protein. In cells that were not treated with geldanamycin, Pim-1 was immunoprecipitated with Hsp90, and Hsp90 was immunoprecipitated with Pim-1. As expected, in geldanamycin-treated cells, neither protein coimmunoprecipitated with the other because the interaction was disrupted or prevented by the action of geldanamycin on the Hsp90 ATP-binding site (Fig. 5A).

Hsp70 has been reported to play a role in the degradation of proteins, such as Bcr-Abl (29, 37). Therefore, we wanted to investigate the possible contribution of Hsp70 to Pim-1 degradation. The same study was carried out with immunoprecipitation of Pim-1 and blotting for Hsp70. It was found that Pim-1 and Hsp70 do associate (Fig. 5B). As expected, geldanamycin did not disrupt the binding of Pim-1 to Hsp70, as it is a specific inhibitor of Hsp90 alone. This also suggests that binding of Pim-1 to Hsp70 is not dependent on the presence of Hsp90. A slight increase in the association of Pim-1 with Hsp70 seems to occur with geldanamycin treatment, suggesting that the loss of Hsp90-mediated protection of Pim-1 may actually promote its association with Hsp70. A reprobe of the blot for Pim-1 confirms that Pim-1 was precipitated in equal amounts. The above results indicate that Pim-1 binds to both Hsp90 and Hsp70. These three proteins, and perhaps others, may exist transiently as complexes inside cells.

Hsp70 Is Associated with Ubiquitylated Forms of Pim-1

Knowing that the inhibition of Hsp90 binding caused a decrease in Pim-1 levels and that Pim-1 increasingly bound to

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Hsp70 under these circumstances, we wanted to investigate whether Hsp70 was associated with Pim-1 during proteasomal inhibition. K562 cells were treated with PS-341 to preserve ubiquitin-conjugated Pim-1 for immunoprecipitation. A Western blot done with antibodies to Hsp70 shows that Hsp70 was bound to Pim-1 in both PS-341-treated and mock-treated samples but more so with proteasomal inhibition (Fig. 6A). A reprobe of the blot for Pim-1 confirms that it was immunoprecipitated in equivalent amounts. These results suggest that Hsp70 is associated with Pim-1 when it is preserved by proteasomal inhibition.

Furthermore, Pim-1 was seen to coprecipitate with both Hsp70 and Hsp90 in cells from normal rat lymphocytes (Fig. 6B). When compared with the amounts of Hsp70 and Hsp90 bound to Pim-1 in K562 cells, it seems that there is more association between Pim-1 and Hsp90 in tumor cells. This could be due to higher amounts of available Hsp90 in the tumor cells or to a Hsp90 with increased binding capacity. K562 is a cell line, and although it may not be representative of all tumors, the result is intriguing because it could help to explain the pro-oncogenic capacity of Pim-1.

Hsp90 Protects Pim-1 from Degradation by the Ubiquitin-Proteasome Pathway

The above results demonstrating Pim-1 association with Hsp70 with the use of PS-341 prompted us to investigate whether this binding occurred primarily with ubiquitylation. To show that Hsp70 is associated with ubiquitylated Pim-1, and Hsp90 is not, fractions containing ubiquitylated Pim-1 were compared with those with nonubiquitylated Pim-1. K562 cell lysates pretreated with PS-341 were depleted of ubiquitylated proteins through immunoprecipitation. The supernatant containing proteins that were not conjugated to ubiquitin was then immunoprecipitated for Pim-1. The sample containing total cellular ubiquitylated proteins was eluted from the protein A-agarose and reimmunoprecipitated with anti-Pim-1 to select only ubiquitin-conjugated Pim-1. A third sample, nonfractionated, was immunoprecipitated for total Pim-1 protein. As seen by Western blotting, the lane with ubiquitylated Pim-1 did not contain Hsp90, but the other lane shows this chaperone bound to the fraction of Pim-1 that was not tagged for degradation (Fig. 6C). These results indicate that Hsp90 associates with nonubiquitylated Pim-1. This is consistent with the half-life data that indicate that Hsp90 protects Pim-1 from degradation. In contrast, Hsp70 seems to be associated mainly with ubiquitin-conjugated Pim-1.

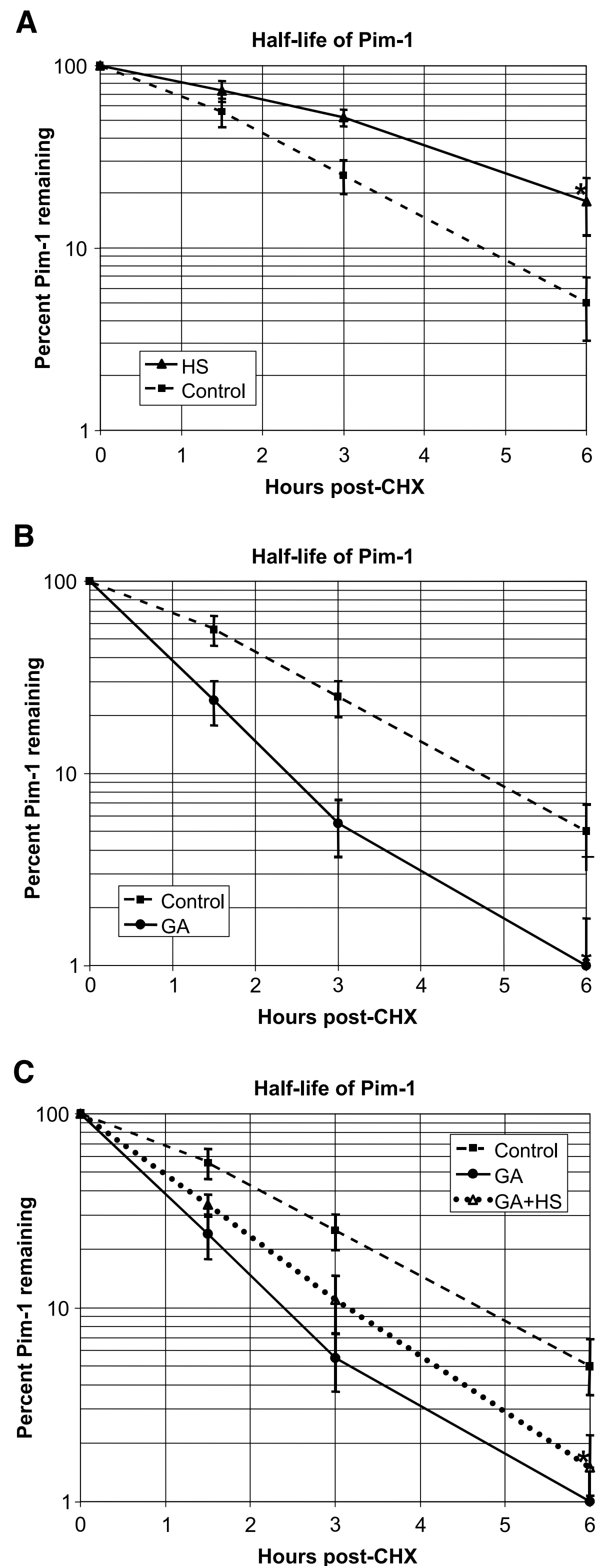

FIGURE 4. Pim-1 protein levels increase with heat shock, except in the presence of Hsp90 inhibition. **A.** Densitometric analyses of the heat shocked and control blots in Fig. 1B were done with the Quantity-One program. A graph was constructed based on these values. A *t* test done on the values at 6-hour cycloheximide confirms a significant difference between heat shocked and control (*, $P = 0.0255$). **B.** Densitometric analyses of the geldanamycin-treated and control blots in Fig. 1B were done with the Quantity-One program. A graph was constructed based on these values. A *t* test done on the values at 6-hour cycloheximide confirms a significant difference between geldanamycin and control (*, $P = 0.0215$). **C.** Densitometric analyses of the heat shock + geldanamycin and control blots in Fig. 1B were done with the Quantity-One program. A graph was constructed based on these values. A *t* test done on the values at 6-hour cycloheximide confirms a significant difference between heat shocked + geldanamycin and control (*, $P = 0.0315$). For comparison, data from the geldanamycin-treated cells in **B** were included on this graph.

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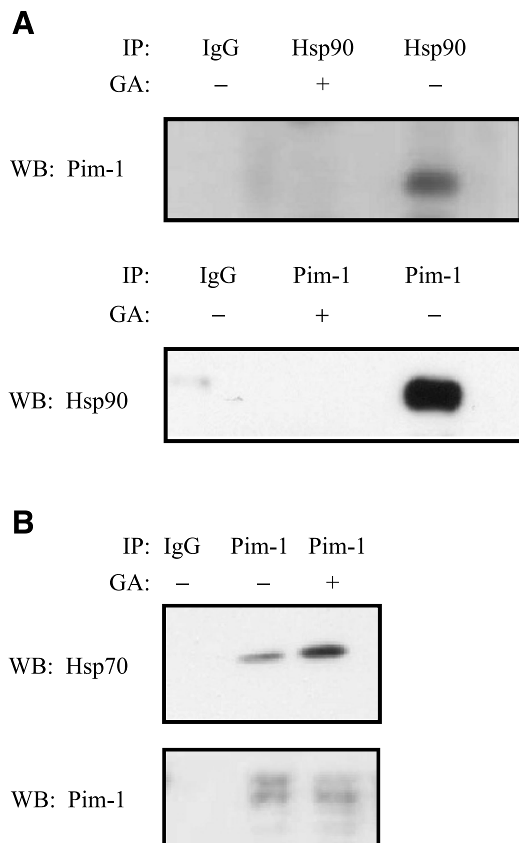

FIGURE 5. Pim-1 binds to Hsp90 and Hsp70. **A.** K562 cells were pretreated with 2 μmol/L geldanamycin for Hsp90 inhibition or DMSO vehicle for 2 hours and lysed in immunoprecipitation buffer. Protein lysate (400 μg) was immunoprecipitated with rat monoclonal anti-Hsp90 or mouse monoclonal anti-Pim-1. Immunocomplexes were separated by 9% SDS-PAGE and Western blotting was done with monoclonal antibodies to Pim-1 and Hsp90. **B.** Cells were treated as in **A.** Samples were immunoprecipitated with Pim-1 monoclonal antibodies, proteins were run on 9% SDS-PAGE, and immunoblotting was done with anti-Hsp70 and anti-Pim-1 polyclonal antibodies. Blots are representative of at least three independent experiments.

Pim-1 Uncomplexed with Hsp70 and Hsp90 Remains Kinase Active

To date, no reports have been made in which Pim-1 has a kinase-inactive state in tumor cells. Indeed, the latest information from crystal structure analyses indicates that Pim-1 is constitutively active (14). Therefore, the regulation of Pim-1 protein levels by Hsp70 and Hsp90 prompted us to investigate whether this might also be a mechanism for regulating Pim-1 kinase activity. K562 lysate was serially immunoprecipitated for Hsp90, Hsp70, and Pim-1. Western blotting for Pim-1 showed that it was present in each fraction, although not in equivalent amounts, and that Pim-1 unbound to either Hsp70 or Hsp90 does exist after Hsp70 and Hsp90 immunodepletion (Fig. 7A). To test the kinase activity of Pim-1, an equivalent amount of recombinant Pim-1 was bound as described to recombinant Hsp70, Hsp90, or left free. The complexes were used in a luminescence-based kinase assay that measures ATP levels. A peptide containing the Pim-1 phosphorylation consensus sequence from $p21^{cip1/waf1}$ (3) was used as the substrate for the isolated Pim-1 complexes, which contained the exogenous Hsp as confirmed by Western blot (data not shown). Luminescence decreases as ATP is used; therefore, a drop in ATP level from input is detected by the assay. Pim-1 kinase activity was detected in each case, at levels that were not significantly different from one another, indicating that Hsp70 and Hsp90 may not inhibit Pim-1 (Fig. 7B). Hsp70 and Hsp90 themselves did not measurably deplete ATP (data not shown). These results underscore the importance of the regulation of Pim-1 protein levels in tumor cells, as the principle way of reducing Pim-1 activity must be reduction of its protein levels by degradation. Similar results were obtained by the coimmunoprecipitation of endogenous protein complexes (data not shown), so the presence of other factors that may be present in the complexes does not change this trend. Thus, association with either chaperone does not seem to suppress Pim-1 kinase activity.

Pim-1 Is Involved in Cell Survival

K562 cells were treated with STI-571, a drug that specifically causes apoptosis in Bcr-Abl-positive leukemias. As STI-571 causes eventual down-regulation of other Hsp90 clients (Akt and Bcr-Abl) as well as Pim-1, it was necessary to show the influence of Pim-1 on cell survival by expression of wild-type and kinase-dead Pim-1. K562 cells were transfected with vectors expressing wild-type green fluorescent protein (GFP)-Pim-1, kinase-dead GFP-Pim-1, or GFP alone and treated with 10 μmol/L STI-571 and geldanamycin or DMSO vehicle for 3 hours. Flow cytometry was done with supravital staining on cells emitting green fluorescence. Figure 7C shows that survival of green-gated cells as measured by propidium iodide exclusion was approximately equal whether wild-type GFP-Pim-1 or GFP alone was expressed. This is probably because K562 cells express very high levels of endogenous Pim-1, and the addition of more is superfluous. Cells expressing kinase-dead GFP-Pim-1 displayed less survival than vector only; thus, the presence of nonfunctional Pim-1 is detrimental to cell survival and may contribute to apoptosis. When the cells expressing wild-type Pim-1 or vector were treated with geldanamycin, there was a significant amount of death comparable with the untreated cells expressing kinase-dead GFP-Pim-1. In this case, endogenous Pim-1 and other Hsp90-dependent proteins were destabilized by geldanamycin, and the loss of Pim-1 contributed to cell death. Other survival-related proteins that are Hsp90 dependent may factor into the lower survival, but we can observe that the death induced by kinase-dead Pim-1 was not prevented by the existence of these other factors in the geldanamycin-untreated sample. In contrast, geldanamycin caused close to the same amount of death in cells expressing only endogenous wild-type Pim-1 (GFP vector only) as in those expressing kinase-dead Pim-1. This indicates that the presence of Pim-1 does have an influence over cell survival that is intimately tied to its stability as regulated by Hsp90. Statistical analysis by *t* test yields a *P* that is <0.05 for cells treated with geldanamycin and/or expressing kinase-dead Pim-1.

Discussion

Recent work from this laboratory and others has shown that Pim-1 acts as a survival factor under many circumstances (3, 5,

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8, 38-41). It has also been shown that the binding of Pim-1 to Hsp90 can prolong its half-life (13). The work presented here shows that in tumor cells Pim-1 can bind not only to Hsp90 but also to Hsp70 and that association with the former protects it from degradation by the ubiquitin-proteasome pathway. Binding to Hsp70, conversely, is associated with the degradation of Pim-1. To date, no mutated forms of Pim-1 have been reported. Furthermore, Pim-1 has a constitutively active conformation (14), which strengthens the case that it is increased levels of Pim-1, and not necessarily malfunction of the kinase, that can make it a contributing factor in neoplasia. In knockout mice, the only known effect of an absence of Pim-1 is erythrocytic microcytosis (42). Mice deficient for all Pim kinases display reduced body size and impaired responses to hematopoietic growth factors (43). There seem, therefore, to be compensatory pathways that allow normal cells, but not some tumor cells, to survive the loss of Pim-1, which suggests that this kinase may be a good target for cancer therapy. Because cells must maintain a balance between survival and apoptosis, and because Pim-1 promotes cell survival, the precise post-translational control of Pim-1 levels must be necessary to prevent tumorigenicity.

Pim-1 seems to be active as a kinase whether it is bound to or free of Hsp70 and Hsp90 (Fig. 7B). Hsp70 binding does not seem to cause Pim-1 to become inactive, but the process of Hsp70 and ubiquitin-conjugating enzymes acting to ligate ubiquitin to Pim-1 may affect Pim-1 activity. Ubiquitylated Pim-1 would, however, be shuttled to the proteasome and degraded. Presumably, if Hsp90 acts to protect Pim-1 from degradation under cellular stress, Pim-1 would be expected to have the potential to remain active in its role as a survival kinase. This may be the reason for its sequesteration from the degradation machinery. Whether there are any changes in Pim-1 conformation that trigger Hsp70 association, ubiquitylation and shuttling to the proteasome remains a subject for further investigation.

Proteasomal involvement in Pim-1 degradation was established with the use of PS-341, a specific inhibitor of the 26S proteasome. It was shown that Pim-1 levels increase in the presence of PS-341 (Fig. 1A and B). The finding that the half-life of Pim-1 increases in response to PS-341 provides further verification that Pim-1 is degraded by the 26S proteasome. That there was not a total conservation of Pim-1 in cells treated with PS-341 suggests that other degradation pathways for Pim-1 may exist, such as the lysosomal pathway. Hsc73/Hsp70 has been shown by Cuervo et al. (44) to chaperone ubiquitylated substrates to lysosomes for degradation by cathepsins under certain circumstances; thus, it is also possible that Pim-1 may

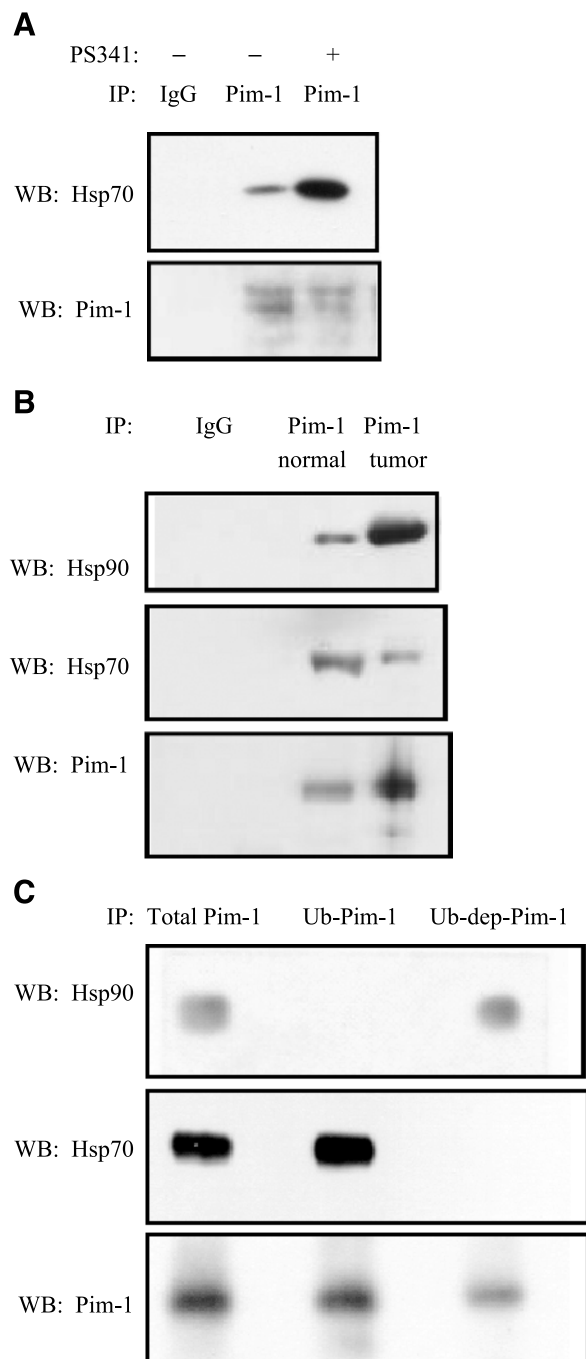

FIGURE 6. Ubiquitylated Pim-1 is associated with Hsp70 and nonubiquitylated Pim-1 with Hsp90. **A.** K562 cells were treated for 18 hours with PS-341 for accumulation of ubiquitylated Pim-1 and lysed. Pim-1 was immunoprecipitated from protein lysate (400 μg) with monoclonal antibodies. Complexes were run on 9% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Western blotting was done with polyclonal antibodies to Hsp70 and Pim-1. Preimmune IgG was used as a negative control. **B.** K562 tumor cells and normal rat lymphocytes isolated from spleen were lysed in immunoprecipitation buffer and proteins (400 μg) were immunoprecipitated for Pim-1. Complexes were run on 9% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Western blotting was done for Hsp90, Hsp70, and Pim-1. Preimmune IgG was used as a negative control. **C.** Cells were lysed in immunoprecipitation buffer and lysates were split in half. One tube (400 μg protein) was immunoprecipitated with anti-Pim-1 monoclonal antibodies (*Total Pim-1*). The other tube (400 μg protein) was immunoprecipitated with anti-ubiquitin polyclonal antibodies. Total cellular ubiquitylated proteins were eluted from the beads and resuspended in immunoprecipitation buffer and then immunoprecipitated with anti-Pim-1 monoclonal antibodies to obtain only ubiquitylated Pim-1 (*Ub-Pim-1*). The ubiquitin-depleted supernatant was immunoprecipitated with anti-Pim-1 monoclonal antibodies (*Ub-dep-Pim-1*). Following separation by 9% SDS-PAGE, Western blotting was done with anti-Pim-1, anti-Hsp90, and anti-Hsp70 antibodies. **A** and **B.** Blots representative of at least three independent experiments.

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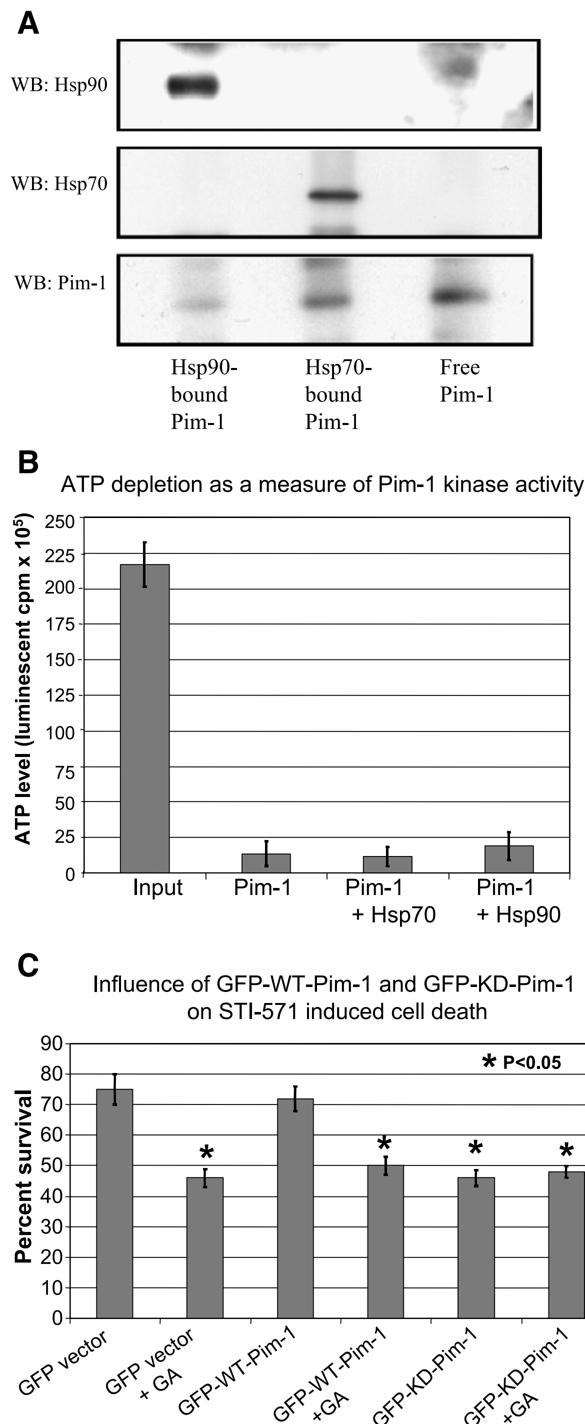
be degraded lysosomally when necessary. In this work, lysosomal inhibitors do not cause a substantial buildup of cellular ubiquitylated proteins, nor do Pim-1 levels accumulate in the presence of such inhibitors. We believe that although there may be cellular conditions that would result in lysosomal degradation of Pim-1 the ubiquitin-proteasome pathway, in most situations, is the main route for destruction of Pim-1 in tumor cells.



Because some proteins may be targeted to the proteasome without being ubiquitylated, as observed by the binding of ornithine decarboxylase to antizyme (45), it was necessary to show that Pim-1 can be ubiquitylated *in vitro* using recombinant GST-Pim-1 and exogenous ubiquitin. As both ubiquitylation and degradation by the proteasome are ATP-dependent processes, it is necessary to add ATP or an analogue to the *in vitro* system. In this case, we used AMP-PNP instead of ATP, because this ATP analogue is β,γ nonhydrolyzable. The activation of ubiquitin moieties, causing a release of AMP and PPi, still occurs, but proteolysis, requiring β,γ cleavage, cannot (33). We show that Pim-1 is extensively ubiquitylated *in vitro* (Fig. 3A) and *in vivo* (Fig. 3B). Clear ubiquitin laddering patterns suggest that the protein is conjugated to ubiquitin chains of varying lengths at one or more discrete locations. A streak or smear of ubiquitin may indicate that the protein is conjugated to both monoubiquitins and polyubiquitins at many locations (34). The immunoprecipitations shown provide the insight that this may be the case for Pim-1.

The data showing that Pim-1 protein levels increase with heat shock treatment (Fig. 1A) were surprising, as total cellular protein degradation increases in this situation (46), whereas translation, in general, decreases (47). The current study shows that the half-life of Pim-1 also increases with heat shock (Figs. 1B and 3A), which led us to investigate the potential protection of Pim-1 by molecular chaperones. Hsp70 and Hsp90 are housekeeping proteins and are not necessarily induced by heat shock but are present in cells ubiquitously (27). However, they are found to bind to client proteins under cellular stresses, such as heat shock (48), so these were good candidates for the protection of Pim-1. Treatment of cells with geldanamycin before heat shock prevented the accumulation of Pim-1 protein (Figs. 1B and 3C), providing further evidence for the involvement of Hsp90 as the protector.

Pim-1 was found to associate with Hsp70 as well as with Hsp90. Both of these chaperones have sometimes been found to associate with the proteasome through adaptor proteins acting as E3 ubiquitin ligases, such as CHIP and Bag-1 (49, 50), and thus may actually shuttle substrates to the degradation machinery. Under the circumstances tested, however, this is not the case for Hsp90, as the half-life of Pim-1 is longer when in association with Hsp90, and Pim-1 is not ubiquitylated when bound to this chaperone. The normal half-life of Pim-1 in the

FIGURE 7. Pim-1 kinase remains active when bound to Hsp70 or Hsp90 and acts as a survival factor. **A.** Hsp90, Hsp70, and Pim-1 were serially immunoprecipitated from the same cell lysate (800 μg protein), run on 9% SDS-PAGE, and immunoblotted for all three proteins. **B.** Recombinant Pim-1 was bound to an excess of Hsp70 or Hsp90. Free and bound Pim-1 were precipitated and incubated with 0.1 μmol/L ATP and 400 μmol/L p21 peptide for 5 minutes at 30°C and measured for luminescent counts per minute. A drop in remaining ATP levels (*Y axis*) indicates the use of ATP in kinase activity. Columns, mean of three experiments; bars, SD. **C.** K562 cells were transfected with vectors encoding GFP, wild-type GFP-Pim-1, or kinase-dead GFP-Pim-1. Half of each was treated with geldanamycin and half with DMSO vehicle and all cells with 10 μmol/L STI-571 for 3 hours. Propidium iodide was added 1.5 hours before flow cytometry. Cells were gated on those displaying green fluorescence and measured for red fluorescence. Exclusion of propidium iodide was considered indicative of living cells. Columns, mean of three experiments; bars, SD. *, $P < 0.05$, statistically significant difference from control.

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tumor cell lines tested is 1.7 hours long compared with the 10 to 15 minutes of earlier reports in primary cells (11) yet short enough to suggest that Pim-1 can exist in the free, unprotected form in untreated cells. Thus, Pim-1 is eventually degraded even when Hsp90 is available and thus either has been naturally released from the chaperone or is shuttled to the proteasome in the presence of Hsp90. We find the former situation to be more likely for Pim-1, as the ubiquitylated forms of Pim-1 do associate with Hsp70 instead of Hsp90, even when geldanamycin has not been added to force the release of Hsp90 from its client proteins.

Some proteins, such as nuclear hormone receptors, have been shown to bind Hsp70 and Hsp90 at the same time (51). Whether Pim-1 may bind both Hsps at once is not clear at present. Experiments showing the coimmunoprecipitation of Pim-1 with Hsp70 and Hsp90 cannot rule out that an individual Pim-1 protein may bind to both chaperones simultaneously. This complex would most likely be transient, given the different functions of Hsp70 and Hsp90 under the circumstances tested.

That Pim-1 is degraded by the ubiquitin-proteasome pathway is an important finding. Many proteins involved in cell cycle regulation, signal transduction, survival, and apoptosis are short lived and regulated through concerted degradation. Binding of Pim-1 to Hsp70 occurs primarily when Pim-1 is conjugated to ubiquitin, suggesting that Pim-1 degradation may be regulated by Hsp70 and associated E3 ubiquitin ligases. This indicates that the role of Hsp70 in this case is opposed to that of Hsp90. The continuous flux of Pim-1 between Hsp90 and Hsp70 could explain in part how its cellular availability is controlled, which is of critical importance considering that it is constitutively active (14). One possible strategy for inducing apoptosis in cancer cells would be to reduce the expression of survival factors like Pim-1. Pim-1 may thus be targeted for degradation by treatments that induce its binding to Hsp70 and its ubiquitylation. Furthermore, geldanamycin has been shown to bind the Hsp90 in tumor cell lines like K562 with a much stronger affinity than the Hsp90 in normal cells (30). A simple disruption of Pim-1 binding to Hsp90, as with geldanamycin or its analogues, may be useful in rapidly reducing the levels of Pim-1 in tumors, thereby contributing to apoptosis. To our knowledge, this work is the first that delineates a specific post-translational control of Pim-1, which is important information regarding the potential targeting of Pim-1 for degradation in tumor cells.

Materials and Methods

Cell Culture

K562 and BV173 cells were purchased from American Type Culture Collection (Manassas, VA). K562 and BV173 cell lines are Philadelphia chromosome–positive human chronic myelogenous leukemias. They were grown in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% newborn calf serum (Sigma, St. Louis, MO), 2 mmol/L L-glutamine, and 100 units/mL streptomycin and penicillin at 37°C in a humidified atmosphere with 5% CO_2. Suspension cultures were maintained at a density of 5×10^5 to 8×10^5 cells and split at a 1:4 dilution every 3 days to ensure active cycling of cells. Cell viability was checked using trypan blue exclusion during cell growth. Normal lymphocytes were isolated from the spleen of a male Fischer-344 rat. A cell suspension was created with a mesh screen and then centrifuged on a Ficoll gradient according to the manufacturer's protocol. Cells were taken from the interface and washed in PBS.

Chemical Treatments

Geldanamycin was purchased from AG Scientific (San Diego, CA) and dissolved in DMSO. For cell treatment, it was used at 2 μmol/L (29). PS-341 was obtained from Millennium Pharmaceuticals (Cambridge, MA) and dissolved in DMSO and used at 1 μmol/L (27). STI-571 was obtained from Novartis (Basel, Switzerland) and dissolved in water for use at a concentration of 10 μmol/L. Lysosomal inhibitors chloroquine (used at 100 μmol/L) and ammonium chloride (used at 2.5 mmol/L; ref. 29) were obtained from Sigma and dissolved in cell culture medium.

Whole Cell Lysate Analysis

Cells were washed in PBS and lysed in ice-cold radioimmunoprecipitation assay buffer [137 mmol/L NaCl, 25 mmol/L Tris-HCl (pH 8), 2 mmol/L EDTA, 10% glycerol, 1% Igepal, 0.5% deoxycholate, 1× protease inhibitor cocktail (Calbiochem, La Jolla, CA)]. Cells were allowed to lyse briefly on ice before sonication. The resulting preparation was a mixture of cytoplasmic, nuclear, and membrane fractions. Insoluble pellets were removed by centrifugation. Protein concentration was measured by absorbance in a Bradford assay (Bio-Rad, Hercules, CA) at wavelength 595 nm on an Ultraspec 2000 (Pharmacia, New York, NY) spectrophotometer. Laemmli loading buffer was added to the samples and boiled for 10 minutes. Equal amounts of protein (30 μg) were loaded onto SDS-PAGE gels. Densitometric analysis was done on Western blot films using a Bio-Rad Fluor-S MultiImager and the Quantity-One software.

Immunoprecipitations

PBS-washed cells were lysed in immunoprecipitation lysis buffer [25 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1% Igepal, 1× protease inhibitor cocktail], vortexed, and incubated on ice for 20 minutes. Lysates were cleared by centrifugation at 12,000 rpm for 10 minutes at 4°C. The resulting supernatants were removed to fresh tubes, and proteins (400 μg) were precipitated for 1 to 2 hours with the appropriate antibodies at 4°C with rotation. Protein A/G-agarose (Boehringer Mannheim, Indianapolis, IN) was added and lysates were rotated for 1 additional hour. Precipitated complexes were washed four times in immunoprecipitation buffer, boiled in Laemmli buffer, and run on SDS-PAGE.

Protein Half-life Determination

Methods were adapted from the Wang et al. (31) and Anwar et al. (32) studies. In brief, cycloheximide was added at a concentration of 30 μmol/L to cells in RPMI 1640. Aliquots of equal cell number were taken at various time points post-treatment. Proteins were prepared for SDS-PAGE and immunoblotting as described.

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Ubiquitylation Assays

Cells were lysed in cold ubiquitylation buffer [50 mmol/L Tris-HCl (pH 8), 5 mmol/L $MgCl_2$, 5 mmol/L $CaCl_2$, 2 mmol/L DTT, 5 mmol/L *N*-ethylmaleimide]. Cleared lysates were aliquoted into tubes, and assay components GST-Pim-1, 10 μg ubiquitin (Sigma), and 4 mmol/L AMP-PNP (Sigma) were added as indicated. Mixtures were incubated at 37°C for 30 minutes, and GST-Pim-1 was precipitated by the addition of glutathione-Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ) for 1 hour at 4°C. Beads were washed four times in ubiquitylation buffer and samples were subjected to SDS-PAGE.

Immunoblots

Protein was transferred from SDS-PAGE gels to polyvinylidene difluoride membrane (Millipore, Bedford, MA) with a semidry blotter. Membranes were blocked in 5% nonfat dry milk in PBS containing 0.1% Tween 20. Primary Pim-1 antibodies used were our laboratory's polyclonal to GST-Pim-1 and anti-Pim-1 monoclonal antibody. Other primary antibodies include a rat monoclonal anti-Hsp90 and polyclonal anti-Hsp70 from Stressgen (Victoria, BC, Canada) and a monoclonal anti-actin and polyclonal anti-ubiquitin from Sigma. All antibodies recognize human proteins. The use of antibodies from different species aids in the reduction of cross-reactivity on Western blots of immunoprecipitated samples. Membranes were incubated for 2 hours in PBS-Tween 20 containing primary antibody, washed extensively in PBS/Tween 20/milk, and then incubated for 1 hour in a secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL). Membranes were washed again, with the last wash in plain PBS to reduce background from Tween 20. Proteins were detected with the Pierce Pico West chemiluminescence kit and Hyperfilm (Amersham Biosciences).

Transfection by Electroporation

Suspension cells in logarithmic growth were collected by centrifugation and resuspended at 4×10^7 cells/mL in serum-free RPMI 1640. Cells (300 μL) were added to a 4 mm cuvette and mixed with 20 μg plasmid DNA. Electroporation was done at 950 μF and 200 V with a time constant of 21 to 29 ms. Cells were incubated on ice for 20 minutes, expanded into 10 mL RPMI 1640 with serum, and analyzed after 24 to 48 hours.

Flow Cytometric Measurement of Cell Survival

Cells were transfected with constructs that produced a protein in which GFP is fused to the COOH terminus of Pim-1: *pEGFP-wild-type pim-1*, *pEGFP-kinase-dead pim-1*, or *pEGFP* vector. Cells were drug treated and stained with propidium iodide before flow cytometric analysis. Only cells that fell within the gates for green were examined. Uptake of propidium iodide was measured, with exclusion as a characteristic of live cells.

Luminescent ATP-Based Assays for Kinase Activity

Recombinant Pim-1 (0.25 μg) was incubated in binding buffer [10 mmol/L HEPES (pH 7.4), 100 mmol/L KCl, 5 mmol/L DTT, 20 mmol/L Na_2MoO_4, 50 mmol/L ATP] alone or with a 5-fold excess of recombinant Hsp70 or Hsp90 (Stressgen) for 20 minutes at 30°C. Pim-1 was immunoprecipitated with monoclonal antibodies. Immobilized Pim-1-containing complexes were washed four times in kinase buffer without ATP (25 mmol/L HEPES, 10 mmol/L $MgCl_2$, 0.5 μg/mL DTT) and incubated with 0.1 μmol/L ATP and 400 μmol/L p21 peptide (RKRRQTS) in kinase buffer for 5 minutes at 30°C before addition of Kinase-Glo reagent (Promega, Madison, WI) at 1:1 (v/v). The mixture was removed from protein A-agarose beads and added to the wells of a Nunc (Rochester, NY) 384-well plate, and ATP levels were counted on a Wallac (Wellesley, MA) Victor-2 plate reader. Kinase-Glo binds to remaining ATP, so a drop in counts per minute indicates kinase activity.

Statistical Analysis

The statistical significance between the means of the unpaired values was determined by Student's *t* test. Results were considered significant if $P < 0.05$. Statistical analysis was done by using GraphPad software.

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