THE PROTO-ONCOGENE PIM-1 KINASE: ITS POST-TRANSLATIONAL REGULATION AND ITS ROLE IN CELL SURVIVAL VIA A MITOCHONDRIAL PATHWAY

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A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
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DECEMBER 2003
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Chair

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ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Nancy Magnuson, for giving me the opportunity and support to work in her laboratory during my graduate career. Without her guidance, ideas, and teaching I would not have been able to earn this degree. My committee members also deserve appreciation for their ideas and feedback: Drs. Ray Reeves, Diana Stone, Kwan Hee Kim, and Howard Hosick. They provided me with many insights that have proved valuable to my research. The past and present members of the Magnuson laboratory have helped me to learn new techniques and have given suggestions that improved my work. I deeply appreciate the camaraderie of the entire lab. I wish especially to thank the new Dr. Matthew Weaver for being my “idea guy” during graduate school, as well as for the kindness he and his wife Eleni have shown me over the years. To my family I owe gratitude for never doubting what I might accomplish, or else I do not know whether I would made a career of doing what I love. Last, I thank my husband, Steve, for his support, for his flexibility regarding a scientist’s schedule, and for a seemingly endless tolerance for listening to me talk about my data.
THE PROTO-ONCOGENE PIM-1: ITS POST-TRANSITIONAL REGULATION AND ITS ROLE IN CELL SURVIVAL VIA A MITOCHONDRIAL PATHWAY

Abstract

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December 2003

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The serine/threonine kinase Pim-1 has been tied to cell survival and prevention of apoptosis. Part of its role in cancer may therefore be allowing malignant cells to evade pro-apoptotic signals and continue to survive. The studies herein examined the post-translational regulation of Pim-1 through degradation, as well as one mechanism by which Pim-1 may promote survival – the phosphorylation and inactivation of the pro-apoptotic protein Bad. We found that the expression of Pim-1 protein increases with heat shock, and that wild type Pim-1 can prevent apoptosis in human cell lines during heat shock, an apoptosis-inducing treatment. This effect is not seen when kinase dead Pim-1 is expressed. This evidence demonstrates that Pim-1 may be responsible in part for preventing the normal process of apoptosis in cancer cells treated by heat shock. Further study examined how Pim-1 is protected and targeted for degradation by heat shock proteins (Hsp) and the ubiquitin-proteasome pathway in human leukemic tumor cells. We found that the half-life of Pim-1 protein increases with heat shock or by treating the cells
with the proteasome inhibitor PS341. It was determined that Pim-1 is degraded by the ubiquitin-proteasome pathway, and co-immunoprecipitation showed that Hsp70 is associated with Pim-1 under these circumstances. Conversely, Hsp90 was found to protect Pim-1 from proteasomal degradation. A kinase assay demonstrated that unbound Pim-1 kinase remains active, so the heat shock chaperones are not absolutely required for Pim-1 activity. The pro-apoptotic protein Bad (Bcl-2 agonist of death) was found to be a substrate that is affected by Pim-1 kinase activity. Phosphorylation of serine 155 on Bad by Pim-1 was found to prevent Bad from binding to the anti-apoptotic protein Bcl-xL at the mitochondria. Cells expressing wild type Pim-1 in alone or in combination with GST-Bad had high survival rates, while cells expressing kinase dead Pim-1 displayed more apoptosis, particularly in conjunction with GST-Bad expression. These findings tie the previously documented survival activity of Pim-1 kinase to a mitochondrial pathway of apoptosis through phosphorylation of Bad. Thus, the post-translational regulation of Pim-1 participates in control of its cellular levels, which in turn allows for its availability to promote cell survival by prevention of apoptosis.
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CHAPTER ONE

INTRODUCTION
**Discovery of Pim-1**

The proto-oncogene *pim-1* is on pter-q12 of human chromosome 6 (1) and 16.4 of mouse chromosome 17 (2). In 1984, Anton Berns *et al.* (Netherlands Cancer Institute) infected mice with the Moloney Murine Leukemia Virus, which preferentially integrated into this gene in T cells; thus it was named *pim-1* for preferential integration MMLV (3). Human and mouse Pim-1 have 94% homology at the amino acid level (4). Genes designated *pim-2* and *pim-3* have now been found, with 58% and 72% homology to murine *pim-1*, respectively (www.ch.embnet.org/software/LALIGN_form.html). Loss of *pim-1* may be compensated for by *pim-2* (5), however, there is little experimental data on the functions of Pim-2 and Pim-3 to date. A recent paper by Fox *et al.* (6) shows that Pim-2 may contribute to cell survival, making it similar to Pim-1. Whether these proteins phosphorylate all of the same substrates is unknown.

The integration of MMLV into the murine *pim-1* gene occurred primarily in the 3´ UTR, inserting a premature stop codon in front of a destabilizing A/U-rich motif. The result of this was an unusually long-lived *pim-1* transcript (7). The *pim-1* mRNA normally has a half-life of about 140 min., and the loss of the A/U-rich motif increases it to over 3.5 h (8).

Studies in transgenic mice have shown that *pim-1* is a weak oncogene, causing lymphomas in about 10% of the mice (3). When expressed in conjunction with the oncogene c-*myc*, 100% of mice die of lymphomas *in utero* (9, 10). Thus, Pim-1 may not be a transforming factor in itself, but demonstrates synergy with other
proteins, particularly when the functions are complementary, as in the overexpression of both proliferative and anti-apoptotic factors.

Knockout of *pim-1* in mice is not lethal (11). However, one must keep in mind that in this system, cells in early embryonic stages that retain totipotency may allow the selection and survival of stronger cells or those with mutations, and thus it is possible that not all effects of gene knockout are observable. It is likely in the case of Pim-1, though, that there are compensatory kinases that may act as back-ups, phosphorylating the natural targets of Pim-1 and allowing the cells to function normally. In fact, it is difficult to imagine a scenario in which advanced, multicellular organisms could evolve without such back-ups. Candidate compensatory kinases include Pim-2 and Pim-3, or unrelated proteins Protein Kinase A (PKA) and Protein Kinase B (PKB/Akt), both of which are discussed in Chapter Four. Protein Kinase C also phosphorylates some of the same substrates as Pim-1, PKA, and PKB/Akt. Although there are many protein kinases that share preferred phosphorylation consensus sequences with one another, their modes of regulation are dissimilar, they may be differently expressed in various cell types, and they are often activated by separate signal transduction pathways.

Pim-1 knockout mice show one striking characteristic: erythrocyte microcytosis (11). Abnormally small red blood cells may indicate that Pim-1 has something to do with the development of the hematopoietic system, and regulation of the cell cycle in these cells, suggesting that the signal to go through mitosis may supercede the need to pause for growth signals and the doubling of the cytoplasmic volume. Pim-1’s role as a kinase in this case seems to be one of temporal regulation,
stemming from its ability to phosphorylate appropriate cell cycle-related targets. Pim-1 does not appear to be the major driving force behind cell cycle progression. Rather, the emerging picture of Pim-1 in the cell cycle is its regulation of events through phosphorylation of proteins such as Cdc25A phosphatase (12) and p21 (13). In further support of a role for Pim-1 in the cell cycle, a paper was published by Bhattacharya et al. in 2003 (14) that indicates NuMa (nuclear mitotic apparatus protein) is a substrate for phosphorylation by Pim-1. NuMa binds to the mitotic spindle apparatus and aids in chromosome separation. Thus, a possible role for Pim-1 in cell cycle control has been indicated.

A second effect of pim-1 knockout is a lack of responsiveness to IL-3 (15). Interleukin-3 is a cytokine that, after engaging its receptor, induces a signal transduction cascade resulting in cell proliferation and/or survival. Murine myeloid cells in culture that are dependent upon IL-3 will die if it is withdrawn. One consequence of IL-3 treatment is an immediate upregulation of pim-1 transcription and translation (16). These effects indicate that Pim-1 may act as a survival factor, as it is IL-3 responsive, and a decrease in Pim-1 after IL-3 withdrawal causes cell death. Indeed, Lilly et al. (17) have confirmed that forced expression of Pim-1 helps factor-dependent cells survive in the absence of IL-3.

**Cell survival factors and the role of Pim-1**

On the cellular level, two main factors contribute to the formation of tumors: uncontrolled proliferation, and failure of the cells to go through normal apoptosis. At one time, it was thought that a defining characteristic of cancerous cells was
hyperproliferation, but recent evidence shows that some cancers are slow-growing, and thus resistant to therapies that rely on the killing of actively dividing cells. This highlights the importance of anti-apoptotic pathways as contributors to tumor development. Cancerous cells that divide only at a normal rate, but fail to undergo any significant degree of apoptosis, may be dependent upon survival proteins.

An example of a cancer with decreased apoptosis is chronic myelogenous leukemia, in which cells of myeloid lineage do not undergo normal differentiation. While differentiated cells have a limited lifespan, the undifferentiated ones are less likely to undergo apoptosis and are in the body for an extended time. Being undifferentiated, they may also continue to divide. The major contributor to this phenotype is a fusion protein called Bcr-Abl (see below). This constitutively active tyrosine kinase phosphorylates a host of proteins involved in cell survival that may otherwise be only intermittently phosphorylated in healthy cells.

Survival factors counter apoptosis in several ways. Bcl-2 and Bcl-xL proteins are survival factors because they regulate mitochondrial-induced apoptosis through the binding of Bax, Bad, and other pro-death proteins. Neither protein causes hyperproliferation (18,19). Instead, overexpression of Bcl-2 or Bcl-xL allows cells to survive insults that would kill their normal counterparts. This suggests that there is a threshold for apoptosis, so that an increase in the availability of pro-death Bcl-2 family proteins like Bad can cause apoptosis only if they displace enough Bax (by binding Bcl-2 and Bcl-xL) to induce sufficient cytochrome c release. A threshold effect may be the reason why cells express lower levels of Bad than they do Bcl-2 or Bcl-xL.
Pim-1 kinase has been shown to cause survival of cells under many circumstances. In our lab, we have found that expression of wild type Pim-1 (often as GFP-Pim-1) allows a greater percentage of cells to survive than those expressing kinase dead Pim-1. Chapter Two shows a figure (page 47) of the flow cytometric analysis of GFP-Pim-1 expressing cells in which heat shock has been used as an inducer of stress and apoptosis. Cells with wild type Pim-1 have a much better rate of survival than those with kinase dead Pim-1, indicating that Pim-1 is a contributor to anti-apoptosis during heat shock. The kinase dead Pim-1 is believed to act as a dominant negative protein, binding to target substrates without phosphorylating them, as well as having much slower kinetics of release than the wild type Pim-1 would. Therefore, substrates are not available for phosphorylation by other, compensatory kinases such as Akt.

The role of Pim-1 as a survival factor must have one or more underlying mechanisms, and our lab has demonstrated that one of these is the phosphorylation of p21cip-1/waf-1 by Pim-1 (13). The cyclin dependent kinase (Cdk) inhibitor p21 may contribute to cell survival in two ways. First, it binds to Cdns in a complex with cyclins at the G1/S phase of the cell cycle and prevents phosphorylation of target proteins that promote entry into S phase (DNA synthesis) (20). This arrests cell division and allows a cell the time to make repairs, synthesize new proteins, and perhaps to avoid apoptosis. The second way that p21 acts is through the binding of procaspase 3. When p21 becomes phosphorylated (as by Pim-1), it is able to form an association with procaspase 3, shielding it from cleavage-mediated activation (21).
As procaspase 3 is a major regulator of the intrinsic pathway of apoptosis, this association is a potent cell protector.

A second mechanism for Pim-1’s role in cell survival is through the phosphorylation of Bad. As discussed below, the phosphorylation of Bad prevents it from exerting its pro-apoptotic effect. Chapter Four shows data demonstrating that phosphorylation of Bad on serine 155 by Pim-1 prevents its binding to Bcl-xL and allows cell survival.

Previous studies that support a role for Pim-1 in cell survival have not always named a specific mechanism, but have provided the framework for current thought in this area. Pircher *et al.* (22) demonstrated that exogenously expressed Pim-1 prevented genotoxin-induced death of FDCW2 cells, while Krumenacher *et al.* (23) showed that the prolactin-mediated prevention of dexamethosone-induced apoptosis caused upregulation of Pim-1 and Bcl-2. This is in agreement with the report that cultured lymph node cells from mice of an *lpr/lpr* and *Eμ-pim-1* transgenic cross fail to undergo apoptosis in response to dexamethosone (24). In contrast, Pim-1 expression has been found to cooperate with c-Myc in apoptosis of Rat-1 fibroblasts (12). Clearly, the role of Pim-1 in cell survival is dependent upon the co-expression of other oncogenes as well as on the cell lineage being tested. Further substrates for Pim-1 phosphorylation will undoubtedly be discovered. It would not be surprising to find that many of these have a role in cell survival, as it has already been established that Pim-1 prevents apoptosis induced by different mechanisms.
The ubiquitin-proteasome pathway of protein degradation

The major route for ridding cells of misfolded or denatured proteins is through tagging with ubiquitin and proteolysis by the 26S proteasome. In the last 10 years, attention has increasingly focused upon the cell’s use of this system for the degradation of normal, “healthy” proteins whose presence is no longer necessary. Thus, proteins that are repressing proliferation, apoptosis, or other activities may be quickly disposed of.

The 26S proteasome consists of a 20S unit and two 19S units that form a barrel-like structure (25) with chymotryptic, tryptic, and peptidylglutamyl proteolytic activities (26). Proteins destined for degradation are unfolded and threaded into the proteasome for cleavage. Specificity of target proteins is conferred by conjugation to ubiquitin on internal lysine residues, which may occur at one or more sites in the protein (27). Typically, a long chain of ubiquitin molecules forms, but monoubiquitylation has also been observed. Monoubiquitylation is not associated with degradation, suggesting that this is a post-translational modification that, like acetylation and methylation, directs other activities. A 5S subunit of the 19S unit is responsible for the recognition of polyubiquitin chains.

Many inhibitors of the 26S proteasome have been tested, but the most specific so far is PS341 from Millennium Pharmaceuticals (Cambridge, Ma). This molecule contains a boron atom that interacts with the catalytic threonine residue of the proteasome that is essential for its activity (28). In Chapter Three, PS341 is used to show that Pim-1 is a substrate for degradation by the 26S proteasome. Ubiquitylation of Pim-1 still occurs in the presence of the drug, but degradation does
not. In fact, the build-up of ubiquitin-conjugated Pim-1 helps in its visualization on a Western blot.

Like many other proteins involved in cell cycle regulation or survival/apoptosis, Pim-1 appears to be a substrate for the ubiquitin-proteasome pathway of degradation. Finding a way to enhance the ubiquitylation of Pim-1 may increase its turnover, and thus be a valuable tool for inducing apoptosis in tumor cells.

**Heat shock and protein degradation**

Although global translation decreases in cells exposed to mild hyperthermia (29), a small number of proteins are actually upregulated. These include a family of chaperones and their adaptors, collectively known as heat shock proteins (or heat shock cognates) and heat shock-associated proteins. Many heat shock proteins (Hsps) are expressed constitutively in cells, but are further induced during hyperthermia. Other Hsps require heat shock for expression and activity. Two constitutively expressed Hsps, Hsp90 and Hsp70, have been shown to be involved in regulating the stability of cellular proteins and in shuttling them to the 26S proteasome for degradation (30).

Pim-1 is another protein that appears to be upregulated with heat shock. In Chapters Two and Three, it is shown that Pim-1 protein levels increase with heat shock, and that this is due, at least in part, to increased stability. Through the use of geldanamycin, a specific Hsp90 inhibitor, we found that Pim-1 owes its increased stability under hyperthermic conditions to Hsp90. In fact, Hsp90 can be found
bound to Pim-1 even under normal conditions. Hsp90 binds its client proteins for the purpose of protection or refolding, but has occasionally been seen to be involved in protein degradation (30). In the case of Pim-1, Hsp70 plays this role. Ubiquitylated Pim-1 is bound to Hsp70, while unubiquitylated Pim-1 (from immunodepleted fractions) was shown to be associated with Hsp90. Whether there is a brief period of exchange of Pim-1 between the two chaperones remains to be seen. Hsps 70 and 90 are known to associate with one another in many circumstances, so it is difficult to prove through co-immunoprecipitation that Pim-1 is bound to both chaperones simultaneously, as Hsp70 could be binding to Hsp90 independent of a client protein go-between.

New evidence suggests that the Hsp70 adaptor protein, CHIP-1, can act as an E3 ubiquitin ligase (31), so perhaps it is not surprising that Hsp70 is associated with Pim-1 that is targeted for degradation. It may be that the Hsp70 complex itself is catalyzing the conjugation of ubiquitin moieties to Pim-1. Further investigation may show whether there is a specific biochemical change in Pim-1 that marks it for ubiquitin tagging, or whether its degradation is simply a matter of cellular context. For example, I-κB, the inhibitor of NF-κB, is degraded by the ubiquitin-proteasome pathway when it becomes phosphorylated through the appropriate signal transduction pathways (32). This frees NF-κB to promote transcription of particular genes. Similar post-translational modifications may act as ubiquitylation signals for other proteins as well.
Apoptosis and the mitochondrion

The phenomenon of normal cell death during development of an organism has been recognized for over a hundred years, but it was not until the 1960s that more powerful modern microscopy allowed a distinction to be drawn between necrosis and cells specifically programmed for death (33). In the early 1970s, the term apoptosis was proposed, as several scientists studying different organisms realized that they were seeing the same features of cell death, and that an inclusive designation was needed. Typical manifestations of apoptosis are those that show destruction from within, and do not cause inflammation and immune response. During necrosis, macrophages are recruited to the site, where they engulf pieces of dead cells. In tissue culture, where an immune response would not be possible, apoptosis can be recognized biochemically, with fragmentation of the DNA, emergence of phosphatidylserine on the surface of the plasma membrane, activation of protein-cleaving caspases, and the condensation of the cells into small bodies that are phagocytized by nearby living cells.

Work performed in Caenorhabditis elegans was at the forefront of apoptosis research until mammalian studies took the lead with the discovery of Bcl-2 (34), the worm homolog CED-9 not being cloned until several years later (35). Less than 20 years after the cloning of Bcl-2, it is now known that there exists a family of Bcl-2-related proteins whose job it is to regulate the apoptotic process. Pro-survival factors Bcl-2 and Bcl-xL are inserted into the outer mitochondrial membrane via C-terminal hydrophobic regions, where they interact with pro-apoptotic proteins such as Bax (36). By sequestering Bax, Bcl-2 and Bcl-xL prevent its homodimerization, wherein
it uses its own C-terminal hydrophobic region to open pores in the mitochondrial membrane (37). Typically, membrane potential is lost during this process. Cytochrome c is thought to exit through the pores, because it rapidly accumulates outside the mitochondria in a complex with Apaf-1 and procaspase 9. As a result, procaspase 9 is cleaved into its active form, and proceeds to activate other caspases such as 3 and 7. Caspases (cysteine aspartate proteases) are activated by cleavage from the inactive, “pro” form, which allows them to cleave other cellular proteins, promoting apoptosis through the loss of survival factors and structural proteins.

In Chaper Four, a mechanism of action for Pim-1 in anti-apoptosis is described. The pro-apoptotic protein Bad, which has homology to Bcl-2 in what is termed the BH-3 (Bcl-2 Homology 3) region, has in its sequence three putative phosphorylation consensus sites for Pim-1. Serine 155, one of the possible sites, is in Bad’s BH-3 region and therefore regulates its interaction with Bcl-xL (38). Similar to Bax, Bad can bind Bcl-xL, and to a lesser extent, Bcl-2. However, Bad lacks the other three BH domains, and has no C-terminal hydrophobic region. Thus, Bad does not insert into the mitochondrial membrane, and is not able to open pores in it. Rather, Bad promotes apoptosis by displacement of Bax on Bcl-xL (39), releasing it for homodimerization, and Bax opens mitochondrial pores. When Bad is phosphorylated on Ser155, it cannot bind to Bcl-xL. Furthermore, phosphorylation of Bad on Ser112 and Ser136 allow it to bind 14-3-3 structural proteins in the cytoplasm (40), sequestering it away from the mitochondria. This means that there are at least three checks on Bad that would have to be circumvented in order for it to exert its pro-apoptotic effect. It has been known for several years that Akt (PKB)
and PKA can phosphorylate Bad. Akt acts primarily on Ser136, and somewhat on Ser112 (41, 42). PKA acts primarily on Ser112 and Ser155 (43, 44). The role of PKA here seems to be a more thorough one than that of Akt, because its phosphorylation activity would provide a way for Bad to bind to 14-3-3 as well as preventing its association with Bcl-xL.

Pim-1 appears to preferentially phosphorylate serines 112 and 155 as well (Chapter 4). Moreover, Pim-1 does not require activation through specific signal transduction pathways, as do proteins such as PKA and Akt. Thus, the contribution of Pim-1 to cell survival through this mitochondrial pathway may become increasingly important as premalignant cells are isolated from growth factor signals and rely more heavily on constitutively active proteins like Pim-1 for survival. Anti-cancer therapies that target Pim-1 expression or activity may therefore be much more effective than those that are intended for proteins that have already been downregulated in tumor cells.

**Bcr-Abl leukemia cells**

Chronic myelogenous leukemia (CML) is characterized, in over 95% of patients, by a product of chromosomal translocation called Bcr-Abl. A piece of chromosome 22 breaks off at a fragile site and reattaches to chromosome 9. The shortened chromosome 22, identifiable by karyotyping, is called the Philadelphia chromosome. The result of the new connection point on chromosome 9 is a chimeric gene where *bcr* (Breakpoint Cluster Region) is fused to the 5’ end of *abl* (Abelson tyrosine kinase). Normally, the amino-terminus of the *abl* gene product has a
myristate-binding cap that can inactivate the protein by folding over onto the catalytic domain, bringing the Scr-homology-3 domain (SH3) into contact with Abl’s own SH2 domain. Thus, Abl is self-regulating, depending upon myristoylation and the availability of phosphorylated tyrosines in other proteins, which activate its SH2 region (45). The fusion of Bcr to Abl results in a deleted myristoylation cap and causes Abl to be almost constitutively active. Not only can Bcr-Abl not be turned off by normal self-regulation, but the overall kinase activity is much higher than for Abl alone (46). There exists, however, a transient inactive state for the Bcr-Abl fusion protein. The recently developed leukemia drug STI-571 (Gleevec, imatinib) (47) binds to Bcr-Abl (and Abl) in this state and stabilizes the inactive form. Drug-resistant Bcr-Abl tumors are often those that have mutations in the SH-domains of the Abl portion, so that the inactive state is rare and STI-571 has little chance to bind and exert its effect. Two leukemic cell lines with the Bcr-Abl fusion protein are BV173 and K562. Both were used in the experiments in Chapter Three.

The effects of Bcr-Abl have been well-studied, but a full understanding of Pim-1’s contribution to cancer is still elusive. It is evident that Pim-1 may be upregulated (at the transcriptional, translational, and/or post-translational levels) in these and other tumor cells by a number of mechanisms, some of which remain to be discovered. Knowledge of the control of Pim-1 stability and degradation, for instance, may prove valuable in future trials. Identification of substrates for Pim-1, like Bad, could provide a basis for diagnostic testing to determine whether Pim-1-based therapies are indicated.
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*Cancer Res.* 1997 Dec 1;57(23):5348-55.


CHAPTER TWO

PROTO-ONCOGENE PIM-1 PROMOTES SURVIVAL IN TUMOR CELLS FOLLOWING HEAT-INDUCED CELLULAR STRESS

This paper was originally written as a manuscript for submission to the William R. Wiley Research Exposition at Washington State University in 2001. It has been updated in format to be appropriate for use in this thesis. I am the first author on this manuscript. I performed the writing and the majority of the experiments. The flow cytometric experiments were performed by Dr. Matthew S. Weaver. Dr. Zeping Wang made the pEGFP-pim-1 expression vectors. Monoclonal antibodies to Pim-1 were made by Pei-xiang Xing, in the laboratory of Ian F.C. McKenzie (Austin Research Institute, Victoria, Australia).
ABSTRACT

The serine/threonine kinase Pim-1 is found highly expressed in some tumor cells. Pim-1 has been tied to cell survival, and therefore may allow malignant cells to evade pro-apoptotic signals and continue to survive. This study found that the expression of Pim-1 protein increases with heat shock, and that wild type Pim-1 can prevent apoptosis in human cell lines during heat shock, an apoptosis-inducing treatment. This effect is not seen when kinase dead Pim-1 is expressed, as these cells die more rapidly than those containing only wild-type Pim-1. We have found that heat shock also increases the levels of the Pim-1 kinase phosphorylation target p21\textsuperscript{cip1/waf1}. The phosphorylation of p21\textsuperscript{cip1/waf1} has been shown to allow it to interact with procaspase 3 to permit survival and prevent apoptosis. This evidence demonstrates that Pim-1 may be responsible in part for preventing the normal process of apoptosis in cancer cells treated by heat shock. Thus, Pim-1 may be a good target for cancer therapy, because reducing its level in cancer cells would sensitize them to hyperthermia-induced apoptosis.

INTRODUCTION

Lymphocytic and myelogenous leukemias are cancers that start in the bone marrow and lymphatic tissues, and are the leading fatal cancers in people under the
age of 35 (Leukemia and Lymphoma Society). Although over 40 different drugs, plus radiation therapy, are being used to treat these cancers, they rely on the malignant cells being more susceptible to treatment than healthy cells. In the case of leukemias with certain mutations, including a chromosome rearrangement called Bcr-Abl, the malignant cells are very resistant to treatment. Thus, healthy cells will be killed more readily by chemotherapy and radiation therapy than will the cancer cells (1). The therapies are designed to eliminate cells through programmed cell death, a process known as apoptosis. We have chosen heat shock stress as a model because it induces apoptosis in much the same way as many types of chemotherapy and radiation therapy (2). The cells used are immortalized K562 leukemia and Jurkat lymphoma lines. The Pim-1 protein, involved in survival of cells under stress, is readily detected by immunoblot in both of these cell lines, particularly in K562s, which have the Bcr-Abl rearrangement that may cause increased transcription of \textit{pim-1} through Stat5 (3-6). This project hypothesizes that Pim-1 protein prevents apoptosis in cancer cells treated with heat shock, allowing cell survival through phosphorylation of its substrate p21^{cip1/waf1} and p21's subsequent anti-apoptotic activities. Pim-1 may be a good target for cancer therapy, because reducing its level in cancer cells would sensitize them to treatments that induce apoptosis. Pim-1 is normally expressed during the cell division cycle of healthy cells (7). When expressed at inappropriate times or in large amounts in damaged or precancerous cells, Pim-1 can prevent the normal process of apoptosis, allowing malignancy to occur. This project examines how overexpression of Pim-1 can prevent apoptosis in human cell lines during heat shock, a treatment that should induce apoptosis.
Pim-1 protein is found in both the cytoplasm and nuclei of mammalian cells. It has been shown to phosphorylate the cell cycle-related proteins Cdc25A (8), Numa (9), and p21 (10). Pim-1 itself can be phosphorylated, suggesting that it works as part of a signal transduction pathway, helping to mediate events like proliferation, differentiation, or survival during pro-apoptotic stresses. An analysis of the enzyme kinetics shows that Pim-1 phosphorylates p21 more efficiently than any previously reported substrate, including Cdc25A (10). A significant amount of work has shown that multiple forms of cellular stress result in an increase in the expression of p21 protein (11-13). p21-mediated cell cycle arrest occurs in response to damage caused by irradiation, heat shock, and oxidative stresses. The arrest or "stalling" of cells in the Gap 1 (G1) phase of the cell cycle allows them to repair DNA damage before synthesizing new DNA and proceeding to mitosis. p21’s role in cell cycle arrest is controlled on several levels, including reversible phosphorylation, having various Cyclins and Cdks as binding partners, and the shifting localization of the protein between the nuclear and cytoplasmic compartments. p21 is a highly disordered protein (Keith Dunker, personal communication), making it flexible and able to associate with many other proteins. Another role for p21, which also requires different binding partners, is anti-apoptosis. In this capacity, p21 is able to sequester the pro-apoptotic proteins procaspase 3 (14) and ASK-1, preventing them from having their intended effects. During the normal course of apoptosis, a mitochondrially-regulated signal is sent to cleave procaspase-3 into active caspase-3, a protease that degrades vital cellular components (11). Caspase-3 activation is one of the irreversible stages of apoptosis. Under pro-apoptotic conditions, p21 may be
phosphorylated so that it sequesters procaspase-3, preventing its cleavage. The balance between apoptosis and survival is therefore tipped in favor of survival. As an activator of p21, Pim-1 can function as a survival factor.

Withdrawal of interleukin-3 from IL-3-dependent cells induces rapid apoptosis. It has been demonstrated that overexpression of retrovirally transduced wild-type Pim-1 in IL-3-deprived cells prolongs survival, while cells transduced with only the vector undergo apoptosis (15). In lymph node cells from Pim-1 transgenic mice, the forced overexpression of Pim-1 decreases sensitivity to steroid-induced apoptosis (16). These and other studies have shown that Pim-1 is involved in cell survival, but the mechanisms through which it works are only beginning to be discovered.

In this study, we found that Pim-1 protein levels increase with heat shock, which is unusual, as overall protein synthesis decreases under hyperthermic conditions (17). Additionally, p21 levels increase. Pim-1 remains active even after 60 min. heat shock, and continues to co-immunoprecipitate with p21. A kinase assay demonstrates that Pim-1 is still able to phosphorylate p21 peptide at this time. Furthermore, cells transfected with wild type Pim-1 survive heat shock better than cells expressing kinase dead Pim-1. These results indicate that Pim-1 acts as a survival factor during heat shock-induced cellular stress, and that this effect may work through its phosphorylation substrate, p21.
MATERIALS AND METHODS

Cell Culture. Jurkat and K562 cells were purchased from ATCC. K562 cells are a human chronic myelogenous leukemia line. Jurkat cells are human T cell lymphoma line. They were grown in RPMI 1640 medium (GIBCO) containing 10% newborn calf serum (SIGMA), 2mM L-glutamine, and 100U/ml streptomycin and penicillin at 37°C in a humidified atmosphere with 5% CO₂. Cultures were maintained at a density of 5-8 X 10⁵ cells and split at a 2:3 dilution once a day for 2 days to ensure active cycling of cells. Cell viability was checked using Trypan blue exclusion during cell growth and prior to flow cytometry.

Heat shock procedure. Cells were heat shocked 43°C, then either removed at intervals during the process or placed into a 37°C incubator to allow a recovery period of up to 24 hours. For analysis, cells were washed in phosphate-buffered saline and lysed in ice-cold Radioimmunoprecipitation assay (RIPA) buffer: 137 mM NaCl, 25 mM Tris-HCl pH 8, 2 mM EDTA, 10% glycerol, 1% Igepal, 0.5% deoxycholate, plus 1X Protease Inhibitor Cocktail (CALBIOCHEM). Where appropriate, phosphatases were inhibited by addition of 1mM sodium orthovanadate to the lysis buffer. For whole-cell lysates, cells were allowed to lyse on ice before sonication. The resulting preparation was a mixture of cytoplasmic, nuclear, and membrane fractions. Protein concentration was measured by Bradford assay (BIORAD) at wavelength 595 on an Ultraspec 2000 (PHARMACIA) spectrophotometer. Laemmli loading buffer was added to the samples and they were boiled for 10 minutes. Equal amounts of protein were loaded onto 11% SDS-polyacrylamide gels.
**Western blots.** Protein was transferred from SDS polyacrylamide gels to PVDF membrane (MILLIPORE) with a semi-dry blotter. Membranes were blocked in 5% nonfat dry milk in PBS containing 1% Tween-20. Primary antibodies include our laboratory's polyclonal to GST-Pim-1 made in rabbit, a polyclonal to the dephosphorylated N-terminal end of Pim-1 from STRESSGEN, a monoclonal to Pim-1 (P3) from Ian McKenzie (Austin Research Institute, Victoria, AU), a polyclonal to the C-terminal end of p21 from SANTA CRUZ BIOTECHNOLOGY, and a polyclonal antibody to procaspase 3 (Cpp32) from PHARMINGEN. Membranes were incubated for 2 hours in PBS-Tween containing primary antibody, and for one hour in a secondary antibody of goat-anti-rabbit conjugated to horseradish peroxidase (PIERCE). Proteins were detected with the PIERCE Pico West chemiluminescence kit and Hyperfilm (AMERSHAM).

**Immunoprecipitations.** Cells were lysed in Co-ip buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Igepal) with protease inhibitors, and lysates were precleared for 2 hours with preimmune serum and Pansorbin (CALBIOCHEM) at 4°C with rotation. The resulting supernatants were removed to fresh tubes, and proteins were precipitated with antibodies and protein-G-agarose (BOEHRINGER MANNHEIM). Protein complexes were washed, boiled in Laemmlli buffer, and run on SDS-PAGE.

**Protein half-life determination.** Cycloheximide was added at a concentration of 30 µm to cells in RPMI 1640 without serum. Aliquots were taken at various time points post-heat shock. Proteins were prepared for SDS-PAGE and immunoblotting as described above.

**Luminescent kinase assay.** Pim-1 was immunoprecipitated in RIPA from treated cells, then washed extensively in kinase buffer (25 mM HEPES, 10 mM
MgCl₂, 0.5 mM DTT). Pim-1 kinase was incubated with 400 nM p21 peptide [RKRRQTS] and 0.1 mM ATP in kinase buffer for 30 min. at 30°C. An equivalent amount (1:1) of Kinase-Glo reagent (PROMEGA) was added, and the entire volume was transferred to the wells of a white 384-well plate (NUNC) and luminescence was read on a WALLAC VICTOR-2 plate reader. The Kinase-Glo reagent binds to ATP and emits light, so unused ATP is measured in terms of relative light units.

Transfection by electroporation. Cells in logarithmic growth were washed in phosphate buffered saline and resuspended at a density of 1×10⁷ cells/ml in RPMI without serum but containing 10 mM dextrose and 0.1 mM dithiothreitol. Recombinant DNA (10 µg) was added to each 0.4 cm cuvette. Cells were electroporated with a BIORAD GenePulser II at a capacitance of 960 µF and a voltage of 200 with a time constant of 28.0 msec. They were then returned to growth medium for 24 hours before use in experiments. Plasmids used included pEGFP vector, pEGFP-wild type-pim-1, and pEGFP-kinase dead-pim-1 (K67ΔM).

Detection of apoptosis and of caspase-3 activation. For detection of apoptosis by flow cytometry, cells were allowed to take up the dye MitoTracker Red (Molecular Probes) for one hour, a treatment which stains mitochondria with membrane potential. They were then washed in phosphate buffered saline and run through a FACSCALIBUR Flow Cytometer. For detection of caspase-3 activation, cells were allowed to take up the PhiPhiLux dye (ONCOIMMUNIN) for 2 hours, washed in phosphate buffered saline, and run through the flow cytometer. Both stains are detected in the red (FL2) spectrum of fluorescence, and were used in separate experiments.
RESULTS

**Pim-1 protein expression increases following cellular stress by heat shock.** Mild hyperthermia has been shown to decrease global protein translation, as well as increasing proteasome-mediated degradation (17). Therefore, any proteins that remain active and highly expressed under these circumstances may be important to the recovery of the cell from heat shock stress. In order to determine whether Pim-1 protein levels increase during heat shock, K562 and Jurkat cells were incubated for up to 60 min. at 43°C, lysed, subjected to SDS-PAGE, and analyzed by Western blot. As seen in Figure 1, Pim-1 protein levels increased with heat shock in both cell lines tested. During the treatment, Pim-1 protein was induced as quickly as fifteen minutes after the initiation of heat shock. Although heat shock causes cells to eventually arrest in the G1 phase of the cell cycle, the accumulation of Pim-1 was probably not due to cell cycle arrest, because we have observed in cell cycle fractions separated by centrifugal elutriation that Pim-1 levels are relatively low in the G1 phase, and higher in the S and G2 phases (7). Thus, the immediate induction of Pim-1 protein during heat shock suggests that Pim-1 kinase plays a role in the cellular response to heat shock in K562 and Jurkat cells.

**p21 is expressed following cellular stress.** p21 protein has been shown to increase in cells following multiple forms of stress including oxidative, chemical, heat shock, and UV damage. The extent of p21 expression in Jurkat and K562 cells during heat shock was examined by Western blot. A very slight increase in p21 protein following heat shock occurred in Jurkat and K562 cells [Fig. 2], and there did
not appear to be a decrease. These results suggest that p21 continues to be expressed during heat shock, and that some increase in its level may occur.

**Pim-1 retains kinase activity after heat shock.** Mild hyperthermia causes unfolding of some proteins, so it is not reliable to assume that all kinases are still active at this time. To test its activity, Pim-1 was immunoprecipitated from heat shocked and untreated Jurkat and K562 cells and used in a kinase assay with p21 peptide. Figure 3 shows that Pim-1 does indeed retain kinase activity after 60 min. heat shock. Overlapping error bars suggest that there is no significant difference between Pim-1 kinase activity in heat-treated and untreated cells in either cell line.

**Pim-1 and p21 localize to the nucleus during heat shock.** Both p21 and Pim-1 are small enough to readily move between the cytoplasm and nucleus. Both are known to be found throughout the cell, and may localize to specific areas depending upon cellular context. Heat shock is a cellular stress that can affect protein localization, and we wanted to make sure that the locations of p21 and Pim-1 are not mutually exclusive at this time. In order to determine whether the localization of Pim-1 and p21 change during heat shock, and whether those locations are compatible, Jurkat and K562 cells were treated with heat shock and fractionated. The proteins were detected by Western blot. As seen in Figure 4, p21 is both cytoplasmic and nuclear in untreated cells. After heat shock, it appears mostly in the nucleus. Pim-1 is cytoplasmic and nuclear in K562 cells, but mostly nuclear in Jurkat cells. In K562s, Pim-1 appears to be increasingly nuclear with heat shock, whereas with Jurkats, it is already nuclear but levels noticeably increase. As both p21 and Pim-1 are present in the nucleus during heat shock, the potential for their
interaction does exist. Thus, phosphorylation of p21 by Pim-1 may occur under these circumstances.

**Pim-1 associates with p21 during heat shock.** As the localization of p21 and Pim-1 is compatible during heat shock, the two proteins were tested for physical association. Jurkat and K562 cells were heat shocked and p21 was immunoprecipitated. Western blotting was performed with antibody to Pim-1. Pim-1 was seen to increasingly co-immunoprecipitate with p21 in heat shocked Jurkat cells [Fig. 5]. In K562 cells, p21 and Pim-1 appear to associate at some level whether the cells have been heat shocked or not. These data demonstrate that an association, possibly through phosphorylation, occurs between p21 and Pim-1, and that it may be induced by heat shock in Jurkat cells. K562 cells, which express higher levels of Pim-1 constitutively, may experience this event even when not placed under stress.

**Pim-1 protein expression remains high during the recovery period.** After experiencing stresses like heat shock, cells must then either go through apoptosis, or induce survival pathways. It was therefore important to determine whether the increased levels of Pim-1 remain throughout a recovery period of 24 h, or whether Pim-1 is no longer present at this critical time. K562 and Jurkat cells were heat shocked at 43°C for one hour and returned to the 37°C incubator. Aliquots were taken at the indicated times during the recovery period and subjected to SDS-PAGE and Western blotting. As seen in Figure 6, Pim-1 levels continue to increase up to 8 hours in surviving cells, then return to very low levels by 24 hours post-heat shock. Because K562 cells have much higher endogenous levels of Pim-1 protein, less
K562 lysate than Jurkat lysate was loaded onto the gels in order for the Pim-1 from Jurkats to be comparably visible. The continued high expression of Pim-1 for several hours after heat shock-induced cellular stress may indicate that it has a function in allowing recovery or survival.

**Pim-1 protein is stabilized during recovery from heat shock.** The evidence that Pim-1 protein levels remain high during recovery from heat shock prompted us to look at the stability of Pim-1 at this time. Proteins involved in the cell cycle or in control of apoptosis may be quickly translated and then degraded when they are no longer needed, in order to prevent their interference with other cellular processes. To determine whether the increased levels of Pim-1 during heat shock represented stabilized proteins, or whether rapid turnover and fresh translation occurred, we tested the half-life of Pim-1 protein during the period of recovery from heat shock. Cycloheximide was added at a concentration of 30 µm to inhibit new protein synthesis. The half-life of Pim-1 after heat shock was found to be longer than in untreated cells for both cell lines [Fig. 7]. This increase in stability provides some explanation for why Pim-1 levels increase following heat shock.

**Pim-1 activity affects apoptosis induction.** In order to look at Pim-1’s effect on the survival of tumor cells following heat-induced pro-apoptotic stress, we used three plasmid constructs to modify Pim-1 activity levels within the cells. The first was a control plasmid producing only green fluorescent protein (GFP) as a marker, which allowed Pim-1 to remain at its endogenous level. Second, we used a plasmid with *gfp* fused to wild-type *pim-1*. Cells transfected with this plasmid overexpressed the Pim-1 protein with a subsequent increase in Pim-1 function, along
with a fluorescent green tag that permitted the selection of only GFP-Pim-1 expressing cells by flow cytometry. The third plasmid had \textit{gfp} fused to a kinase-dead form of \textit{pim-1}. When expressed in cells, we believe that this fusion protein acts as a dominant negative, lowering the overall Pim-1 activity, because the kinase dead will tightly bind its substrates without phosphorylating them. Constructs were transfected into both Jurkat and K562 cells via electroporation. Cells were then heat shocked for 1 hour at 43°C. They were allowed to recover at 37°C for 23 hours, and allowed to take up MitoTracker Red dye. This dye fluoresces within the red spectrum when there is an established electric potential across the mitochondrial membrane, an indication of a healthy cell. During the early stages of apoptosis, this potential is lost, signaling the beginning of programmed cell death and the loss of fluorescence. Transfected cells were analyzed by flow cytometry. By gating on green fluorescence, only transfected cells, with modified GFP or GFP-Pim-1 activity, were analyzed. These cells were then tested in the red fluorescent spectrum for loss of mitochondrial membrane potential as an indication of apoptosis induced by heat shock. As is seen in Figure 8, a decrease in Pim-1 activity through the use of the kinase dead form of Pim-1 increased cell death in both Jurkat and K562 cells. When wild-type Pim-1 was overexpressed, increased survival was observed in Jurkat cells, which are usually lower than K562 in endogenous Pim-1 protein. There was no effect in K562 cells, a leukemic cell line that already expresses abnormally high levels of endogenous Pim-1. We also found that measuring Mitotracker Red uptake during the recovery period when Pim-1 levels were highest (2-8 h) gave less dramatic results (data not shown), suggesting that the cells may require more time to
either recover or start to go through apoptosis. The enforced expression of wild type or kinase dead Pim-1 is, however, ongoing during the entire 24 hours. These results indicate that Pim-1 activity is acting to increase survival of tumor cells when responding to heat-induced apoptosis.

**Caspase activation is induced by apoptotic stimuli.** Previous research has shown that the activation of caspase-3 is responsible for apoptosis of tumor cells following heat shock (18). To test the activation of caspase-3 in both Jurkat and K562 cells, they were heat shocked for 1 hour at 43°C, then left to recover at 37°C up to 6 hours. These cells were then allowed to take up PhiPhiLux, a fluorescent caspase-3 substrate. When caspase-3 is active in cells, the substrate is cleaved and will become fluorescent. This reaction can then be detected by flow cytometry while looking at the red spectrum of fluorescence. As seen in Figure 9, by 6 hours post heat shock, the beginning of casapase-3 activity is apparent in Jurkat cells. In the K562 cells, which already have high levels of endogenous Pim-1, there is no activation of caspase-3 activity.

**DISCUSSION**

Both Pim-1 and p21 are expressed during heat shock of Jurkat and K562 cells, and Pim-1 levels noticeably increase. Our laboratory has already demonstrated that p21 is a target for phosphorylation by Pim-1 (10). Phosphorylation of p21 is necessary for its anti-apoptotic function in cells, because this allows it to bind and sequester procaspase-3, preventing the formation of the cleavage product caspase-3,
which degrades proteins vital to normal cellular function. As Pim-1 remains kinase-active after 60 min. heat shock, it is still able to phosphorylate available substrates.

It is clear from the co-immunoprecipitation of Pim-1 and p21 that they share a strong interaction in cells. It is of note that they co-immunoprecipitate both before heat shock and after 60 minutes of heat shock in K562 cells, and that this interaction does not necessarily increase as it does in Jurkat cells. The association that was observed between Pim-1 and p21 in Jurkat cells before heat shock showed a minimal interaction compared to that seen after heat shock. These data may indicate that the already high levels of Pim-1 in K562 cells make some degree of its interaction with p21 and other substrates a foregone conclusion, even when the cells are not in a stress-inducing environment. Indeed, such events may contribute to the unusual resistance of K562s to apoptosis. Cells like Jurkats, which express less Pim-1, may actually require pro-apoptotic stress to promote the interaction between Pim-1 and p21.

We have found that Pim-1 protein is stabilized during the recovery period after heat shock, providing an explanation for its accumulation at high levels. There appears to be an immediate response of Pim-1 during the heat shock process, but less so with p21. p21 is known to accumulate during the recovery period from heat shock (13), and we observed that this is also true for Pim-1. Clearly, Pim-1's role in cell survival is not over after the first 60 minutes of heat shock, and may even continue until cells have either recovered from the stress or failed to survive by undergoing apoptosis. Although we can show that Pim-1's half-life lengthens, we have not yet identified the molecular mechanism for stabilization. Typically, protein
degradation increases during recovery from heat shock, so the increased half-life is not due to an overall decrease in proteolysis. A likely hypothesis is that Pim-1 binds to a protein-stabilizing partner, such as heat shock response proteins Hsp90 or Hsp70. Pim-1’s increased half-life does suggest that it is necessary for the survival/recovery process.

The events during the period of recovery following cellular stress are crucial to the survival process. We have shown that altering the activity level of Pim-1 kinase by overexpression of a dominant negative kinase dead Pim-1 (one that can bind its targets but cannot phosphorylate them) has decreased the survival of both Jurkat and K562 cells. Overexpression of additional Pim-1 protein provides increased survival rates in Jurkat cells, but not in K562 cells. The leukemic K562 line, relative to the Jurkat T cell lymphoma line, already expresses very high levels of Pim-1. The probable explanation here is that extra expression of Pim-1 in K562 cells is superfluous and does not have any effect, as K562s are known for having high survival rates under a multitude of circumstances. We have also confirmed that caspase-3 activation occurs in Jurkat cells within 6 hours of recovery from heat shock, but K562 cells did not appear to be subject to this proapoptotic event. It may proceed more slowly in these cells because of the high endogenous levels of Pim-1, or it may prove irrelevant, as these cells readily resist heat shock-induced apoptosis.

Pim-1 has been shown to promote cell survival under many circumstances, and these results also demonstrate a role for Pim-1 in anti-apoptosis. It is likely that Pim-1’s contribution to survival works, at least in part, through the phosphorylation of p21. The evidence that Pim-1 may continue to act as a survival factor during heat
shock suggests that hyperthermia may not be a viable therapeutic solution for tumor
cells that express high levels of Pim-1.
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Figure 1. Pim-1 protein increases during heat shock. Jurkat or K562 cells were heat shocked at 43°C for up to 60 minutes. Cells were lysed and proteins were run on SDS-PAGE. Proteins were transferred to PVDF membrane and analyzed by Western blot with our laboratory’s antibody to GST-Pim-1.
Figure 2. **p21 protein expression with heat shock.** K562 and Jurkat cells were heat shocked for up to 60 min. at 43°C. Lysates were run on SDS-PAGE and Western blotting was performed for p21.
Figure 3. Kinase activity of Pim-1 with heat shock. Jurkat and K562 cells were heat shocked for 1 h at 43 °C or left at 37 °C. Pim-1 was immunoprecipitated from the lysates of each. Antibody with no lysate served as the control (input). Resulting Pim-1 was incubated with p21 peptide and ATP for 30 min., then added to luminescence-producing reagent. Relative light units (RLU) measure ATP, so that a decrease in RLU indicates kinase activity.
Figure 4. Pim-1 and p21 localization during heat shock. Jurkat cells were heat shocked at 45°C for 0, 30, or 60 minutes. Cells were lysed, and the cytoplasmic fractions were removed to fresh tubes. Nuclei were lysed separately. Proteins were analyzed by SDS-PAGE and Western blot with our laboratory antibody to GST-Pim-1 and a polyclonal antibody to p21.
Figure 5. Pim-1 associates with p21 during heat shock. Jurkat and K562 cells were heat shocked at 43°C for 0 or 60 minutes. p21 was immunoprecipitated and complexes were run on SDS-PAGE. Proteins were transferred to PVDF membrane and analyzed by Western blot with monoclonal antibody to Pim-1.
Figure 6. Expression of Pim-1 during heat shock recovery. Jurkat and K562 cells were heat shocked for 1 h at 43°C and returned to the 37 °C incubator. Samples were taken at the indicated times. Western blotting was performed with our laboratory antibody to GST-Pim-1.
Figure 7. **Pim-1 protein is stabilized after heat shock.** K562 cells were heat shocked for 1 hour at 43°C. Cells were returned to the 37°C incubator and treated with cycloheximide to halt protein synthesis at the time points given. Proteins were analyzed by immunoblot.
Figure 8. Effect of wild type and kinase dead Pim-1 on mitochondrial membrane potential. K562 and Jurkat cells were transfected with plasmids encoding wild type GFP-Pim-1, kinase dead GFP-Pim-1, and GFP vector. After 24 h, cells were heat shocked at 43 °C for 1 h and returned to the 37 °C incubator for 23 h. One h prior to analysis, cells were stained with Mitotracker red as an indicator of apoptotic resistance. Red fluorescence was measured by flow cytometry.
Figure 9. Effect of heat shock on caspase 3 activity. Jurkat and K562 cells were heat shocked for 1 h at 43 °C and allowed to recover at 37 °C for 6 h. Cells were incubated with PhiPhiLux substrate and then red fluorescence, a measurement of caspase 3 substrate cleavage, was measured by flow cytometry.
Figure 10. This model demonstrates that heat shock may induce expression of Pim-1, which phosphorylates p21, in turn associating with procaspase 3 and preventing its activation. In this way, Pim-1 may promote cell survival.
This chapter was written as a manuscript that will be submitted to the journal *Oncogene*, and it therefore differs in format from the other chapters. I am the first author on this paper, and performed the experiments and writing. Dr. Zeping Wang made the *pEGFP-pim-1* constructs that I used, and Pei-xiang Xing, a member of the laboratory of Ian F.C. McKenzie (Austin Research Institute, Victoria, Australia) made the Pim-1 monoclonal antibodies that I used.
Pim-1 kinase stability is regulated by heat shock proteins and the ubiquitin-proteasome pathway

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Running title: Regulation of Pim-1 stability

Key words: Pim-1, serine/threonine kinase, heat shock proteins, ubiquitin, 26S proteasome

1Supported in part by NIH grants RO1 CA104470 and T32 AI07025

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Abbreviations used are: Ab, antibody; AMP-PNP, adenosine 5’-(β,γ-imido)triphosphate; Chlq, chloroquine; CHX, cycloheximide; DMSO, dimethylsulfoxide; GA, geldanamycin; GST, glutathione-S-transferase; h, hours; HA, hemagglutinin; HS, heat shock; Hsp, heat shock protein; IgG, immunoglobulin G; IP, immunoprecipitation; mAb, monoclonal antibody; min, minutes; NH₄Cl, ammonium chloride; PVDF, polyvinylidene fluoride; SDS-PAGE, SDS polyacrylamide gel electrophoresis; Ub, ubiquitin; Ub-dep, ubiquitin-depleted; WB, Western blot.
ABSTRACT

Elevated expression of the proto-oncogene *pim-1*, encoding a serine/threonine kinase, has been shown to increase the incidence of lymphomas in Pim-1 transgenic mice. More recently, Pim-1 has been found to be highly expressed in some human leukemias, lymphomas, and carcinomas. The connection of Pim-1 with tumor formation appears to involve its ability to delay or even prevent the normal process of apoptosis, marking it as a "cell survival factor." In malignant cells, the presence of survival factors can outweigh the effects of pro-apoptotic proteins, allowing the cells to live and proliferate unhindered. Because Pim-1 can act as a cell survival factor, it was of interest to understand how cellular levels of Pim-1 protein are maintained in tumor cells. This study examined how Pim-1 is protected and targeted for degradation by heat shock proteins (Hsp) and the ubiquitin-proteasome pathway in K562 and BV173 human leukemic tumor cells. Using cycloheximide treatment, we found that the half-life of Pim-1 protein in these cells increases from 1.7 h to up to 3.1 h when induced by heat shock or by treating the cells with the proteasome inhibitor PS341. We also found that the Hsp90 inhibitor, geldanamycin, prevents the stabilization of Pim-1 by heat shock. Using *in vitro* and *in vivo* ubiquitylation assays, it was determined that Pim-1 is degraded by the ubiquitin-proteasome pathway, and co-immunoprecipitation showed that Hsp70 is associated with Pim-1 under these circumstances. Conversely, Hsp90 was found to protect Pim-1 from proteasomal degradation. A population of unbound Pim-1 also exists in these cells, as demonstrated by immunodepletion of Hsp70 and Hsp90. A luminescence-based kinase assay demonstrated that unbound Pim-1 kinase remains
active, so the heat shock chaperones are not absolutely required for Pim-1 activity. Thus, the importance of this study is that it shows how Pim-1 levels can be altered in cells with the major mode of degradation being the ubiquitin-proteasome pathway. In addition, it shows that Pim-1 degradation and stabilization are regulated in part by binding to Hsps 70 and 90.
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 (Wang et al. a 2001, Dhanasekaran et al. 2001). Although not all of its substrates are known, Pim-1 has been shown to phosphorylate several proteins, including p21\textsuperscript{cip-1/waf-1} (Wang et al. 2002), CDC25A (Mochizuki et al. 1999), PTP-U2 (Wang et al. b 2001), NuMA (Bhattacharya et al. 2002), and PAP-1 (Maita et al. 2000). Of particular relevance to its role in cancer, Pim-1 has been shown to delay or even prevent the normal process of apoptosis, marking it as a "cell survival factor." Although important in normal cellular processes, survival factors may contribute to malignancy by interfering with pro-apoptotic signals, preventing programmed cell death and allowing the next round of mitosis to take place. As an example, Lilly et al. found that factor-dependent 32D cells deprived of IL-3 survived longer in the presence of overexpressed wild type Pim-1, whereas dominant negative Pim-1 increased apoptosis (Lilly et al. 1999). Pim-1 has also been implicated in antiapoptosis through the phosphorylation of p21\textsuperscript{cip1/waf1} (Wang et al. 2002), which is thought to mediate survival by binding to and preventing the activation of procaspase 3 (Suzuki et al. 1998).

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 normally makes \textit{pim-1} transcripts inherently short-lived.
The loss of this element results in longer-lived pim-1 mRNAs, which allows for increased translation. It has also been shown that Pim-1 protein has a short half-life of about 5-10 minutes in primary cells (Saris et al. 1991, Liang et al. 1996). In this study, we have found the Pim-1 protein half-life in tumor cells to be at least 100 min. The half-life of Pim-1 has been found to shorten in response to the heat shock protein 90 inhibitor geldanamycin (Mizuno et al. 2001), 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. 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, or when they are not needed at the level currently expressed, a number of 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 multi-unit 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 (Treier et al. 1994), I-kappa-B (Chen et al. 1995), Mos (Ishida et al. 1993), STAT1 (Kim et al. 1996), cyclin E (Clurman et al. 1996), p27 (Pagano et al. 1995), c-Myb (Bies et al. 1997), pRB (Boyer et al. 1996), and cyclin D1 (Diehl et al. 1997).
Heat shock protein 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 (Connell et al. 2001). 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 (Yufu et al. 1992). Cells expressing high levels of heat shock proteins can become resistant to chemotherapy (Ciocca et al. 1992), suggesting that Hsp90 and other chaperones may protect proteins that are closely involved in tumor cell survival (Neckers 2002).

This study focused on 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 this degradation can be regulated by binding to the chaperones Hsp70 and Hsp90. This association appears to occur on a constant, basal level, and does not require any extracellular induction, although such events may occur with cellular stresses such as heat shock. The binding of Pim-1 to Hsp90 can be disrupted by the use of geldanamycin, a Hsp90-specific inhibitor. 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 mainly when Pim-1 is conjugated to ubiquitin. It was also shown that a fraction of Pim-1 unbound to Hsp70/90 exists in these cells, and that it remains kinase active. These findings suggest that the protein level of Pim-1 in tumor cells is mediated, at least in part, through Hsps 70 and 90, and the ubiquitin-proteasome pathway, and that the amount of Pim-1 protein in cells can be regulated by Hsp70/90 binding. Furthermore, with regard to 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 anti-apoptotic factor, Pim-1 appears to aid in survival of tumor cells, whereas a decrease in its expression or activity would shift the balance toward apoptosis.

RESULTS

Inhibition of the proteasome causes the accumulation of ubiquitin-conjugated proteins. 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 is shown, with similar data being obtained for the BV173 cell line. We have found that the Pim-1 protein has a long half-life ($\geq 1.7$ h) in these and other tumor cell lines, in contrast to the 10 minute half-life reported for the 34 kD Pim-1 in primary cells (Saris et al. 1991). 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.

PS341 is an inhibitor of the ubiquitin-specific 26S proteasome (Adams 2001). 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. First, we wanted to confirm in our cell system that the drug PS341 does function as an inhibitor of the proteasomal degradation of ubiquitin-tagged proteins. It is not always easy to visualize ubiquitylated proteins by Western blot because of the rapid cycle of degradation, so the use of a proteasome inhibitor not only preserves proteasomally degraded proteins, but also facilitates the appearance of ubiquitylated forms of the proteins. As a control, chloroquine and NH₄Cl were added to cells to inhibit lysosomal cathepsins (An et al. 2000). 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. K562 cells were treated with geldanamycin or DMSO vehicle, and with PS341 or chloroquine/ NH₄Cl as indicated. Resulting lanes show total cellular ubiquitylated proteins (Figure 1A). Cells treated with PS341 displayed a buildup of ubiquitin-protein conjugates, while those treated with lysosomal inhibitors and those treated with DMSO alone showed a lesser amount. This confirms in our hands that PS341 specifically inhibits the
degradation of ubiquitin-tagged proteins by the 26S proteasome. While a fraction of total cellular proteins are being ubiquitylated continuously, only the cells treated with PS341 were able to retain the conjugates intact because degradation by the 26S proteasome was inhibited. We also tested the effects of Hsp90 inhibitor geldanamycin, in order to make sure it would not alter the ubiquitylation process of cellular proteins in our system. Geldanamycin changes the physical association of Hsp90 with its substrates by acting as an ATP analog that specifically binds Hsp90 in its ATP binding site, thus the client protein is not protected (Kamal et al. 2003). Treatment with geldanamycin did not appear to have an effect on the amount of ubiquitin-conjugated proteins in the samples. This observation becomes important in later experiments, verifying that any change in Pim-1 levels in geldanamycin-treated samples is a result of its effect on Hsp90, and is not related to a change in degree of ubiquitin tagging.

Next, in order to show that ubiquitin-conjugated proteins are rapidly degraded by the 26S proteasome, the half-life of total cellular ubiquitylated proteins was tested in the presence and absence of PS341. K562 cells were treated with PS341, or DMSO as a control, and protein synthesis was halted with cycloheximide. Initially, a streak of ubiquitin-conjugated proteins from total cellular lysate is apparent. (Fig. 1B) Over 24 hours, in the absence of new protein synthesis, the targeted proteins are eventually degraded. In the presence of PS341, however, the ubiquitylated proteins are not degraded. A reprobe of the blot for the long-lived protein actin shows equal loading. Total cellular ubiquitylated proteins appear to increase in these samples even though protein translation has been stopped. This
accumulation can be attributed to the continued addition of ubiquitin to existing proteins and does not indicate new protein synthesis.

**Inhibition of the 26S proteasome prevents Pim-1 degradation.** In order to show that Pim-1 itself is a target for degradation by the 26S proteasome, K562 cells were treated with PS341 or chloroquine/NH_4 Cl. The resulting Pim-1 Western blot shows the effects of proteasome inhibition on preventing Pim-1 protein turnover (Fig. 2A). The inhibitors of lysosomal degradation, which inactivate cathepsins by raising the pH inside lysosomes (normally pH 5.5), did not cause an 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 for the first 8 h after treatment, but normal levels returned by 16 h. The pretreatment of cells with PS341 or heat shock prior to geldanamycin provides some preservation of Pim-1, although it cannot prevent the action of geldanamycin. These results demonstrate 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 performed. K562 cells were treated with cycloheximide, and PS341 or DMSO vehicle. Samples were taken at the indicated times (Fig. 2B). A Western blot of
Pim-1 confirms that in PS341-treated cells, Pim-1 is more stable than in untreated cells. For consistency, Pim-1 half-life studies were also performed with geldanamycin and heat shock in the same experiment, and will be discussed later. A graph showing the half-life of Pim-1 in PS341-treated and untreated cells is shown in Fig. 2C. The normal half-life of Pim-1 in K562 vehicle-treated cells (“Control”) is approximately 1.7 h. The half-life of Pim-1 in the presence of PS341, when proteasomal degradation is inhibited, is approximately 2.6 h. This result demonstrates that the 26S proteasome is involved, at least to some degree, in Pim-1 degradation.

**Pim-1 can be conjugated to ubiquitin in vitro.** In order to test whether Pim-1 may be tagged with ubiquitin, we performed an *in vitro* ubiquitylation assay using cell lysate and GST-Pim-1 (59 kD), AMP-PNP, and/or ubiquitin in a cell-free system. AMP-PNP is a β,γ-non-hydrolysable ATP analog. It was used to uncouple ubiquitylation from degradation by allowing the addition of ubiquitin molecules in an increasing chain to the GST-Pim-1, while preventing their removal by proteasomal degradation, which uses β−γ cleavage of ATP (Johnston *et al.* 1991). The differing ATP cleavage requirements of these two processes make AMP-PNP a useful analog for this experiment. As seen in Figure 3A, GST-Pim-1 is conjugated to ubiquitin when it is present, and this is an ATP-dependent process. 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. The blots show a form of GST-Pim-1 with slightly altered mobility, consistent with a
monoubiquitylated molecule (Bender et al. 2000), 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 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 non-hydrolysable ATP analog.

Proteasomal inhibition results in ubiquitylated forms of endogenous Pim-1 in vivo. The ubiquitylation of Pim-1 was confirmed with an in vivo experiment. To inhibit protein degradation by the 26S proteasome, K562 cells were pretreated with PS341 prior to Pim-1 (34 kD) immunoprecipitation. As shown in Figure 3B, Western blotting with anti-ubiquitin reveals higher molecular weight forms of Pim-1 that are apparent in both immunoprecipitates, but the lane treated with PS341 shows a larger amount. These ubiquitin-tagged Pim-1 proteins were allowed to accumulate by use of the proteasome inhibitor. A small fraction of cell lysate from the PS341-treated sample was run as a control, and shows ubiquitylated forms of total cellular proteins.

Next, to confirm the presence of Pim-1 in the population of ubiquitin-conjugated proteins, total ubiquitin was immunoprecipitated from K562 cells treated with geldanamycin, PS341, or a combination of the two drugs. Western blotting was performed for Pim-1. Figure 3C demonstrates that Pim-1 bound to ubiquitin is detected primarily in cells that have been treated with geldanamycin to release it
from Hsp90, plus PS341 to prevent immediate degradation by the 26S proteasome. PS341 alone may not cause a detectable build-up of ubiquitin-conjugated Pim-1 unless geldanamycin has been used to destabilize Pim-1, and geldanamycin used in the absence of a proteasome inhibitor may cause a rapid turnover of Pim-1, which is also difficult to detect. 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. These and the above results indicate that 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.

Expression of tagged Pim-1 and tagged ubiquitin allows in vivo visualization of ubiquitin conjugation to Pim-1. When proteins are conjugated to chains of endogenous ubiquitin, visualization on a Western blot may show smears of very high molecular weight. The use of an exogenous, tagged ubiquitin construct may reduce artifacts and show a clearer picture. K562 cells were transfected by electroporation with vectors expressing hemagglutinin-tagged ubiquitin (UbHA) (Hochstrasser et al. 1991) and/or Pim-1 with a FLAG tag on the C-terminal end of the open reading frame. Co-immunoprecipitation and Western blotting were performed with anti-HA or anti-FLAG antibodies, as indicated. The bands indicate FLAG-Pim-1 conjugated to one or more UbHAs (Fig. 3D). Conjugation of the expressed FLAG-Pim-1 to endogenous ubiquitin may also occur, and may affect FLAG-Pim-1 mobility on a gel, but is not shown on an anti-HA Western blot. We found that very high
molecular weight forms of ubiquitylated Pim-1 did not appear in this experiment. Nevertheless, this result confirms that Pim-1 can be conjugated to ubiquitin, and provides the additional assurance of specificity by the use of tagged constructs.

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. 2A). 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 h heat treatment allows them to resist further injury (Luscher et al. 1988), a phenomenon that requires pro-survival activity. In our experiments, K562 cells were heat shocked at 43° C for 1 h prior to the addition of cycloheximide. The Western blot is shown in Figure 2B. The half-life of Pim-1 in heat shocked cells is approximately 3.1 h, more than twice as long as 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 heat shock protein 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 (Mizuno et al. 2001) led us to investigate its possible contribution to Pim-1 stability with regard to the ubiquitin-proteasome pathway. We employed the inhibitor geldanamycin 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 Figure 2B. The normal half-life of Pim-1 without geldanamycin is 1.7 h in these cells (Fig. 4B). When geldanamycin is present, the half-life of Pim-1 decreases to approximately 0.7 h, 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 h heat shock at 43°C seems to be caused, at least in part, by an increase in half-life. Although an increase in Pim-1 translation during heat shock may also be a contributing factor in cells such as HeLa and COS-7, 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 Pim-1’s longer half-life following heat shock may be the protection provided by Hsp90. Indeed, treatment with geldanamycin before heat shock prevented the accumulation of Pim-1 protein (Fig. 4C). In addition, a graph of the half-life experiment in which K562 cells were pretreated with geldanamycin before heat shock (Western blot in Fig. 2B) shows that heat shock no longer prolongs Pim-1 stability when Hsp90 is unable to bind it (Fig. 4D). The half-life of Pim-1 when treated with geldanamycin before heat shock is only 1.0 h, slightly longer than with geldanamycin treatment alone, but much shorter than the 3.1 h half-life of Pim-1 in cells treated only with heat shock.

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4 Weaver M.S., Petersen Shay K., Magnuson N.S. Use of both an internal ribosomal entry site (IRES) and a cryptic promoter provides the pim-1 proto-oncogene with alternative modes of expression under pro-apoptotic and stressful conditions. Submitted.
**Pim-1 binds to Hsp90 and Hsp70 in vivo.** To confirm that Pim-1 does indeed bind to Hsp90 in K562 cells, a co-immunoprecipitation was performed 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 performed with antibodies to the co-immunoprecipitating 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 co-immunoprecipitated with the other because the interaction was disrupted by geldanamycin’s action on the Hsp90 ATP-binding site (Figure 5A).

Heat shock protein 70, in addition to Hsp90, has been reported to play a role in the stability of several proteins, such as p53 (King *et al.* 2001) and ApoB (Gusarova *et al.* 2001). Therefore, we wanted to investigate the possible contribution of Hsp70 to Pim-1 stability or degradation. The same study was carried out with antibodies to Hsp70 instead of Hsp90. 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 upon the presence of Hsp90. A slight increase in the association of Pim-1 with Hsp70 appears to occur with geldanamycin treatment, suggesting that the loss of Hsp90-mediated protection of Pim-1 may actually promote its association with Hsp70. 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.
Heat shock protein 70 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 Hsp70 under the same circumstances, we wanted to investigate whether Hsp70 was associated with Pim-1 during proteasomal inhibition. K562 cells were treated with PS341 to preserve ubiquitin-conjugated Pim-1 for immunoprecipitation. A Western blot performed with antibodies to Hsp70 shows that Hsp70 was bound to Pim-1 in both the PS341-treated and mock-treated samples, but more so with proteasomal inhibition (Figure 6A). The treated cells may express more Pim-1 because of the use of PS341, but these results suggest that Hsp70 is associated with Pim-1 when it is preserved by proteasomal inhibition.

Hsp90 protects Pim-1 from degradation by the ubiquitin-proteasome pathway. The above results demonstrating Pim-1 association with Hsp70 with the use of PS341 prompted us to investigate whether this binding occurred primarily with ubiquitylation. In order to show that Hsp70 is associated with ubiquitylated Pim-1, and Hsp90 is not, fractions containing ubiquitylated Pim-1 were compared to those with non-ubiquitylated Pim-1. K562 cell lysates briefly pretreated with PS341 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 re-immunoprecipitated 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. 6B). These results indicate that Hsp90 associates with nonubiquitylated Pim-1. This is consistent with the half-life data that indicates that Hsp90 protects Pim-1 from degradation. In contrast, Hsp70 appears to be associated mainly with ubiquitin-conjugated Pim-1.

K562 cells were then transfected by electroporation with pRC/CMV-ha-ubiquitin. After 24 hours, cells were split into four wells and treated overnight with either DMSO vehicle or with geldanamycin and/or PS341, and Pim-1 was immunoprecipitated. In the DMSO-treated fraction, probing for HA shows little ubiquitylation of Pim-1 (Fig. 6C), indicating normal ubiquitin conjugation and proteasomal degradation. When cells were treated with geldanamycin or with a geldanamycin/PS341 combination, ubiquitylation of Pim-1 was observed. In these samples, Pim-1 association with Hsp90 was forcibly disrupted, allowing Pim-1 to be conjugated to multi-ubiquitin chains, but is best visualized in the lane where PS341 is present to prevent rapid degradation by the proteasome. Cells treated with PS341 alone show minimal conjugation of Pim-1 to HA-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. This study shows that Pim-1, when released from the protection of Hsp90, may be targeted for proteasomal degradation. Reprobing of the membranes
confirmed that Hsp70 preferentially immunoprecipitated with the samples containing the most ubiquitylated forms of Pim-1.

**Pim-1 uncomplexed with Hsp70 and Hsp90 remains kinase-active.** The strict regulation of Pim-1 protein levels prompted us to investigate whether this might also be a mechanism for regulating Pim-1 kinase activity. K562 lysates were serially immunoprecipitated for Hsp90, Hsp70, and Pim-1. Western blotting for Pim-1 showed that it was present in each fraction, and that Pim-1 does exist unbound to either Hsp70 or Hsp90 (Fig. 7A).

Next, the kinase activity of Pim-1 was tested for each circumstance. An equivalent amount of recombinant Pim-1 was bound 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 (Wang *et al.* 2002) was used as the substrate for the isolated Pim-1 complexes. Luminescence decreases as ATP is used, and 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). Notably, chaperone-bound Pim-1 did not appear to experience enhanced activity, either. Hsp70 and Hsp90 themselves had no measurable kinase activity toward the p21 peptide (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
co-immunoprecipitation of endogenous protein complexes (data not shown), so the presence of other factors does not change this trend. Thus, permanent association with either chaperone does not appear to be necessary for Pim-1 kinase activity.

**Heat shock and proteasome inhibition provide protection from apoptosis.** The above data that show Pim-1 protein stabilization by heat shock and proteasome inhibition prompted us to investigate whether these treatments can prevent early apoptotic events in tumor cells. K562 cells were treated with STI-571, a drug that specifically causes apoptosis in Bcr-Abl positive leukemias. A fluorescence-based assay was performed for caspase 3/7 activity. As seen in Figure 8A, cells treated with STI-571 and DMSO vehicle are positive for caspase3/7 activity, and this effect is augmented by the addition of geldanamycin (GA). Cells that were pretreated with heat shock or PS-341 have considerably less caspase 3/7 activity, indicating protection from apoptosis. Overall, the presence of STI-571 augmented the onset of caspase activity. As Pim-1 is reduced by geldanamycin, but stabilized by heat shock and PS-341, part of the protective effect may be due to Pim-1 survival activity.

**Pim-1 is involved in cell survival.** As STI-571 causes eventual down-regulation of other Hsp90 clients (Akt, 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 GFP-Pim-1, kinase dead GFP-Pim-1, or GFP alone, and treated with STI-571 and geldanamycin (GA) or DMSO vehicle. Flow cytometry was performed with supravital staining on cells emitting green fluorescence. Figure 8B shows that
survival of green-gated cells as measured by PI 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, showing that Pim-1 does contribute to cell survival. When the cells were treated with geldanamycin, there was very little difference in survival for the cells with kinase dead GFP-Pim-1. In this case, endogenous Pim-1 and other Hsp90-dependent proteins were being destabilized by geldanamycin, but 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 wild type Pim-1 (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. The fact that the cells expressing wild type GFP-Pim-1 survived slightly better under geldanamycin treatment than the other cells may suggest that GFP-Pim-1 fusion protein is not degraded as rapidly as endogenous Pim-1.

DISCUSSION

Recent work from this laboratory and others has shown that Pim-1 acts as a survival factor under many circumstances (Wang et al. 2002, Wang et al. a 2001, Lilly et al. 1999, Wang et al. c 2001, Lilly et al. 1997, Moroy et al. 1993, Shirogane et al. 1999). It has also been shown that the binding of Pim-1 to Hsp90 can prolong its half-life (Mizuno et al. 2001). The work presented here demonstrates that in
tumor cells, Pim-1 can bind not only to Hsp90, but also 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. Figure 9 shows a model demonstrating Pim-1’s association with Hsp90 and with Hsp70/ubiquitin. It is interesting that no mutated forms of Pim-1 have been reported, which strengthens the case that it is increased levels of Pim-1, and not 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 (Laird et al. 1993). There appear, therefore, to be compensatory pathways that allow normal cells, but not some tumor cells, to survive the loss of Pim-1, which suggests this kinase may be a good target for cancer therapy. Because cells must maintain a balance between survival and apoptosis, and because Pim-1 functions as a survival factor, the precise post-translational control of Pim-1 levels must be necessary to prevent tumorigenicity.

Pim-1 appears to be active as a kinase whether it is bound to or free of Hsp70 and 90 (Fig. 7B). 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 PS341, a specific inhibitor of the 26S proteasome. It was demonstrated that Pim-1
levels increase in the presence of PS341 (Fig. 2A,B). The finding that the half-life of Pim-1 increases in response to PS341 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 PS341 suggests that other degradation pathways for Pim-1 may exist. However, the 26S proteasome appears to be a major regulator of Pim-1 turnover under the circumstances tested in this study.

Because some proteins may be targeted to the proteasome without being ubiquitylated, as observed by the binding of ornithine decarboxylase to antizyme (Murakami et al. 1992), it was necessary to demonstrate 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 analog to the in vitro system. In this case, we used AMP-PNP instead of ATP, because this ATP analog is β,γ-nonhydrolysable. The activation of ubiquitin moieties, causing a release of AMP and PPi, still occurs, but proteolysis, requiring β,γ cleavage, cannot (Johnston et al. 1991). In Fig. 3A, we show that Pim-1 is extensively ubiquitylated in vitro.

Pim-1 is also ubiquitylated in vivo, both with endogenous components and with tagged expression constructs (Fig. 3B,C). Ubiquitin-tagging of Pim-1 was confirmed with the expression of transfected FLAG-Pim-1 and HA-ubiquitin, and co-immunoprecipitation of the two tags. The laddering effect of ubiquitylated Pim-1 was clear, although the visibility of the antibody heavy chains obscures part of the laddering. Clear laddering patterns such as these 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 mono- and polyubiquitins at many various locations (Mimnaugh et al. 1999). When HA-tagged ubiquitin is expressed in cells, this does not supercede the ability of endogenous ubiquitin to bind its substrates, but the visualization by anti-HA Western blot shows only the forms of Pim-1 that have HA-ubiquitin, and does not show whether any naturally occurring ubiquitin is also bound to Pim-1. The immunoprecipitations with endogenous Pim-1 and ubiquitin (Fig. 3B) provide the insight that Pim-1 may be conjugated not only to ubiquitin chains of varying lengths, but perhaps also in varying locations on the protein, resulting in a much tighter laddering pattern than that seen on the HA-ubiquitin blots.

The data showing that Pim-1 protein levels increase with heat shock treatment (Fig. 4A,B) was surprising, as total cellular protein degradation increases in this situation (Fujimoro et al. 1997), while translation, in general, decreases (Blagosklonny et al. 2002). Of note, Pim-1 translation has been found to increase with heat shock through the use of an internal ribosomal entry site. However, the current study shows that Pim-1’s half-life also increases with heat shock, which led us to investigate the potential protection of Pim-1 by molecular chaperones. Heat shock proteins 70 and 90 are housekeeping proteins and are not necessarily induced by heat shock, but are present in cells ubiquitously (Neckers 2002). However, they are found to bind to client proteins under cellular stresses such as heat shock (Morimoto et al. 1998), so these were good candidates for the protection of Pim-1. Treatment of cells with geldanamycin prior to heat shock prevented the accumulation

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5 Ibid.
of Pim-1 protein (Fig. 4D,E), providing further evidence for the involvement of Hsp90 as the protector.

Pim-1 was found to associate with Hsp70 as well as 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 (Gusarova et al. 2001, Demand et al. 2001), 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. When cells were treated with geldanamycin to prevent the protection of Pim-1 by Hsp90, ubiquitylation occurred (Fig. 6C). Cells do not require heat shock in order for Pim-1 to associate with heat shock proteins 70 and 90, but the normal half-life of Pim-1 in the tumor cell lines tested is 1.7 h, long compared to earlier reports in primary cells (Saris et al. 1991), but it is short enough to suggest that Pim-1 exists in the free, unprotected form in untreated cells. Thus, Pim-1 is eventually degraded even when Hsp90 is available, and thus has either been naturally released from the chaperone, or is shuttled to the proteasome in its presence. 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 have been shown to bind Hsp70 and Hsp90 at the same time (Nollen et al. 2002). Whether Pim-1 may be one of these is not clear at this time.
Experiments showing the co-immunoprecipitation of Pim-1 with Hsps 70 and 90 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. Hsc73/Hsp70 has been shown by Cuervo et al. (1998) to chaperone ubiquitylated substrates to lysosomes for degradation by cathepsins under certain circumstances, through binding of the KFERQ motif (Olson et al. 1991), and thus it is also possible that Pim-1 may be degraded lysosomally when necessary. Although Pim-1 does contain a putative KFERQ motif (amino acids 252-257: QVFFRQ), this present work demonstrates that lysosomal inhibitors do not cause a substantial build-up 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.

Binding of Pim-1 to Hsp70 occurs primarily when Pim-1 is conjugated to ubiquitin, suggesting that the control of Pim-1 degradation by the proteasome 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 would explain in part how its cellular availability is controlled. Furthermore, the previously documented contribution of Pim-1 to cell
survival is upheld by the data showing that geldanamycin does not increase STI-571-
induced cell death when kinase dead Pim-1 is expressed (Fig. 8B). The ability of
cancer cells to adapt to environmental stress is well-documented. One possible
strategy for inducing apoptosis in cancer cells would be to reduce the expression of
survival factors. Pim-1 may thus be targeted for degradation by treatments that
induce its binding to Hsp70 and its ubiquitylation. Geldanamycin has recently been
shown to bind the Hsp90 in tumor cell lines like K562 with a much stronger affinity
than the Hsp90 in normal cells (Kamal et al. 2003). A simple disruption of Pim-1
binding to Hsp90, as with geldanamycin or less toxic analogs, may be useful in
rapidly reducing the levels of Pim-1, 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, BV173, and COS-7 cells were purchased from ATCC. K562 and BV173 cell lines are Philadelphia chromosome positive human chronic
myelogenous leukemias. They were grown in RPMI 1640 medium (Gibco)
containing 10% newborn calf serum (Sigma), 2mM L-glutamine, 100 U/ml
streptomycin and penicillin at 37°C in a humidified atmosphere with 5% CO2.
Suspension cultures were maintained at a density of 5-8 X 10^5 cells and split at a 1:4
dilution every three days to ensure active cycling of cells. Cell viability was checked
using Trypan blue exclusion during cell growth. COS-7 cells were maintained in
DMEM medium (Gibco) containing 10% newborn calf serum (Sigma), 2mM L-
glutamine, 100 U/ml streptomycin and penicillin at 37°C in a humidified atmosphere with 5% CO₂. Cultures were passaged at a 1:5 dilution every 4 days.

*Chemical treatments.* Geldanamycin was purchased from AG Scientific and was dissolved in DMSO. For cell treatment, it was used at 2 µM (An et al. 2000). PS341 was obtained from Millennium Pharmaceuticals (Cambridge, MA) and was dissolved in DMSO and used at 1 µM (27). STI-571 was obtained from Novartis (Basel, Switzerland) and was dissolved in water for use at a concentration of 10 µM. Lysosomal inhibitors chloroquine (100 µM) and ammonium chloride (2.5 mM) (An et al. 2000) were obtained from Sigma and were dissolved in cell culture medium.

*Whole cell lysate analysis.* Cells were washed in phosphate-buffered saline and lysed in ice-cold Radioimmunoprecipitation assay (RIPA) buffer: 137 mM NaCl, 25 mM Tris-Cl pH 8, 2 mM EDTA, 10% glycerol, 1% Igepal, 0.5% deoxycholate, plus 1X Protease Inhibitor Cocktail (Calbiochem). Where appropriate, phosphatases were inhibited by addition of 5mM sodium orthovanadate to the lysis buffer. 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 (BioRad) at wavelength 595 nm on an Ultraspec 2000 (Pharmacia) spectrophotometer. Laemmli loading buffer was added to the samples and they were boiled for 10 minutes. Equal amounts of protein or an equal number of cells, as appropriate, were loaded onto SDS-polyacrylamide gels.

*Immunoprecipitations.* PBS-washed cells were lysed in IP lysis buffer (25 mM Tris-Cl pH 8, 150 mM NaCl, 1% Igepal, 1X protease inhibitor cocktail),
vortexed, and incubated on ice for 20 min. Lysates were cleared by centrifugation at 12,000 rpm for 10 min. at 4°C. The resulting supernatants were removed to fresh tubes, and proteins were precipitated for 1-2 hours with the appropriate antibodies at 4°C with rotation. Protein-A or G-agarose (Boehringer Mannheim) was added and lysates were rotated for 1 additional hour. Precipitated complexes were washed 4X in IP buffer, boiled in Laemmli buffer, and run on SDS-PAGE.

*Protein half-life determination.* Cycloheximide was added at a concentration of 30 µm to cells in RPMI 1640 without serum. Aliquots of equal cell number were taken at various time points post-treatment. Proteins were prepared for SDS-PAGE and immunoblotting as described.

*Ubiquitylation assays.* Cells were lysed in cold ubiquitylation buffer (50 mM Tris-Cl pH 8, 5 mM MgCl₂, 5 mM CaCl₂, 2 mM DTT, 5 mM N-ethylmaleimide). Cleared lysates were aliquoted into tubes, and assay components GST-Pim-1, 10 µg ubiquitin (Sigma), and 4 mM AMP-PNP (Sigma) were added as indicated. Mixtures were incubated at 37°C for 30 minutes, then GST-Pim-1 was precipitated by the addition of glutathione sepharose 4B beads (Amersham Biosciences) for 1 h at 4°C. Beads were washed 4X in ubiquitylation buffer and samples were subjected to SDS-PAGE.

*Immunoblots.* Protein was transferred from SDS polyacrylamide gels to PVDF membrane (Millipore) with a semi-dry blotter. Membranes were blocked in 5% nonfat dry milk in PBS containing 1% Tween-20. Primary Pim-1 antibodies used were our laboratory's polyclonal to GST-Pim-1 made in rabbit and an anti-Pim-1 monoclonal antibody. Other primary antibodies include a rat monoclonal anti-Hsp90
and polyclonal anti-Hsp70 from Stressgen, monoclonal anti-FLAG from Stratagene, polyclonal anti-ubiquitin from Sigma, and monoclonal anti-hemagglutinin from Cell Signaling Technology. 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 containing primary antibody, washed extensively in PBS/Tween/milk, then incubated for one hour in a secondary antibody conjugated to horseradish peroxidase (Pierce). 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 x 10^7 cells/ml in serum-free RPMI 1640. 300 µl cells was added to a 4 mm cuvette and mixed with 20 µg plasmid DNA. Electroporation was performed at 950 µF and 200 V with a time constant of 21-29 msec. Cells were incubated on ice for 20 min., then expanded into 10 ml RPMI with serum and analyzed after 24-48 h. DNA constructs were pBK/CMV-pim-1-flag, resulting in a fusion protein with the FLAG tag at Pim-1’s C-terminus, and pRC/CMV-ha-ubiquitin, resulting in hemagglutinin-tagged ubiquitin. The ha-ubiquitin insert was subcloned from YEp112 (gift of Dr. Mark Hochstrasser, Yale University) into pRC/CMV for use in mammalian cells.

Flow cytometric measurement of cell survival. Cells were transfected with constructs that produced a protein in which GFP is fused to the C-terminus of Pim-1. pEGFP-wild type-pim-1, pEGFP-kinase dead-pim-1, or pEGFP vector and
incubated for 48 h. Cells were drug-treated and stained with propidium iodide prior to flow cytometric analysis. Only cells that fell within the gates for green were examined. Uptake of PI was measured, with exclusion as a characteristic of live cells.

Assay for caspase 3/7 activity. Cells were drug-treated as indicated and 20 μl of each suspension were plated in quadruplicate into a 384-well plate. At the appropriate time points, 20 μl Apo-One reagent (Promega) were added to the wells and cleavage of the caspase 3/7 substrate was measured as fluorescence on a Wallac Victor-2 plate reader.

Luminescent ATP-based assays for kinase activity. Recombinant Pim-1 (0.25 μg) was incubated in binding buffer (10 mM HEPES, pH 7.4, 100 mM KCl, 5 mM DTT, 20 mM Na2MoO4, 50 mM ATP) alone or with a fivefold excess of recombinant Hsp70 or 90 (Stressgen) for 20 min. at 30°C. Pim-1 was immunoprecipitated with monoclonal antibodies. Immobilized Pim-1-containing complexes were washed 4X in kinase buffer without ATP (25 mM HEPES, 10 mM MgCl2, 0.5 μg/ml DTT) and incubated with 0.1 μM ATP and 400 μM p21 peptide (RKRRQTS) in kinase buffer for 45 min. at 30° C prior to addition of Kinase-Glo reagent (Promega) at 1:1 (vol:vol). The mixture was removed from protein-A agarose beads and added to the wells of a Nunc 384-well plate, and ATP levels were counted on a Wallac Victor-2 plate reader. Kinase-Glo binds to remaining ATP, so a drop in cpm indicates kinase activity.
ACKNOWLEDGMENTS

We thank Dr. Mark Hochstrasser, Yale University, for the \textit{ha-ubiquitin} expression vector YEp112.
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Figure 1. (A-B) Proteasome inhibition causes an increase in total cellular ubiquitylated proteins.
A. K562 cells were treated for 12 h with 2 µM geldanamycin (GA) or an equivalent volume of DMSO vehicle, and with 1 µM PS341 or 100 µM chloroquine + 2.5 mM NH₄Cl. Lysates were subjected to 7% SDS-PAGE and Western blotting was performed with polyclonal antibodies to ubiquitin and actin.
B. Half-lives of cellular ubiquitylated proteins. K562 cells were treated with 1 µM PS341 or DMSO vehicle for up to 24 h, plus 30 µM CHX to halt translation. Cells were harvested at the times indicated, lysates run on 7% SDS-PAGE, and Western blotting was performed with polyclonal antibodies to ubiquitin. Both A and B are representative of three independent experiments.
Figure 2. (A-B) Pim-1 protein levels and half-life increase with proteasomal inhibition. A. K562 cells were treated with 1 μM PS341 or 100 μM chloroquine + 2.5 mM NH₄Cl for 4, 8, or 16 h. K562 cells were treated with 2 μM geldanamycin, 1 μM PS341, pretreated with 1 h heat shock at 43°C, or a combination of these for 4, 8, or 16 h. Cell lysates were run on 11% SDS-PAGE and Western blotting was performed with polyclonal Pim-1 and actin antibodies. The blot is representative of four independent experiments.

B. For the half-life studies, K562 cells were given 30 μM CHX in serum-free media to halt translation. Treatments included 1 μM PS341, 2 μM geldanamycin (GA), or an equivalent volume of DMSO vehicle (Control). Heat shock (HS) was performed by pretreating cells for 1 h at 43°C and adding DMSO vehicle. All results are compared to the same control. Cells were harvested at the times indicated and lysates separated by 11% SDS-PAGE. Western blotting was performed with anti-Pim-1 polyclonal antibodies. The results of a representative study are shown; at least three other experiments yielded similar results.
Figure 2. (C) Pim-1 protein levels and half-life increase with proteasomal inhibition. Densitometric analysis of the PS341 and Control blots in Fig. 2B was performed with the Quant-One program. A graph was constructed based on these values.
Figure 3. (A-B) Pim-1 is ubiquitylated in vitro and in vivo. A. Using COS-7 cell lysates as a source of ubiquitylation enzymes, an in vitro assay was performed with exogenous GST-Pim-1, ubiquitin, and/or AMP-PNP. Reactions were incubated at 37°C for 30 min. and the reaction stopped with the addition of cold RIPA buffer. GST-Pim-1 (59 kD) was isolated on glutathione B sepharose. Samples were run on 7% SDS-PAGE and Western blotting was performed with anti-ubiquitin and anti-Pim-1 polyclonal antibodies. Two additional experiments yielded similar results.

B. For in vivo ubiquitylation of Pim-1, K562 cells were treated for 16 h with PS341 to allow ubiquitylated forms of Pim-1 to accumulate. Pim-1 was immunoprecipitated with monoclonal antibodies, run on 9% SDS-PAGE, and immunoblotting was performed with polyclonal antibodies to ubiquitin. The blot is representative of at least three independent experiments.
Figure 3. (C-D) Pim-1 is ubiquitylated in vitro and in vivo.  C. K562 cells were treated for 2 h with geldanamycin, PS341, both, or DMSO vehicle. Total ubiquitin was immunoprecipitated and Western blotting was performed with monoclonal anti-Pim-1. The blot was reprobed with anti-Hsp70. Similar results were found in two additional experiments.

D. K562 cells were transfected with pBK/CMV-pim-1-flag and/or pRC/CMV-ha-ubiquitin constructs. FLAG- or HA- fusion proteins were immunoprecipitated with FLAG or HA antibodies and proteins were run on 11% SDS-PAGE. The PVDF membrane was cut in half and Western blotting was performed with antibodies to HA or FLAG, as indicated. Two other experiments yielded similar results.
Figure 4. (A-B) Pim-1 protein levels increase with heat shock, except in the presence of Hsp90 inhibition.  
A. Densitometric analysis of the Heat Shocked (HS) and Control blots in Fig. 2B was performed with the Quant-One program.  A graph was constructed based on these values.
B. Densitometric analysis of the Geldanamycin-treated (GA) and Control blots in Fig. 2B was performed with the Quant-One program.  A graph was constructed based on these values.
Figure 4. (C-D) Pim-1 protein levels increase with heat shock, except in the presence of Hsp90 inhibition. C. K562 cells were pretreated with 30 μM CHX and 2 μM GA or DMSO vehicle and heat shocked in a 43°C water bath for the times indicated. Cells were lysed, run on 11% SDS-PAGE, and immunoblotting was performed with anti-Pim-1 polyclonal antibodies and actin polyclonal antibodies as a control. The blot is representative of three independent experiments.

D. Densitometric analysis of the Heat Shock + Geldanamycin and Control blots in Fig. 2B was performed with the Quant-One program. A graph was constructed based on these values. For comparison, the data from the heat shocked and the geldanamycin-treated cells in 4B and 4C was included on this graph.
Figure 5. (A-B) Pim-1 binds to Hsp90 and Hsp70. A. K562 cells were treated with 2 µM geldanamycin (GA) for Hsp90 inhibition or DMSO vehicle and lysed in IP buffer. Samples were immunoprecipitated with rat monoclonal anti-Hsp90 or mouse monoclonal anti-Pim-1. Immunocomplexes were separated by 9% SDS-PAGE and Western blotting was performed with monoclonal antibodies to Pim-1 and Hsp90.

B. Cells were treated as in A. Samples were immunoprecipitated with Pim-1 monoclonal antibodies or polyclonal Hsp70 antibodies, proteins run on 9% SDS-PAGE, and immunoblotting performed with anti-Hsp70 and anti-Pim-1 polyclonal antibodies. In both A and B, the blots are representative of at least three independent experiments.
Figure 6. (A-C) Ubiquitylated Pim-1 is associated with Hsp70 and non-ubiquitylated Pim-1 with Hsp90.  

A. K562 cells were treated for 18 h with PS341 for accumulation of ubiquitylated Pim-1, lysed, and Pim-1 was immunoprecipitated with monoclonal antibodies. Complexes were run on 9% SDS-PAGE and transferred to PVDF membrane. Western blotting was performed with antibodies to Hsp70.  

B. Cells were lysed in IP buffer and lysates split in half. One tube was immunoprecipitated with anti-Pim-1 monoclonal antibodies (“Total Pim-1”). The other tube was immunoprecipitated with anti-ubiquitin polyclonal antibodies. The ubiquitin-depleted supernatant was immunoprecipitated with anti-Pim-1 monoclonal antibodies (“Ub-dep-Pim-1”). The total cellular ubiquitylated proteins were eluted from the beads and resuspended in IP buffer, then immunoprecipitated with anti-Pim-1 monoclonal antibodies to obtain only ubiquitylated Pim-1 (“Ub-Pim-1”). Following separation by 9% SDS-PAGE, Western blotting was performed with anti-Pim-1, anti-Hsp90, and anti-Hsp70 antibodies.  

C. K562 cells were transfected by electroporation with pRC/CMV-ha-ubiquitin. Cells were split into four wells and treated for 18 h with DMSO vehicle, GA, PS341, or GA + PS341. After lysis in IP buffer, endogenous Pim-1 was immunoprecipitated with monoclonal antibodies and immune complexes were run on 11% SDS-PAGE. Western blotting was performed with polyclonal anti-HA or with polyclonal anti-Hsp70. For A, B, and C, at least two other experiments yielded similar results.
Figure 7 (A-B). Pim-1 kinase remains active when not bound to Hsp 70 or Hsp90.  A.  Hsp90, Hsp70, and Pim-1 were serially immunoprecipitated from the same cell lysate, 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 µM ATP and 400 µM p21 peptide for 45 minutes at 30°C and measured for luminescent CPM.  A drop in remaining ATP levels (y axis) indicates the use of ATP in kinase activity.  Data represent the mean of 3 experiments ± SD.
A

**Effect of treatments on STI571-induced apoptosis**

![Graph showing Caspase 3/7 activity (RFLU) for different treatments.](image)

B

**Influence of GFP-WT-Pim-1 and GFP-KD-Pim-1 on STI-571 induced cell death**

![Bar chart showing percent survival for different treatments.](image)

**Figure 8 (A-B).** STI-571-induced cell death is decreased by treatments that stabilize Pim-1 and is not altered by Pim-1 destabilization when KD-Pim-1 is expressed. **A.** K562 cells were treated with 1 µM STI-571 and DMSO vehicle, 2 µM geldanamycin, 1 µM PS341, or 1 h heat shock at 43°C. Caspase 3/7 activity was measured at 0 and 4 hours by relative fluorescent light units (RFLU).

**B.** 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 (GA) and half with DMSO vehicle. Propidium iodide was added 1.5 h prior to flow cytometry. Cells were gated on those displaying green fluorescence, and the incorporation of propidium iodide was measured as indicative of cell death.
Figure 9. Model. Pim-1 is protected from degradation while in association with Hsp90, but binding to Hsp70 facilitates degradation of Pim-1. The model indicates that Pim-1 also exists free of interaction with Hsp90 or Hsp70/ubiquitin.
CHAPTER FOUR

PIM-1 KINASE CONTRIBUTES TO CELL SURVIVAL THROUGH PHOSPHORYLATION OF BAD ON SERINE 155 AND PREVENTION OF BCL-XL BINDING.

This chapter was written as a manuscript that is being prepared for submission to *Biochemistry Journal*. It therefore differs in format from the other chapters. I am the first author and I performed the experiments and the writing. Dr. Zeping Wang made the pEGFP-pim-1 constructs that I used, and Pei-xiang Xing, a member of the laboratory of Ian F.C. McKenzie (Austin Research Institute, Victoria, Australia) made the Pim-1 monoclonal antibodies that I used.
Pim-1 kinase contributes to cell survival through phosphorylation of Bad on serine 155 and prevention of Bcl-xL binding.

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Running title: Phosphorylation of Bad by Pim-1

1Supported in part by NIH grants RO1 CA104470 and T32 AI07025

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Key words: Pim-1, serine/threonine kinase, Bad, apoptosis, Bcl-xL
Abbreviations used are: Ab, antibody; Akt, AKR mouse thymoma; Apaf-1, apoptotic protease activating factor 1; Bad, Bcl-2 associated death promotor or Bcl-2 agonist of death; Bax, Bcl-2 associated X protein; Bcl-2, B cell leukemia 2; Bcl-xL, B cell leukemia X long; BH, Bcl-2 homology; CHX, cycloheximide; DMSO, dimethylsulfoxide; GM-CSF, granulocyte-macrophage colony stimulating factor; GST, glutathione-S-transferase; h, hours; HA, hemagglutinin; HS, heat shock; IgG, immunoglobulin G; IL-3, interleukin 3; IP, immunoprecipitation; mAb, monoclonal antibody; min, minutes; PI3 Kinase, phosphatidylinositol-3 kinase; PKA, protein kinase A, PKB, protein kinase B; PVDF, polyvinylidene fluoride; SDS-PAGE, SDS polyacrylamide gel electrophoresis; Ser, serine; WB, Western blot.
ABSTRACT

The pro-apoptotic protein Bad (Bcl-2 agonist of death) has been shown to be inactivated by phosphorylation on serine residues 112, 136, and 155. While serines 112 and 136 are involved in sequestration of Bad in the cytoplasm by 14-3-3 proteins, serine 155 resides in Bad’s BH-3 domain and its phosphorylation prevents Bad from binding to the anti-apoptotic protein Bcl-xL at the mitochondria. Akt and PKA are two kinases that contribute to the phosphorylation of Bad on these residues, and both recognize preferred phosphorylation consensus sequences similar to that of Pim-1. In this work, we show that Pim-1 preferentially phosphorylates serines 112 and 155 of Bad in a peptide kinase assay. It was also found that overexpression of wild type Pim-1 causes the phosphorylation of Bad on Ser155, an event that is blocked by kinase dead Pim-1. Furthermore, phosphorylation of Ser155 by Pim-1 inhibits the binding of GST-Bad to Bcl-xL and prevents apoptosis. In the presence of kinase dead Pim-1, GST-Bad associates with Bcl-xL and promotes apoptosis. Cells expressing wild type Pim-1 alone or in combination with GST-Bad had high survival rates, while cells expressing kinase dead Pim-1 displayed more apoptosis, particularly in conjunction with GST-Bad expression. It was determined that inhibitors of PKA (H-89) and Akt (wortmannin) have very little effect on the kinase activity of Pim-1, and that phosphorylation of Bad by Pim-1 occurs even when PKA and Akt are inactive. Bad is therefore a substrate for Pim-1, independent of PKA and Akt, two kinases with similar activity. These findings tie the previously documented survival activity of Pim-1 kinase to a mitochondrial pathway of apoptosis through phosphorylation of Bad.
INTRODUCTION

Pim-1 is a serine/threonine kinase that is expressed at high levels in some leukemias, lymphomas, and carcinomas. It has been shown to phosphorylate the proteins p21cip-1/waf-1 [1], Cdc25A [2], Numa [3], PTP-U2S [4], PAP-1 [5], and Bad [6], which indicates that Pim-1 may be involved in the regulation of cell cycle-related events and apoptosis. Through the overexpression of wild-type and kinase dead Pim-1, it has been found that Pim-1 can prevent apoptosis under various pro-apoptotic conditions, whereas lack of Pim-1 allows quicker and more efficient cell death [7]. Therefore, one primary function of Pim-1 appears to be its use by the cell as a survival factor. Of the known Pim-1 substrates, there are two that have been shown to be intimately involved in apoptosis and cell survival: p21 and Bad.

The Bcl-2 family of proteins, whose major site of action is at the mitochondria, are potent regulators of cell survival and apoptosis. Pro-survival proteins include Bcl-2, and Bcl-xL, while Bad, Bax, and Bid promote apoptosis. The homology of these proteins to Bcl-2 is described in terms of shared domains, namely BH1, BH2, BH3, and BH4. Bcl-2, Bcl-xL, and Bax contain C-terminal hydrophobic regions that anchor them to the outer mitochondrial membrane, where they can form heterodimers with other proteins. Bad, a BH3-only protein, lacks this domain and can localize either to the cytoplasm when phosphorylated, where it is sequestered by 14-3-3 proteins [8], or to the mitochondria when unphosphorylated for heterodimerization with Bcl-xL, and to a lesser extent, Bcl-2 [9]. Under normal, anti-apoptotic conditions, Bcl-2 and Bcl-xL are already heterodimerized with Bax [10]. The binding of Bcl-xL or Bcl-2 with Bax prevents Bax from exerting its
apoptotic effect. Bad can break this association by binding to Bcl-xL and Bcl-2, freeing Bax and allowing the formation of pro-apoptotic Bax homodimers using its now-available hydrophobic regions [11]. Newly formed Bax homodimers allow the release of cytochrome c from the mitochondria, probably by opening membrane channels. Transmembrane potential is eventually lost, however, evidence suggests that the mitochondria can maintain membrane potential even during the release of cytochrome c [12], supporting the notion that proteins such as Bax are indeed responsible for membrane permeabilization by the creation of pores. Cytochrome c promotes the formation of the apoptosome, a complex that also includes Apaf-1 (apoptotic protease activating factor-1) and procaspase 9, resulting in the proteolytic processing of procaspase 9 to active caspase 9, which in turn activates other procaspases. A cell full of active caspases loses structural and membrane integrity, leading to the classic hallmarks of apoptosis.

Cellular stresses such as the removal of growth factors from factor-dependent cells have been shown to cause translocation of pro-apoptotic Bad to the mitochondria [8]. A search for the mechanism of stress-induced cell death through Bad has led to the description of Bad-phosphorylating kinases such as Protein Kinase B (PKB/Akt), Protein Kinase A (PKA), Pim-2 [13], and now Pim-1 [6]. Bad can be phosphorylated on serine residues 112, 136, and 155 [14] by a combination of Akt and PKA. The phosphorylation of Bad on serines 112 and 136 causes its association with cytoplasmic 14-3-3 proteins [8], while phosphorylation of Bad on serine 155 prevents its binding to Bcl-xL [15]. Serine 155 resides in the BH-3 region of Bad, which regulates its binding to Bcl-xL (and Bcl-2 to a lesser degree) [16,17],
connecting this phosphorylation event directly to apoptosis. A more recent study implicates Ser170 of Bad in anti-apoptosis, but phosphorylation of this residue does not prevent Bad binding to Bcl-xL [18], so the mechanism by which it acts is unknown. Bad dephosphorylation, occurring in the absence of the active forms of the kinases or in the presence of protein phosphatases 2A and 2C [19,20], allows Bad to translocate to the mitochondria and promote apoptosis by the displacement of Bax from Bcl-xL. The amino acid sequence surrounding serines 112, 136, and 155 of Bad comprise attractive consensus sequences for phosphorylation by Pim-1. Furthermore, Pim-1 may be available under circumstances when Akt, PKA, and other kinases are not active. Unlike these kinases, Pim-1 does not appear to require phosphorylation through a specific signal transduction pathway to become active. Active Pim-1 exists constitutively in Jurkat and other cell lines. Phosphorylation of Bad in a context-dependent manner could explain the pro-survival activity of Pim-1 in relation to Bcl-2 family proteins.

This study found that Pim-1 is able to phosphorylate all three aforementioned serines of Bad, particularly Ser112 and Ser155. In the interest of tying the survival activity of Pim-1 to the prevention of Bad/Bcl-xL association, phosphorylation of Bad Ser155 was examined in Cos-7 cells expressing wild type or kinase dead Pim-1. Indeed, wild type Pim-1 allowed phosphorylation of Bad on Ser155 and prevented its binding to Bcl-xL, while kinase dead Pim-1 had the opposite effect. Furthermore, apoptosis occurred at lower levels in the presence of wild type Pim-1, even when pro-apoptotic Bad was overexpressed. These results are important in the study of
cell survival mechanisms and tumorigenesis, because they indicate that Pim-1 plays a role in allowing malignant cells to escape apoptosis.

**MATERIALS & METHODS**

*Cell lines and maintenance:* Jurkat and Cos-7 cells were purchased from ATCC. The Jurkat human T cell lymphoma line was maintained in RPMI 1640 with 10% FBS, 100 U/ml penicillin and streptomycin, and 2 mM l-glutamine at 37°C in a 5% CO₂ humidified incubator. Cos-7 African monkey kidney cells were grown in DMEM with 10% NCS, 100 U/ml penicillin and streptomycin, and 2 mM l-glutamine. Actively cycling cultures were split 1:4 every three days.

*Transfection:* Cos-7 cells were transfected by the DEAE-dextran method with chloroquine. Plasmids used were *pEGF-gst-bad* and the Pim-1 constructs *pEGFP-vector*, *pEGFP-wt-pim-1*, or *pEGFP-kd-pim-1* (K67ΔM). Briefly, cells were seeded 24 hours before transfection and grown to 50% confluence. After a wash in PBS, DEAE-dextran-chloroquine containing 10 µg plasmid DNA was added dropwise to the cultures, along with 5 ml serum-free medium. This mixture was incubated on the cells for 4 hours, followed by DMSO shock and refeeding in complete medium. Cells were trypsinized and harvested after 48 hours.

*Western blotting:* Cells or immunocomplexes were boiled in Laemmli loading buffer and run on SDS-PAGE. After transfer to PVDF membrane on a semi-dry blotter, nonspecific binding was blocked with 1% BSA in PBS-Tween-20 for 1 hour.
Membranes were incubated in primary antibody for 2 hours. Antibodies included polyclonal anti-Bad and anti-phosphoserine155 Bad (Cell Signaling Technology), polyclonal anti-GFP “Living Colors” (Clontech), monoclonal anti-Bcl-xL (Oncogene), and a monoclonal generated to Pim-1 (McKensie). The membranes were washed 3X for 10 min. each in PBS-Tween-20 and incubated in secondary antibody (Pierce) for an additional hour. Three washes were performed in PBS and membranes were treated with Pierce Pico West chemiluminescence and exposed to Hyperfilm (Amersham).

**Immunoprecipitation:** Cells were lysed in ice-cold TNSEV buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Igepal, and 2 mM EDTA, with addition of fresh 5 mM sodium orthovanadate as a phosphatase inhibitor and 1X protease inhibitor cocktail from Calbiochem), sonicated, and cleared by centrifugation at 12,000 rpm for 10 min. Supernatants were removed to fresh tubes and incubated with primary antibodies for 1-3 h at 4°C with rotation. Twenty µl protein-A-agarose beads were added to precipitate the protein-antibody complexes. After 1 h additional rotation, beads were washed 4X in TNSEV or kinase buffer (25 mM HEPES, 10 mM MgCl₂, and 0.5 mM DTT).

**Subcellular fractionation:** Cells were washed in PBS and suspended in Sucrose Buffer (10 mM Tris-HCl pH 7.5, 300 mM sucrose, and 1X protease inhibitor cocktail). Lysates were centrifuged for 15 min at 10,000 rpm, and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in Sucrose
Buffer plus 1% CHAPS and 150 mM NaCl and sonicated. Following centrifugation for 30 min at 10,000 rpm, the supernatant was collected as the mitochondrial fraction.

**Luminescence-based kinase assay:** Immunoprecipitated kinases (Akt, PKA, or Pim-1) from 500 µg Jurkat lysate or 0.25 µg bacterially expressed GST-Pim-1 (wild type or mutant K67ΔM) from pGEX-2T were incubated with 400 nM peptide and 0.1 µM ATP in kinase buffer (25 mM HEPES, 10 mM MgCl₂, 0.5 mM DTT) in a total volume of 20 µl for the indicated times in a 30°C water bath. An equivalent volume of Kinase-Glo reagent (Promega) was added and mixtures were transferred to a white 384-well plate. Luminescence (no filter) was read on a Wallac Victor-2 plate reader. A drop in ATP levels, indicating phosphorylation activity, was expressed in terms of relative light units (RLU).

**Peptides:** Peptides to serines 112 and 136 of murine Bad were made as in Masters *et al.* [21]. Serine 112: AMETRSRHSSYPAGTEE, Serine 136: LSPFRGRSRSAPPNLWA. Peptide to serine 155 was made with the phosphorylatable serine in the same respective location: RYGRELRRMSDFEGSF. Other peptides were: p21: RKRRQTS, Histone H2B: KKRRRESE.

**GST-pulldown:** GST or GST-Pim-1 were isolated from a bacterial expression system on glutathione sepharose 4B beads. Precleared lysates from Jurkat cells (500 µg) were incubated with the beads for 1 hour at 4°C with rotation. Precipitated
complexes were washed 4X in TNSEV buffer, resuspended in Laemmli loading buffer, and run on SDS-PAGE. Western blotting was performed for Bad protein.

*Survival and apoptosis assays:* For number of live cells per milliliter, transfected cells were harvested and incubated with trypan blue at 10% of total volume. Cells excluding the dye were counted on a hemocytometer. For measurement of caspase 3/7 activity, transfected cells were pipetted into a white 384-well plate (Nunc) and incubated with an equal volume of Apo-One reagent (Promega) containing a substrate that is cleaved by caspases 3 and 7. Fluorescence of the cleaved substrate was measured on a Wallac Victor-2 plate reader and is expressed in terms of relative fluorescent light units (RFLU).

**RESULTS**

**Phosphorylation consensus sequences in Bad may be substrates for Pim-1.**

Figure 1 shows the amino acid sequences of human (H) and mouse (M) Bad. The phosphorylation sites of Ser112, Ser136, and Ser155 are named for their position in the mouse protein. These correspond to Ser75, Ser99, and Ser118 in human Bad, respectively. Commercial antibodies made to these sites that recognize human as well as mouse Bad bear the names of the murine numbering system. For the sake of simplicity, this work will do the same.

Serines 112, 136, and 155 are close to the ideal phosphorylation consensus sequence for Pim-1, which is \((R/K)_3\)-X-S/T-X’, where X’ is neither a basic nor a large hydrophobic residue \([22]\). The preferred consensus sequence for Akt is
RXRXRX-S/T [23], and for PKA it is RXS, RRXS, KRXXS, or RXXS [24]. Thus, all three of these kinases may potentially phosphorylate Bad. Experimental results are necessary to show which sites are actually phosphorylated. PKA and Akt have been studied in some detail for the phosphorylation of Bad, but the prospective capability of Pim-1 to do the same is now being explored.

**PKA and Akt inhibitors do not affect Pim-1 activity.** PKA is a major phosphorylator of Ser155 of Bad, and Akt is a major phosphorylator of Ser136 [14]. Both appear to contribute to the phosphorylation of Ser112 [14], although this may be context-dependent. Both PKA and Akt themselves require activation by phosphorylation, and thus one may be at times more active than the other, due to differing survival signals received by the cell. Pim-1 activity, however, has been shown to be regulated primarily by abundance, and does not appear to need a signal from a specific pathway for its kinase activity. In fact, recent work from our laboratory shows that Pim-1 can phosphorylate itself under certain conditions (Zhang and Magnuson, unpublished results). Signals such as those provided by GM-CSF and IL-3 result in an increase in Pim-1 transcription and translation [25].

Akt is activated through phosphorylation by phosphatidylinositol-3 kinase (PI3-K), which is sensitive to the compound wortmannin. Binding of wortmannin to PI3-K prevents the phosphorylation of Akt, keeping it inactive. Protein levels of Akt remain the same after a short treatment with wortmannin, but phosphorylation of Akt is lost [26]. The inhibitor H-89 works by binding directly to PKA, which then is unable to phosphorylate its substrates [27]. It was determined that 2 h treatment with
0.01 μM wortmannin or 10 μM H-89 was sufficient to inhibit their activity in Jurkat cells (data not shown).

Phosphorylation of Bad may be necessary to prevent apoptosis under circumstances when Akt or PKA is not active. Pim-1-mediated Bad phosphorylation may be of primary importance at this time. To assure that Pim-1 kinase activity is not significantly affected by either wortmannin or H-89, and thus can remain active when Akt and PKA are not, luminescent kinase assays were performed. Jurkat cells were treated with wortmannin or DMSO vehicle for 2 h prior to lysis. Pim-1 and Akt were immunoprecipitated and used in an assay with Histone H2B peptide a common substrate. Wortmannin prevented Akt from phosphorylating Histone H2B peptide, as shown by no decrease in ATP levels from “Input” (Figure 2A). The same concentration of wortmannin had only a small effect on Pim-1, as Pim-1 continues to exhibit kinase activity as measured by a decrease in ATP. It is important to note that cells may not express identical endogenous levels of Pim-1 and Akt, so the activity of these two kinases cannot be compared to one another, only themselves. The results in Figure 2A show that wortmannin slightly inhibits Pim-1’s activity, indicating either that PI3-K may contribute somewhat to Pim-1 activation, or that wortmannin may have a direct effect on Pim-1. However, the fact that Pim-1 retains most of its activity in the presence of wortmannin suggests that Pim-1 is not dependent upon PI3-K for its activity. Therefore, Pim-1 may remain active in circumstances where Akt is either blocked or has not been activated.

Next, PKA and Pim-1 were immunoprecipitated from Jurkat cells treated for 2 h with H-89 or DMSO vehicle, and used in a kinase assay with Histone H2B
peptide. PKA activity was almost entirely inhibited by H-89, but Pim-1 was not inhibited at this concentration, as demonstrated by the proximity of the error bars (Figure 2B). Again, the activity of these two kinases must be compared only internally and not one to another. Pim-1 and PKA have some substrates in common, but these results show that Pim-1 may be available for phosphorylating targets when PKA is not.

**Pim-1 phosphorylates Bad peptides.** A submitted work by Lilly *et al.* [6] shows that Pim-1, like Pim-2, can phosphorylate Bad on Ser112. The consensus sequences surrounding Bad serines 136 and 155, however, also appear to be favorable for Pim-1. In order to determine which serine, 112, 136, or 155, provides the best phosphorylation consensus sequence for Pim-1, we generated 17-mer peptides based on those of Masters *et al.* An *in vitro* luminescent kinase assay was performed with wild type GST-Pim-1 and each peptide, with peptides to p21 and Histone H2B as controls. Figure 3A shows that all three peptides were phosphorylated to some extent by wild type GST-Pim-1, and that Ser112 and Ser155 may be more favorable than Ser136. A time course confirms that Ser155 and Ser112 are the most efficiently phosphorylated by Pim-1 (Fig. 3B). These results suggest that Bad may be a good substrate for Pim-1 kinase, and that Ser112 and Ser155 are the most probable phosphorylation sites.

As phosphorylation on Bad Ser155 directly prevents its association with Bcl-xL, we were interested in confirming the results in 2A and 2B with Bad Ser155 peptide rather than Histone H2B peptide. Jurkat cells were pretreated with the
inhibitors H-89 and wortmannin, and immunoprecipitated Pim-1 was used in a kinase assay. Figure 3C demonstrates that phosphorylation of Ser155 by Pim-1 is only slightly inhibited by H-89 or wortmannin, as compared to DMSO vehicle, but overlapping error bars indicate that any inhibition is not significant. Pim-1, therefore, may phosphorylate Bad on Ser155, and this may also occur under circumstances when PKA and Akt are inhibited or unavailable.

**Bad associates with Pim-1.** In order to assay for the physical association of Pim-1 with Bad, GST and GST-Pim-1 bound to glutathione sepharose beads were incubated with lysate from untreated Jurkat cells. Western blotting from this GST-pulldown experiment revealed that endogenous Bad does interact with Pim-1 (Fig. 4A). Furthermore, the addition of 25 – 250 μM Ser155 peptide competes with this binding, as seen with the disappearance of Bad in the GST-pulldown. These data suggest that Pim-1 kinase and Bad associate, and that their interaction takes place, at least in part, at Bad’s phosphorylation site on Ser155.

Next, Jurkat cells transfected with the Pim-1 constructs pEGFP-wt-pim-1 and pEGFP-kd-pim-1 were examined for the in vivo phosphorylation of Bad on Ser155. As seen in Fig. 4B, Bad is phosphorylated on Ser155 in the presence of wild type Pim-1, but not in the presence of kinase dead Pim-1. This indicates that Pim-1 is responsible, at least in part, for the phosphorylation of Bad on Ser155.

**Pim-1 phosphorylates Bad on Ser155 in vivo.** To show an in vivo phosphorylation of GST-Bad by Pim-1, Cos-7 cells were transfected with pEGF-gst-bad and the Pim-
1 constructs pEGFP-gfp, pEGFP-wt-pim-1, or pEGFP-kd-pim-1. Levels of PKA and Akt are very low or undetectable in Cos-7 cells (data not shown), so we did not expect interference from these two kinases. To be sure of their inactivity, the transfected cells were pretreated with H-89 and wortmannin. Cos-7 cells expressing Bad and GFP vector did not show phosphorylation of Bad on Ser155 (Fig. 5A). Neither did cells expressing kinase dead GFP-Pim-1. Wild type GFP-Pim-1, however, phosphorylated GST-Bad on Ser155. This result confirms our above findings and shows that Ser155 of Bad is a substrate for Pim-1, and that this event occurs in vivo.

**Pim-1 is localized to the cytoplasm rather than the mitochondria for interaction with Bad.** Phosphorylation of Bad by Pim-1 on Ser155 would prevent association of Bad with Bcl-xL on the mitochondria. To determine whether this event is likely to occur in the cytoplasm or at a mitochondrial location, Jurkat cells were fractionated, and the cytoplasmic and mitochondrial (and other membrane) fractions were immunoprecipitated for Pim-1 and Bad, with Western blotting of the associating protein. The two proteins appeared to co-immunoprecipitate from the cytoplasm, and not from the mitochondria (Fig. 5B). Thus, it is probable that Pim-1 phosphorylates Bad in the cytoplasm.

**Phosphorylation of Bad by Pim-1 prevents its binding to Bcl-xL and promotes cell survival.** The pro-apoptotic effect of Bad is carried out through its displacement of Bax, which occurs by Bad binding to Bcl-xL. Ser155 of Bad resides in the BH-3
domain responsible for the binding of Bad to Bcl-xL. When Ser155 is phosphorylated, Bad cannot bind to Bcl-xL and promote apoptosis. Pim-1 may contribute to the prevention of apoptosis through phosphorylation of Bad on Ser155. Therefore, it was important to show that phosphorylation by Pim-1 prevents Bad from binding to Bcl-xL. To test this hypothesis, Cos-7 cells were transfected with pEGF-gst-bad and the Pim-1 constructs pEGFP-vector, pEGFP-wt-pim-1, or pEGFP-kd-pim-1 and serum starved to encourage programmed cell death. Cells were harvested and GST-Bad was isolated on glutathione sepharose 4B beads and run on SDS-PAGE. Bcl-xL was detected by Western blotting. Fig. 6A shows that Bcl-xL does not co-precipitate with Bad in the presence of overexpressed wild type Pim-1. Kinase dead Pim-1 allows the interaction of Bad and Bcl-xL, moreso than when only the vector is expressed. This may be because the kinase dead Pim-1 binds to its substrate, Bad, without phosphorylation but with delayed kinetics of release.

Transfected Cos-7 cells expressing GFP kinase dead Pim-1, GFP wild type Pim-1, or each of these plus GST-Bad, were measured for cell survival by trypan blue exclusion. In each case, the presence of wild type Pim-1 resulted in more live cells than kinase dead Pim-1, but when Bad was also expressed, fewer live cells were counted (Fig. 6B). These results indicate that Pim-1’s contribution to cell survival works at least in part through its phosphorylation of Bad. Expression of Bad may lower the threshold for apoptosis.

In order to show that the decrease in surviving cells was indeed due to apoptosis, Cos-7 cells expressing GST-Bad and GFP-Pim-1 constructs were used in
an assay for caspase 3/7 activity. Kinase dead Pim-1 caused the highest level of caspase activation, and cells expressing wild type Pim-1 had the lowest (Fig. 6C). This effect was also apparent when cells were transfected only with Pim-1 constructs and not GST-Bad. Interestingly, the expression of Bad in conjunction with kinase dead Pim-1 and vector increased their caspase 3/7 activity, but in the case of wild type Pim-1, this pro-apoptotic activity was low, even in the presence of Bad. These results demonstrate that the pro-apoptotic activity of Bad can be prevented by phosphorylation by Pim-1 kinase, and that Pim-1’s role as a survival factor is due in part to its inactivation of Bad.

**DISCUSSION**

Pim-1 has been repeatedly implicated in cell survival, but the mechanisms by which it acts must be delineated in order to understand how Pim-1 counters apoptosis. One way that Pim-1 appears to be involved in cell survival is through the phosphorylation of the pro-apoptotic protein Bad. Phosphorylation of Bad on Ser112 and Ser136 has been shown to promote its binding to 14-3-3 proteins [8], and Lilly et al. [13] have shown that Pim-2 can phosphorylate Bad on Ser112. However, the control of Bad’s binding to Bcl-xL is of primary importance in the apoptotic process. We have shown here that Pim-1 can phosphorylate Bad on Ser155, in the domain that controls its interaction with Bcl-xL.

GST-Pim-1 and immunoprecipitated Pim-1 from Jurkat cells can phosphorylate peptides corresponding to serines 112, 136, and 155 of Bad (Fig. 3A). The phosphorylation of Ser136 by Pim-1 appears to be less favorable (Fig. 3B).
Whether phosphorylation on a particular site is performed efficiently may be influenced by cellular context, as well as by sequential phosphorylation of other residues. Chiang et al. found that there was a specific order to the dephosphorylation of Bad by PP2A [19], so it is reasonable that phosphorylation may be subject to a similar process of biochemical favorability.

Co-expression of GST-Bad and wild type GFP-Pim-1 in Cos-7 cells showed that Pim-1 can phosphorylate Bad \textit{in vitro} on Ser155 (Fig. 5A). Similarly, endogenous Bad was phosphorylated on Ser155 in Jurkat cells in the presence of wild type GFP-Pim-1 but not kinase dead (Fig. 4B). This data, showing \textit{in vivo} phosphorylation of Bad as a consequence of Pim-1 expression, taken together with physical association and the \textit{in vitro} kinase assays, allows the conclusion that Bad Ser155 is a substrate for Pim-1.

The phosphorylation of Bad on Ser155, in blocking its interaction with Bcl-xL, helps to prevent apoptosis. This event provides an explanation for Pim-1’s ability to promote cell survival. In cells transfected with GST-Bad and with GFP-Pim-1, wild type and kinase dead, the wild type Pim-1 prevented GST-Bad from associating with Bcl-xL. Cells expressing kinase dead Pim-1 did show binding between GST-Bad and Bcl-xL (Fig. 6A), as did those transfected with GST-Bad and only the GFP vector. As Bad is detected at very low endogenous levels in most cell types, as compared to Bcl-2 and Bcl-xL, its pro-apoptotic effect may be one of threshold, and not an all-or-nothing effect. It may only be necessary for a certain percentage of the Bcl-2 or Bcl-xL in a cell to bind Bad and release Bax in order to
begin an apoptotic cascade. Indeed, overexpression of Bad by transfection has been shown to increase cell death [28].

An assay for caspase 3/7 activity in the cells expressing GST-Bad and GFP-Pim-1 demonstrated that the interaction between Bad and Bcl-xl does in fact have a direct effect on apoptosis, as the prevention of Bad from binding to Bcl-xl through phosphorylation increased cell survival (Fig. 6C). Expression of the Pim-1 and vector constructs alone displayed less caspase activity and more cell survival than when GST-Bad was co-expressed, indicating that Pim-1’s survival activity works, at least in part, through the phosphorylation of Bad. A model (Fig. 7) summarizes this work.

ACKNOWLEDGEMENTS

We thank Dr. Mike Lilly (Loma Linda University) for the pEGB-bad expression vector, and Gerhard Munske (Washington State University) for making the peptides.
REFERENCES


### Sequence alignment of human and murine Bad

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*Underlined areas show these serine residues.*

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**Figure 1. Putative Pim-1 phosphorylation sites on Bad protein.** Murine and human Bad were aligned with LALIGN (www.ch.embnet.org/software/LALIGN_form.html). Sites for serine phosphorylation in mouse 112, 136, and 155 Bad correspond to serines 75, 99, and 118 in human Bad. Underlined areas show these serine residues.
Figure 2 (A-B). Effect of Akt and PKA inhibitors on Pim-1 activity.  
A. Jurkat cells were pretreated with 0.01 μM wortmannin for 2 h prior to lysis. Akt and Pim-1 were immunoprecipitated and used in a luminescent kinase assay with Histone H2B peptide. A drop in ATP levels (RLU) indicates kinase activity.  
B. Jurkat cells were pretreated with 10 μM H-89 for 2 h prior to lysis. PKA and Pim-1 were immunoprecipitated and used in a luminescent kinase assay with Histone H2B peptide.
Figure 3 (A-B). Kinase activity of Pim-1 toward p21, H2B, and Bad peptides. A. GST-Pim-1 (0.25 µg) was incubated with ATP and 400 nM peptide for 30 min. at 30°C. Luminescence was used to measure ATP levels in relative light units (RLU).

B. The assay in (A) was repeated using the three Bad peptides, and samples were incubated for 0, 15, 30, or 60 min to show decline in ATP levels over time.
Figure 3 C. Kinase activity of Pim-1 toward Bad peptide. Jurkat cells were pretreated for 2 h with 0.01 µM wortmannin, 10 µM H-89, or an equivalent volume of DMSO. Pim-1 was immunoprecipitated and used in a luminescent kinase assay with ATP and 400 nM Bad Ser155 peptide. A drop in ATP levels (RLU) indicates kinase activity.
**Figure 4 (A-B). Association of endogenous Bad with Pim-1.**

A. Bacterially expressed GST-Pim-1 bound to glutathione sepharose beads was incubated with lysates from untreated Jurkat cells for 1 h at 4°C. The indicated concentrations of Bad Ser155 peptide were added as a competitor to binding. Western blotting was performed for Bad.

B. Jurkat cells were transfected with *pEGFP-kd-pim-1* or *pEGFP-wt-pim-1*. Western blotting was performed for GFP (Pim-1) and Phosphoserine 155 Bad. A reprobe for total Bad confirms the presence of unphosphorylated forms or those phosphorylated on other residues.
A

GFP construct: KD vector WT

GFP

Total GST-Bad

Phospho-Ser155 GST-Bad

B

Fraction: Cytoplasmic Membrane

IP: Bad
WB: Pim-1

IP: Pim-1
WB: Bad

Pim-1

Bad

Figure 5 (A-B). Phosphorylation of GST-Bad by Pim-1. A. Cos-7 cells were transfected with pEGB-bad and either pEGFP-kd-pim-1, pEGFP-wt-pim-1, or pEGFP vector. Western blotting was performed for GFP (Pim-1 or vector) or Phosphoserine 155 Bad. A reprobe for total Bad confirms the presence of unphosphorylated forms or those phosphorylated on other residues.
B. Jurkat cells were separated into cytoplasmic and membrane fractions and immunoprecipitated for either Pim-1 or Bad. Western blotting was performed for Bad or Pim-1 co-precipitating proteins.
Figure 6 (A-B). Effect of Bad phosphorylation by Pim-1 on Bcl-xl binding and cell survival.  A. Cos-7 cells were transfected with pEGB-bad and either pEGFP-kd-pim-1, pEGFP-wt-pim-1, or pEGFP vector.  GST-Bad was isolated on glutathione sepharose beads, and Western blotting was performed for Bad and Bcl-xl.
B. Cells from (A), as well as cells transfected with Pim-1 constructs and no Bad, were analyzed by trypan blue exclusion.  Only live, adherent cells were collected.  The graph shows the number of live cells per milliliter in each sample.
Figure 6C. Effect of Bad phosphorylation by Pim-1 on cell survival. Live cells from (A), as well as cells transfected with Pim-1 constructs and no Bad, were assayed for caspase 3/7 activity by incubating whole cells with reagent (caspase 3/7 substrate and buffer) in a 384-well plate. Relative fluorescent light units (RFLU) resulting from substrate cleavage were measured on a plate reader.
Figure 7. Model: A proposed model for cell survival when Pim-1 phosphorylates Bad on Ser155 and binding to Bcl-xl is prevented.
Proto-oncogenes encode proteins that are involved in normal cell regulation. When disregulated, though, their normal functions may lead to tumorigenesis. No mutated forms of Pim-1 have been reported, but it is often found overexpressed in cancer cells. The factors controlling Pim-1’s expression are therefore important for understanding how Pim-1 contributes to the malignant phenotype.

Wild type Pim-1 is closely associated with the survival of cells in culture. Survival rates increase when Pim-1 overexpressing cells encounter pro-apoptotic stimuli, including genotoxins (1), growth-factor withdrawal (2), and lpr/lpr background (3). The use of a kinase dead Pim-1, in which a crucial residue in the ATP-binding domain is mutated (K67∆M), typically results in increased cell death. In our hands, transfection of cells with wild type Pim-1 often causes slightly more death than transfection with an empty vector, but still not nearly as much death as is caused by kinase dead Pim-1. Additionally, the creation of stably transfected Pim-1 cell lines is very difficult. This may indicate that cells can only tolerate so much Pim-1 expression without having experienced other changes that would allow such expression to continue. It is interesting that Eμ-pim-1 transgenic mice are susceptible to malignancies of the hematopoietic system. If Pim-1’s contribution to tumor formation is by acting as a survival factor, there is clearly incentive for the elimination of superfluous Pim-1 on the organismal level, in order to prevent cancer from forming. This seems to be kept in check on the cellular level when possible. Transfected cells may eliminate their expression vectors or die, but tumor cell lines already expressing high levels of Pim-1 (K562, BV173, etc.) have also accumulated many other changes, which may allow Pim-1 to contribute to survival rather than
having the cells go through apoptosis. Thus, tumor cell lines permit biochemical studies of Pim-1 translational and post-translational regulation that may be more challenging in normal cells. Microarray studies demonstrate that gene expression profiles in various immortalized cell lines are more similar to each other than they are to patient-derived cancers of the same tissue origin (Scott Ness, personal communication). Any trials that are eventually performed to down-regulate Pim-1 expression may therefore benefit from being tested on a wide array of cell types, including normal and malignant samples that are not immortalized into cell lines. Future experiments may reveal whether healthy cells are more likely to down-regulate Pim-1 than are tumor cells, and by what mechanism they do so.

In Chapters Two and Three, the regulation of Pim-1 levels is explored by examination of tumor cells treated with a mild heat shock. Heat shock, or hyperthermia, is a treatment that induces apoptosis by creating cellular stress (4). Many proteins inside the cell are denatured by this process. Heat shock chaperones aid in refolding, and the proteasome degrades those proteins that are beyond repair. Pim-1 is one protein that is upregulated during heat shock, and we have shown that it retains kinase activity. It may also continue to phosphorylate substrates like p21. Although we do not know whether Pim-1 is unfolded during heat shock, we have shown in Chapter Three that it binds to Hsp90 under this circumstance. In fact, at least a fraction of Pim-1 appears to be bound to Hsp90 even in untreated cells. This may be the only fraction of Pim-1 that remains after heat shock, and is stabilized, or else all Pim-1 in the cell may bind to Hsp90 upon heat shock. Whether p21 is also
bound to a chaperone is unknown, but because p21 is a highly disordered protein, its nature is to change folding conformation depending upon cellular context.

Heat shock also causes p21 to bind procaspase 3 (Cpp32), preventing its activation by cleavage (5). This aids in preventing apoptosis. Heat shocked cells may thus avoid apoptosis when p21 is able to bind Cpp32, an event that is caused by phosphorylation of p21. Because p21 is a substrate for Pim-1, this ties Pim-1 to anti-apoptosis by the prevention of caspase activation. Another mechanism for Pim-1 in cell survival with a similar result was presented in Chapter Four; the phosphorylation and inactivation of pro-apoptotic Bad by Pim-1. Activation of Bad would also cause apoptosis at the mitochondria through the eventual cleavage of procaspases. Cellular cross-talk may mean that many paths lead from Pim-1 to cell survival through a mitochondrial mechanism.

Heat shock protein 90 appears to stabilize Pim-1 protein in K562 and BV173 tumor cells (Chapter 3). As Pim-1 has not been reported to have any mutations in these or other cells, it is reasonable to suppose that Pim-1 may also bind to Hsp90 in healthy cells. However, it is unknown whether Hsp90 contributes substantially to Pim-1 stability in this case. Modifications of Hsp90 itself may exist in some tumor cell lines. A recent report (6) demonstrates that the inhibitor geldanamycin binds to the ATP binding site of Hsp90 in tumor cells much more efficiently than it does in normal cells, suggesting that Hsp90 itself may be somehow altered. Another theory is that tumor cells may become dependent upon high levels of Hsp90 for their survival. Proteins like Pim-1 being kept at high expression levels by Hsp90 may explain in part how these cancer cells continue to survive despite the checkpoint
controls used to eliminate unhealthy cells. It is possible that the excess or altered Hsp90 in tumor cells stabilizes Pim-1 expression moreso than it would in healthy cells. Certainly Pim-1 is not the only protein found highly expressed in tumor cells, nor is it the only one that binds to Hsp90. However, Pim-1 has repeatedly been shown to be involved in cell survival. Furthermore, signal transduction pathways such as the ones constitutively activated by Bcr-Abl (another Hsp90 client) may actually upregulate Pim-1, perhaps through Stat5-mediated transcription (7). It may not be necessary to dissect cause and effect in cases such as these; the only way that Bcr-Abl itself contributes to malignancy is through its tyrosine kinase activity that activates the pathways that induce anti-apoptotic events. Pim-1 would certainly qualify as one of these.

Heat shock protein 70 is associated with ubiquitylated Pim-1, suggesting that it may not stabilize Pim-1, but may instead shuttle it to the 26S proteasome for degradation. Hsp70 has been shown also to stabilize some proteins, but this does not appear to be the case for Pim-1. The specificity of Hsp70, or any other chaperone, is influenced by its binding partners. Hsp70 is known to have cofactors that act as E3 ubiquitin ligases (8), which help to form bonds between ubiquitin chains and the internal lysines of proteins targeted for degradation. It would be interesting to know, in the case of severe heat shock that leads to necrosis, whether Pim-1 associates with Hsp70 rather than Hsp90, and is degraded. There would be little reason for the stabilization of survival proteins once the cell is committed to death.

In Chapter Four, we delineate a mechanism for Pim-1 kinase in cell survival: the phosphorylation of Bad. Bad is a BH-3 only protein related to Bcl-2, but with
quite the opposite effect. While overexpression of Bcl-2 (or Bcl-xL) leads to cell survival and high resistance of pro-apoptotic stimuli, overexpression of Bad causes rapid apoptosis, even without external treatment (9). In all cell types we have seen, either Bcl-2 or Bcl-xL is present at much higher levels than Bad. Bad appears to exist at low levels and is difficult to detect by Western blot. Indeed, nearly all studies of Bad to date utilize expression vectors in order to raise Bad to experimentally detectable levels. Unfortunately, this increases death in the very cells the experimenter is examining. It is reasonable to assume that Bad is naturally at lower levels than Bcl-2 or Bcl-xL because there is a threshold effect for apoptosis, as opposed to an all-or-nothing effect. Not every molecule of Bcl-2 or Bcl-xL must be bound to Bad in order for apoptosis to occur. Binding of Bad releases Bax for homodimerization and the opening of pores in the mitochondrial membrane, so only enough Bax needs to be displaced by Bad that some cytochrome c is released. The level of membrane porousness and release of cytochrome c may be the critical factors in whether or not a cell goes through apoptosis. Therefore, as long as the majority of Bad molecules are phosphorylated by Pim-1 or similar kinases, there is enough Bad sequestered away from Bcl-2 and Bcl-xL to prevent Bax from exerting its effect to any dangerous degree.

It is necessary at this time to point out that the maintenance of cell survival is not always a desirable event. Particularly in tumor cells, a main focus of current research is on how to prevent cell survival and sensitize cells to apoptosis. Knowing how Pim-1 permits survival is essential to this work. Many studies, including those herein, have demonstrated that expression of kinase dead Pim-1 actually induced
apoptosis in tumor cells. Whether it does the same in normal, healthy cells is unknown, but the effect of \textit{pim-1} knockout in mice is nonlethal. Of course, this may be due to the selection of hardier cells early in embryogenesis. Nonetheless, it will be interesting to discover whether Pim-1 can be targeted for inhibition or destruction to the detriment of tumor cells, while leaving healthy cells intact. As is the case with Hsp90, cancers often become dependent upon the increased expression of certain proteins, otherwise they would not be able to become growth-factor independent, or to be multidrug resistant. Elimination of such proteins may remove a necessary foundation from tumor cells, and allow them to become more responsive to chemo- and radiation therapies.
References:


