The PIM-2 Kinase Phosphorylates BAD on Serine 112 and Reverses BAD-induced Cell Death*

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Hematopoietic growth factors mediate the survival and proliferation of blood-forming cells, but the mechanisms through which these proteins produce their effects are incompletely known. Recent studies have identified the pim family of kinases as mediators of cytokine-dependent survival signals. Several studies have identified substrates for the pim-1 kinase, but little is known about the other family members, pim-2 and pim-3. We have investigated potential functions for the pim-2 kinase in factor-dependent murine hematopoietic cells. We find that pim-2 mRNA and protein expression are regulated by cytokines similarly to pim-1. Three PIM-2 protein isoforms are produced in cytokine-treated cells. All three forms are active kinases, and the short (PIM-2(34 kDa)) form is the most active at enhancing survival of FDCP1 cells after cytokine withdrawal. This pro-survival function involves inhibition of apoptosis and caspase activation. Enforced expression of PIM-2(34 kDa) kinase does not appear to regulate expression of BCL-2, BCL-xL, BIM, or BAX proteins. However, the kinase can phosphorylate the pro-apoptotic protein BAD on serine 112, which accounts in part for its ability to reverse Bad-induced cell death. Our results indicate that pim-2 functions similarly to pim-1 as a pro-survival kinase and suggest that BAD is a legitimate PIM-2 substrate.

Among the signaling intermediates implicated in hematopoietic cell survival is the pim-1 serine/threonine kinase (1). pim-1 expression is regulated by hematopoietic growth factors (2, 3). Furthermore, the kinase enhances factor-independent survival of hematopoietic cells, in part through a bcl-2-dependent pathway (4, 5). The pim-1 gene product is a true oncoprotein, in that its enforced expression in transgenic mice leads to an increased incidence of tumors (6, 7). Potential PIM-1 substrates include proteins active in cell cycle regulation and transcription, such as Cdc25 (8), PAP-1 (9), HP1 (10), NFATc1 (11), PTP-U2S (12), and the c-myc transcriptional co-activator p100 (13).

pim-1 belongs to a kinase family that has three members: pim-1 (1), pim-2 (14), and pim-3 (15). These related enzymes show substantial homology, but differ in their tissue expression (16). It is unknown to what extent the various family members differ in their biochemical effects. The pim-2 gene was identified as a frequent site for retroviral insertion in experimental lymphomas, both in normal and pim-1-deficient mice (14). The pim-2 gene also encodes a cytoplasmic serine threonine kinase whose expression is regulated by hematopoietic cytokines (14–17). Like the Pim-1 kinase, there are multiple isoforms of PIM-2 protein (three in the mouse and potentially two in humans) due to the use of the alternative translation start codon, CTG (18).

Functional similarity between pim-1 and pim-2 gene products has been inferred from studies of transgenic mice. Both pim-1 and pim-2 induce lymphomas alone or in synergy with c-myc (6, 14). Furthermore, the relatively weak phenotype associated with disruption of the pim-1 gene (19) suggests that its functions may be largely assumed by related molecules, such as the highly homologous pim-2 gene. Little is known, however, of the biochemical and molecular events through which the PIM-2 kinase may act. We have therefore sought to characterize the effect of PIM-2 protein in immortalized hematopoietic cells and identify potential molecular events modulated by this kinase.

Our data indicate that PIM-2 kinase inhibits apoptosis induced by various stimuli. Furthermore, we implicate phosphorylation of BAD as a possible mechanism through which the enzyme may inhibit apoptosis. These data demonstrate that enforced expression of the pim-2 gene produces effects similar to those identified for pim-1 in immortalized hematopoietic cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The IL-3-dependent murine hematopoietic cell lines FDCP1 (obtained from Dr. Scott Boswell, Indiana University) and 32Dcl3 (obtained from Dr. Irv Bernstein, Fred Hutchinson Cancer Research Center) were used for this study. Cells were cultured in RPMI1640 medium with 10% (v/v) iron-supplemented calf serum, and 10% (v/v) medium conditioned by the WEHI-3B cell line (a convenient source of IL-3). Jurkat cells (ATCC) were grown in RPMI 1640 medium

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§§ The abbreviations used are: IL, interleukin; GST, glutathione S-transferase; CS, calf serum; neo, neomycin; SCF, stem cell factor; ELISA, enzyme-linked immunosorbent assay; Chaps, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; Mops, 4-morpholinepropanesulfonic acid.

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with 10% iron-supplemented calf serum. All cells were maintained at 37 °C in 5% CO₂. HeLa, NIH3T3, and U2OS cells were maintained in McCoy’s medium plus 10% iron-supplemented calf serum.

**Plasmids and cDNA Clones**—The cDNA for the short form of murine PIM-2 protein (PIM-2(34 kDa)) was cloned from an FDCP1 cell cDNA library by PCR, using the published sequence for primer design. The cDNAs for long (PIM-2(40 kDa)) and medium (PIM-2(38 kDa)) forms were cloned by a similar approach from a murine spleen library (Stratagene). The cDNA for kinase-inactive PIM-2 was prepared by introducing a K61A mutation into the cDNA for both short and long forms of murine PIM-2 (long or short PIM-2(K61A)), by a PCR-based technique. In each case the start codon was changed to ATG, and was preceded by an idealized Kozak sequence. All cDNAs were ligated into the mamma-

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**Western Blotting**—Cells were collected, washed in phosphate-buff-
ered saline, and lysed in either 1% Triton lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM Na₃VO₄, 20 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin) by vortexing, or in Chaps lysis buffer (50 mM Pipes/NaOH, pH 6.5, 2% Chaps, 0.5 mM EDTA, 20 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) by three freeze/thaw cycles. Lyseate protein was then measured (BCA method; Pierce), and lysates were mixed with an equal volume of 2× Laemmli buffer. Equal amounts of total protein were added to each well for electrophoresis in 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were blocked, then incubated with primary antibodies, followed by incubation with horseradish peroxidase-linked secondary antibodies. Antibody-antigen complexes were detected using chemiluminescence (Pierce). The following primary antibodies were used: anti-PIM-2 (described above, Anti-BAD (Santa Cruz Biotechnology), anti-BCL-2 (Santa Cruz Biotechnology). Antibody-antigen complexes were detected using chemiluminescence (Pierce). The following primary antibodies were used: anti-PIM-2 (described above, Anti-BAD (Santa Cruz Biotechnology), anti-BCL-2 (Santa Cruz Biotechnology), anti-BIM (Chemicon), anti-procaspase 3 (Santa Cruz Biotechnology), anti-active caspase 3 (Cell Signaling), goat anti-BAD (Santa Cruz Biotechnology); for transfected PIM-2 proteins, mouse monoclonal anti-BAD (BD Transduction Laboratories; for endogenous BAD), and anti-phospho-

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**Construction of Stable Cell Lines**—FDCP1 and 32Dc13 cell lines stably expressing Pim-2 constructs were prepared by electroporation. Ectopic expression of the PIM-2 isoforms was verified by immunoblotting (see below).

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**FDCP1 cells stably expressing neo or pim-2(34 kDa) constructs were further transfected by electroporation with pEGF/Bad and pPKG/puro to produce additional cell lines. Puromycin-resistant clones were examined for GST/BAD expression by immunoblotting. Positive clones were then utilized for biochemical and cell growth experiments.

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**Cell Survival and Apoptosis Assays**—For enumeration of surviving cells, the cells were seeded complete medium and analyzed by FACS analysis for expression of the dsRed marker transgene.

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Expression of pim-2 mRNA and Protein Is Regulated by Cytokines in a Similar Pattern to That of pim-1—The ability of pim-2 to functionally compensate for the lack of pim-1 (14) suggests also that the expression of these two genes might be regulated in a similar fashion. To test this possibility, we cultured FDCP1 cells without IL-3 for 4 h and then stimulated them with a variety of cytokines for an additional 2 h. Northern blotting showed that both pim-1 and pim-2 mRNAs were induced by IL-3 and to a lesser extent by IL-4 (Fig. 1). By contrast, stem cell factor and IL-1α were unable to induce expression of either message.

To determine if PIM-2 protein levels correlated with pim-2 mRNA expression, we developed a specific anti-PIM-2 monoclonal antibody. The 1D12 antibody identified PIM-2 protein with no detection of PIM-1 in immunoblots of transiently transfected HeLa cells (Fig. 2). FDCP1 cells expressed three PIM-2 forms, as expected (14), with the intermediate form being most abundant. PIM-2 protein levels closely paralleled the changes in mRNA (Fig. 1). FDCP1 cells deprived of cytokine had little PIM-2. IL-3 treatment induced a marked increase in the kinase protein. Prolonged exposure of the blot showed that IL-4 minimally induced expression of PIM-2, but stem cell factor and IL-1α failed to promote expression of the kinase (not shown).

Transfection of Murine Hematopoietic Cells with pim-2 Expression Plasmids—To identify potential biological effects mediated by pim-2, we expressed the various isoforms, as well as an inactive kinase, in two factor-dependent murine hematopoietic cells, FDCP1 and 32Dcl3. In each case enforced expression resulted in a marked increase in the corresponding isoform protein (Fig. 3A; 32D cell data not shown). Levels of the PIM-2 (38 kDa) protein in both cell lines were somewhat lower than for the other two forms. Expression of the kinase-dead long PIM-2(K61A) mutant was also detected. Expression of the transgene products did not decrease after IL-3 withdrawal in the FDCP1-derived cells or in 32Dcl3-based cell lines.

The half-life of the expressed transgene proteins in stably transfected FDCP1 cells was measured after cycloheximide blockade of new protein synthesis (Fig. 3, B and C). The PIM-2 (38 kDa) protein exhibited an extremely short half-life (10–15 min), whereas the PIM-2 (40 kDa) was somewhat longer (about 30 min). In contrast, PIM-2 (34 kDa) had a half-life in excess of 60 min. Endogenous PIM-2 proteins were not seen due to the short exposure times for the films.

PIM-2 Enhances Survival of Hematopoietic Cells after IL-3 Withdrawal or Doxorubicin Treatment—Both FD/neo and 32D/neo cells died in the absence of IL-3. Cells expressing pim-2 transgenes also died, but at a slower rate (Fig. 4; 32D cell data not shown) with some viable cells persisting for several days. The three isoforms of PIM-2 showed different survival effects. The shortest form (PIM-2(34 kDa)) was the most active isoform at inhibiting cell death, resulting in little change in cell number for up to 70 h of cytokine deprivation. However, the long and medium forms of PIM-2 were less active at delaying cell death. As expected, a kinase-dead K61A mutant did not promote factor-independent survival and actually appeared to enhance death during the initial observation period. In this respect the K61A mutant acts similarly to a dominant-negative PIM-1 protein, which also increases cell death during cytokine withdrawal (5).

The pim-1 kinase has been implicated in resistance to genotoxic stresses such as ionizing radiation and cytotoxic drugs (20). We found that enforced expression of the active PIM-2 (34 kDa) kinase also enhanced resistance to doxorubicin in FDCP1 cells, leading to a 2-fold increase in the ID₅₀ for that agent (data not shown), compared with FD/neo cells.

PIM-2 Inhibits Apoptosis and the Activation of Caspase 3 Associated with IL-3 Deprivation—Cell death in hematopoietic cells following cytokine withdrawal is thought to result from apoptosis, or programmed cell death. We have previously demonstrated that the pim-1 kinase inhibits the onset of apoptosis...
following IL-3 removal in FDCP1 cells (4). Thus we questioned if the survival effects of PIM-2 protein resulted from decreased apoptosis. Because the greatest pro-survival effects were associated with enforced expression of the PIM-2 (34 kDa) protein, subsequent studies focused on this kinase isoform. Apoptotic cells were measured by flow cytometry in FD/neo and FD/Pim-2(34 kDa) cells following IL-3 withdrawal. FD/neo cells had an increased proportion of cells within which were annexin V-positive and propidium iodide-negative, consistent with an increase in apoptosis (Fig. 5 A).

A mitochondria-mediated mechanism of caspase activation is involved in apoptosis induced by genotoxic drugs and growth factor withdrawal. We have examined whether overexpression of PIM-2 can regulate the activation of caspase 3 (Fig. 5 B). IL-3 deprivation led to a decrease in the 34-kDa caspase 3 precursor in FD/neo cells, accompanied by a concomitant increase in the 17-kDa active fragment. In contrast, cleavage of caspase 3 precursor was remarkably inhibited by the overexpression of PIM-2 in FD/Pim-2(34 kDa) cells. These data demonstrate that pim-2 prevents cells from undergoing apoptosis by inhibiting caspase 3 activation.

**PIM-2 Phosphorylates BAD Both in Vitro and in Vivo and Inhibits Apoptotic Effect of BAD**—To study the mechanism of how pim-2 inhibits caspase 3 activation, we examined its effects on the expression or activity of several members of the Bcl-2 family. We found that pim-2 promotes the phosphorylation of BAD both in vitro and in vivo which inhibits its apoptotic effect.

**Fig. 3.** Immunoblots of pim-2 transgene expression in stably-transfected FDCP1 cells. A, transgene expression. Lane 1, untransfected FDCP1 cells cultured without IL-3 x 6 h; lane 2, untransfected cells maintained in IL-3; lanes 3–7, transfected FDCP1 cells cultured without IL-3 x 6 h. B, half-life estimation for PIM-2 proteins. Transfected FDCP1 cells growing in IL-3 were treated with cycloheximide (20 μg/ml) for the indicated periods. Equal numbers of cells were then lysed and analyzed for PIM-2 proteins by immunoblotting with anti-PIM-2 monoclonal antibody. C, decay curves for PIM-2 proteins. Video densitometry was performed on bands of immunoblots in B.
BCL-2 family of survival proteins. FD.neo and FD/Pim-2(34 kDa) cells were examined by immunoblotting after culturing them in the presence or absence of IL-3. Neither IL-3 withdrawal nor enforced expression of Pim-2 led to changes in expression of BCL-2 or BAX. By contrast, IL-3 withdrawal was associated with decreased expression of BCL-xL and increased expression of BIM, but PIM-2 did not affect their expression any further (data not shown).

We have previously seen that the PIM-1 protein can antagonize the effects of BAX protein expression, independently of bcl-2 expression (5). This suggested that PIM-1 could regulate the activity of other pro-apoptotic BCL-2 family proteins, such as BAD (5). Indeed we have seen direct evidence that PIM-1 kinase can phosphorylate BAD.2 We therefore asked if the PIM-2 kinase could phosphorylate, and thereby inactivate the BAD protein, as well. IL-3 removal leads to rapid dephosphorylation of BAD, and onset of apoptosis in some factor-dependent hematopoietic cells (21). The phosphorylation consensus sequences for pim kinases are similar to those of AKT and cAMP-dependent (protein kinase A) kinases (22). Because these kinases phosphorylate BAD, it seemed possible that PIM-2 could do so as well.

PIM-2 proteins were purified by immunoprecipitation from U2OS cells transfected with expression plasmids for either wild-type or kinase inactive PIM-2(34 kDa) proteins. The enzymes were then assayed in an immunocomplex kinase assay for their ability to phosphorylate recombinant GST-BAD protein. Anti-PIM-2 immunoprecipitates from wild-type kinase transfecants were able to phosphorylate GST-BAD, whereas anti-prostate-specific antibody immunoprecipitates (isotype-matched negative control antibody) failed to do so (Fig. 6A). Recombinant GST protein alone was not phosphorylated by the immunoprecipitated kinase. To characterize the reaction further, we subjected PIM-2-phosphorylated GST-BAD protein to immunoblotting with anti-phospho-BAD antibodies (data not shown). PIM-2 appears to selectively phosphorylate GST-BAD on serine 112, because the phosphorylated substrate did not react with antibodies specific for serine 136 or serine 155 phosphorylation.

Because the PIM-2 isoforms differed in their ability to support survival of cytokine-deprived FDCP1 cells, we directly determined their relative kinase activity. Proteins for the three PIM-2 isoforms were expressed by in vitro translation then purified by immunoprecipitation. When used in an in vitro kinase reaction, all were able to phosphorylate GST-BAD on serine 112. The kinase activity of the 40-kDa form toward that substrate was about 22% lower than that of the 38-kDa form, a significant difference (p = 0.03 for no difference). Not surprisingly, the K61A mutant 34-kDa protein had no detectable kinase activity, again suggesting that the minimal activity seen in panel B was due to coprecipitating kinases. The kinase-dead long (40-kDa) form also lacked detectable ability to phosphorylate BAD (data not shown).

We have previously noted that PIM-1 protein can phosphorylate BAD.2 To compare the relative efficiency of the two kinases for the same substrate, we prepared 6-His-PIM-2(34 kDa) protein along with 6-His-PIM-1(33 kDa). The freshly prepared kinases were utilized immediately in an ELISA-format recombinant kinase reaction with GST-BAD as the substrate. The recombinant PIM-2 protein phosphorylated GST-BAD in the ELISA format. Time-course experiments showed that the initial reaction rate typically was linear for at least 18 min (Fig. 7A). As expected, with longer incubation the reaction rate declined. A series of time-course experiments was performed, with differing quantity of substrate in the wells. The initial reaction rate (measured over the first 12 min) was then compared with substrate concentration by Lineweaver-Burk analysis (Fig. 7B). The resulting linear plot confirmed that the Pim-2 enzyme demonstrated kinetics consistent with Michae-
lis-Menten predictions. It was also apparent from the Lineweaver-Burk plot that the lines for PIM-2 and PIM-1 were parallel, and therefore the slopes ($K_m/V_{max}$) were equal. This suggests that PIM-1 and PIM-2 may be equivalent kinases for phosphorylating GST-BAD protein. True $V_{max}$ values could not be calculated from the ELISA-derived data, because the reaction rate is described in terms of change of color ($d(A_{450})/dt$) rather than the more traditional millimoles/liter/min. The apparent $V_{max}$ and $T_m$ values can be used only as relative indices to compare PIM-1 and PIM-2 but not for comparisons with values for other kinases, as recorded in the literature. Non-linear regression analysis of the Michaelis-Menten data showed no significant differences in the estimated $V_{max}$ and $T_m$ for the two kinases in either of the two experiments (data not shown).

We next examined the phosphorylation of BAD by PIM-2 in vivo, using a transient transfection system. U2OS and 3T3 cells were transfected with expression plasmids for PIM-2(34 kDa) and GST-BAD, alone or in combination (Fig. 8). In the presence of PIM-2, GST-BAD was heavily phosphorylated on serine 112 in both cell lines. Interestingly, in U2OS cells there was additional phosphorylation of serine 136 and serine 155. In contrast, in 3T3 cells only serine 112 was phosphorylated in the presence of PIM-2. Expression of the $pim-2$ transgene product was much higher in the U2OS cells, however.

We also stably co-transfected FD/neo and FD/Pim-2(34 kDa) cells with a GST-BAD mammalian expression construct and a puromycin resistance plasmid. A GST-BAD fusion protein was expressed, because we were unable to isolate stable lines expressing wild-type BAD proteins. Presumably the GST-BAD fusion protein has somewhat less death-promoting activity than the wild-type BAD protein, allowing stably expressing...
clones to be identified. Puromycin-resistant clones were examined for expression of GST-BAD by immunoblotting with an anti-BAD antibody. Several clones of FD/Pim-2(34 kDa)/Bad and FD/neo/Bad cells were isolated, and three clones of each were pooled. Enforced expression of GST-BAD in FD/neo cells enhanced death, compared with FD/neo/puro cells (Fig. 9). However, enforced expression of PIM-2 protein was able to overcome the exaggerated death effect of GST-BAD.

We therefore examined the extent of GST-BAD phosphorylation in the stable transfectants after IL-3 withdrawal (Fig. 10A). FD/neo/Bad cells grown in IL-3 demonstrated phosphorylation of GST-Bad on serine 112. Phosphorylation decreased after withdrawal of IL-3. The FD/Pim-2(34 kDa)/Bad cells had markedly increased phosphorylation of GST-BAD, which changed little after IL-3 withdrawal. Phosphorylation of BAD on serine 136 or serine 155 was not detected under these conditions.

Finally we sought to determine if ambient amounts of PIM-2 could phosphorylate endogenous BAD (Fig. 10B). FDCP1 cells stably expressing either neo, PIM-2(34 kDa) protein, or long PIM-2(K61A) protein were deprived of IL-3 for 10 h. Whole cell lysates were then used for immunoblotting. The endogenous BAD protein was detected in all samples. In three independent experiments, BAD showed slight but reproducible phosphorylation on serine 112 in FD/neo cells. FD/Pim-2(34 kDa) had greatly increased phosphorylation of endogenous BAD. In contrast, cells with kinase-dead PIM-2 had no detectable phosphorylation of endogenous BAD.

PIM-2 Inhibits BAD-induced Cell Death in Part through Phosphorylation of BAD on Serine 112—To determine if phosphorylation of BAD on serine 112 played a role in the ability of PIM-2 to inhibit BAD-induced cell death, we sought to co-express PIM-2 and a mutant GST-BAD that could not be phosphorylated on serine 112. However, we were unable to isolate FDCP1-based cell lines with these characteristics. The GST-BAD(S112A) proteins were invariably truncated (data not shown). We therefore chose to transiently co-express PIM-2 and GST-BAD or GST-BAD(S112A) in Jurkat T-cells (Fig. 11). This cell line was used because it can be transfected more efficiently than FDCP1 and has previously been used for studies of BAD kinases. Cells were also transfected with an expression plasmid for the fluorescent protein DsRed (Clontech) to
Fig. 10. Phosphorylation of BAD proteins by PIM-2 in stably transfected FDCP1 cells. A, phosphorylation of GST-BAD in FD.neo/Bad and FD/Pim2(34 kDa)/Bad cells. Pools of stable transfectants (three clones each) were cultured with or without cytokine for 6 h, as indicated. Whole cell lysates were then prepared, and 50 μg of lysate protein was loaded on each lane. Blots were then probed with the indicated antibodies. B, phosphorylation of endogenous BAD by exogenous, endogenous PIM-2. FDCP1 cells, stably transfected with neo, PIM-2(34 kDa), or kinase-dead long PIM-2(K61A), were deprived of IL-3 and cultured for 10 h. Whole cell lysates were then prepared and used for immunoblotting as indicated. 250 μg of whole cell lysate was used for each lane of the blot.

Fig. 11. Dependence on S112 phosphorylation for reversal of GST-BAD-induced death by PIM-2. Jurkat cells were transiently transfected with the indicated plasmid. pCDNA3 plasmid was included as needed to ensure that each transfection contained the same amount of total plasmid DNA. 24 h later they were analyzed by FACS analysis for expression of the transfection marker protein dsRed. Results are normalized (mean percentage of FL2-high cells from transfections with dsRed plasmid alone = 1.0). For each experiment, measurements were made in triplicate, and averaged. Each bar represents the mean ± S.E. from three independent experiments.

mark transfected cells. Twenty-four hours later the transfected cells were analyzed by flow cytometry. Between 1.35 and 4.29% of cells transfected with a DeRed plasmid alone were highly red fluorescent. Adding the GST-BAD expression plasmids markedly reduced the number of red fluorescent cells, demonstrating that the two GST-BAD proteins equally induced cell death in the transfected cells. In contrast, co-transfection with a pim-2 expression plasmid increased the number of red fluorescent cells, reflecting the ability of the kinase to promote survival of the transfected population. Cells were also transfected simultaneously with plasmids encoding PIM-2(34 kDa) and GST-BAD or GST-BAD(S112A), along with the DeRed expression plasmid. Enforced expression of PIM-2(34 kDa) protein was markedly better at preventing cell death in the presence of GST-BAD protein, compared with its ability to reverse the effects of the GST-BAD(S112A) protein (p = 0.026 for no difference). These data demonstrate that antagonism of GST-BAD by PIM-2(34 kDa) depends at least in part on the ability of the kinase to phosphorylate GST-BAD on serine 112.

DISCUSSION

IL-3, granulocyte-macrophage colony-stimulating factor, and related growth factors induce proliferation and differentiation of hematopoietic cells and protect them from apoptosis. We have shown that this family of cytokines selectively induces the expression of pim family kinases in hematopoietic cells. The pim kinases show tissue-specific expression (16). Few studies have examined functional or biochemical redundancy within the family, however. pim-1 and pim-2 appear to be regulated similarly (2, 3, 17). Because these kinases share functional activities (6, 14, 17), it is likely that they may also act through similar biochemical pathways. A recent report documented that both PIM-1 and PIM-2 can phosphorylate the SOCS-1 protein (23). With this exception, however, no studies have identified potential PIM-2 substrates. We now report that PIM-2 can phosphorylate and inactivate BAD and that its efficiency as a BAD kinase resembles that of PIM-1. Although we cannot conclude that PIM-1 and PIM-2 are equally efficient for all substrates, these data do suggest that any unique roles for the different kinases may result from their variable tissue expression (16).

Three PIM-2 isoforms are apparent in FDCP1 and 32Dcl3 cells. The endogenous PIM-2(38 kDa) protein is more abundant than the other isoforms. This parallels the results reported from in vitro translation of the pim-2 cDNA (14). In contrast, when expressed as a transgene, PIM-2(38 kDa) was the least abundant form. Under these conditions protein expression likely reflects in part kinase stability, with the 38-kDa form of PIM-2 being less stable, and consequently expressed at a lower level. In aggregate these data suggest that transcriptional and translational regulation may both play an important role in the relative levels of the various isoforms in the natural state. In the pim-2 mRNA sequence, the translational start site AUG for PIM-2(34 kDa) is preceded by UCCUC, whereas the alternative start codon CUG for PIM-2(40 kDa) is preceded by UUGGGG. For PIM-2(38 kDa) the start codon is preceded by UCCUCC, whereas the alternative start codon CUG for PIM-2(40 kDa) is preceded by UUGGGG. For PIM-2(38 kDa) the start codon is preceded by UCCUCC, whereas the alternative start codon CUG for PIM-2(40 kDa) is preceded by UUGGGG. For PIM-2(38 kDa) the start codon is preceded by UCCUCC, whereas the alternative start codon CUG for PIM-2(40 kDa) is preceded by UUGGGG. For PIM-2(38 kDa) the start codon is preceded by UCCUCC, whereas the alternative start codon CUG for PIM-2(40 kDa) is preceded by UUGGGG. For PIM-2(38 kDa) the start codon is preceded by UCCUCC, whereas the alternative start codon CUG for PIM-2(40 kDa) is preceded by UUGGGG. For PIM-2(38 kDa) the start codon is preceded by UCCUCC, whereas the alternative start codon CUG for PIM-2(40 kDa) is preceded by UUGGGG.
The biological and biochemical effects of these three isoforms differ as well. When the pure proteins were examined with an in vitro kinase assay, the 34- and 38-kDa forms showed similar activity, whereas the 40-kDa form was noticeably less active. As expected the K61A mutant enzymes were inactive. It is not clear why the 40-kDa form is less active than the shorter enzymes. Possibly the additional sequences in the 40-kDa form constitute an autoinhibitory sequence. Because the 40- and 34-kDa forms were expressed at similar levels in the stably transfected FDCP1 cells, it is likely that the intrinsic differences in kinase activity accounts for the impaired survival effect of the longer kinase. The 34- and 38-kDa PIM-2 proteins showed similar ability to phosphorylate BAD in vitro, yet the 38-kDa enzyme was slightly less active at prolonging survival in cytokine-deprived FDCP1 cells. In this situation, the lower level of expression of PIM-2/38 kDa in the stably transfected cells, compared with that of PIM-2/34 kDa, may account for the superior survival effect of the latter kinase.

The mechanism through which pim-2 acts to promote cell survival and inhibit programmed cell death have not been previously characterized. Our previous studies of the pim-1 kinase documented the involvement of bcl-2 family members (5). Because of the strong homology between the two kinases, they may act through similar biochemical processes. We have previously observed that the PIM-1 kinase can phosphorylate BAD and report here that the PIM-2 kinase can act similarly. PIM-2 kinase, immunoprecipitated from transfected cells, was able to directly phosphorylate a GST-BAD substrate in vitro, on serine 112. In contrast, in U2OS cells transiently transfected with both pim-2 and gst-bad expression plasmids, the substrate was phosphorylated on serine 112, serine 136, and serine 155. These additional phosphorylations may be the result of PIM-2 activating other kinases, rather than a direct effect of PIM-2 protein itself. Certainly the in vitro kinase reactions, which are known to encourage promiscuous phosphorylations, did not show detectable serine 136 or serine 155 phosphorylation. In addition, we have seen that co-transfection of a dominant-negative abt construct, along with pim-2 and gst-bad plasmids, markedly decreases the serine 136 phosphorylation of the substrate (data not shown). We feel that this potential transactivation of AKT (and serine 155 kinases) by PIM-2 is unlikely to be of physiological significance. No serine 136 or serine 155 phosphorylation was seen in an NIH3T3 transient transfection system, in which expressed levels of the transgenes were lower than those seen in U2OS cells. Furthermore we found no evidence of serine 136 or serine 155 phosphorylation in stable FDCP1 cells co-expressing the two cDNAs. Thus it appears that serine 112 is the preferred phosphorylation site on BAD, for the PIM-2 kinase. Minimal phosphorylation at other sites likely does not mediate a significant part of the PIM-2 effect.

Serine 112 of BAD appears to be the preferred phosphate acceptor site for kinase reactions involving both PIM-1 and PIM-2. The sequence surrounding serine 112 includes several positively charged amino acids, but only distantly resemble a consensus PIM phosphorylation site (22). Several other known PIM-1 substrates also have atypical phosphorylation sites; thus it is not clear how comprehensive the described consensus sequences are for predicting PIM substrates. PIM-2 now joins several other enzymes as known kinases for BAD (serine 112). These include protein kinase A (24), JNK1 kinase (25), p90/RSK kinase (26), and PAK1 kinase (27). It is not clear if ambient levels of all of these other kinases can phosphorylate endogenous BAD protein. Because our data show that enforced expression of a kinase-inactive PIM-2 protein both decreases phosphorylation of endogenous BAD and shortens cell survival relative to that of neo-expressing cells (which retain partial BAD phosphorylation), we can identify BAD as a legitimate substrate for PIM-2 kinase expressed at ambient levels, as well as when overexpressed in a test system.

Enforced expression of PIM-2 was able to overcome the death-promoting effects of GST-BAD in both stable and transiently transfected cells. However, our inability to isolate FDCP1 stably co-expressing PIM-2 and a mutant GST-BAD(S112A) suggested that phosphorylation of GST-BAD by the PIM-2 kinase on serine 112 was important for this reversal. This was directly confirmed in the transient transfection experiments. Enforced expression of either GST-BAD or GST-BAD(S112A) similarly reduced the number of successfully transfected cells, whereas PIM-2 increased marker transgene expression. Co-expression of the cDNAs reversed the death-enhancing effects of both of the GST-BAD constructs. However, reversal of the GST-BAD-induced death was much more efficient than correction of the GST-BAD(S112A) toxicity. While confirming the need of PIM-2 to phosphorylate BAD on serine 112 for maximal effects, these data also suggest that other molecular targets may be involved. Indeed some studies question the role of BAD in survival of cytokine-deprived hematopoietic cells (28, 29). The temporal correlation between decreased phosphorylation of endogenous BAD in the presence of enforced expression of kinase-dead PIM-2, and enhanced cell death (Fig. 4, 10), suggest that BAD plays some role in survival of these cells, and that it likely mediates part of the survival effect of PIM-2. We anticipate, however, that pim kinases regulate a variety of survival pathways. Recently, PIM-1 has been found to phosphorylate and regulate the activity of the p21/Waf1 protein, a key mediator of p53-dependent programmed cell death (30). We have performed a large number of genetic screens for PIM substrates and partners and have found many candidates, several of which can be implicated in survival responses. Thus it is likely that many additional interactions between PIM family kinases and survival pathways will be identified in the future.

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