

Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser¹¹² gatekeeper site

Teija L.T. Aho^{a,c}, Jouko Sandholm^a, Katriina J. Peltola^{a,c}, Harri P. Mankonen^{a,1},
Michael Lilly^b, Päivi J. Koskinen^{a,*}

^aTurku Centre for Biotechnology, University of Turku/Åbo Akademi University, Tykistökatu 6 B, 20520 Turku, Finland

^bCenter for Molecular Biology and Gene Therapy, Loma Linda University School of Medicine, Loma Linda, CA 92354, USA

^cTurku Graduate School of Biomedical Sciences, Finland

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Abstract Constitutive expression of the Pim-1 kinase prolongs survival of cytokine-deprived FDCP1 cells, partly via maintenance of Bcl-2 expression. Here, we show that Pim-1 colocalizes and physically interacts with the pro-apoptotic Bad protein and phosphorylates it in vitro on serine 112, which is a gatekeeper site for its inactivation. Furthermore, wild-type Pim-1, but not a kinase-deficient mutant, enhances phosphorylation of this site in FDCP1 cells and protects cells from the pro-apoptotic effects of Bad. Our results suggest that phosphorylation of Bad by Pim-1 is one of several mechanisms via which the Pim-1 kinase can enhance Bcl-2 activity and promote cell survival.

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1. Introduction

The *pim-1* proto-oncogene encodes a serine/threonine kinase with three alternatively translated murine isoforms of 33, 34 and 44 kDa [1]. *pim-1* is mainly expressed in cells of hematopoietic origin, where its transcription can be induced by multiple cytokines such as interleukins IL-2, IL-3 and IL-6 [2,3]. We have previously demonstrated that the Pim-1 kinase can in turn participate in cytokine-induced signal transduction by regulating activities of several transcription factors such as c-Myb [4], NFATc1 [5] and signal transducer and activator of transcription 5 [6].

Pim-1 has been shown to protect hematopoietic cells from cell death caused by cytokine withdrawal, glucocorticoids or genotoxins [7–9]. While the anti-apoptotic mechanisms of Pim-1 are still largely unknown, we have previously shown that the Pim-1-promoted survival of IL-3-deprived FDCP1 cells is as-

sociated with sustained expression of *bcl-2* mRNA and protein levels, and that coexpression of antisense *bcl-2* reduces the protective effects of the shorter Pim-1 isoforms [10]. These results suggest involvement of additional mechanisms, possibly targeting other Bcl-2 family members, such as Bad, which is a pro-apoptotic protein regulated by phosphorylation [11]. Unphosphorylated Bad binds and thereby inactivates anti-apoptotic family members, primarily Bcl-X_L but also Bcl-2. Cytokine-dependent phosphorylation of Bad on Ser¹¹², Ser¹³⁶ and/or Ser¹⁵⁵ by several kinases such as Akt, protein kinase A or Rsk impairs its binding to Bcl-X_L and leads to sequestration of Bad from the surface of mitochondria to the cytosol by the 14-3-3 protein [12–18]. Bad phosphorylation is a reversible phenomenon antagonized by several protein phosphatases such as PP2A [19]. Thus, diverse signaling pathways can converge at the level of Bad phosphorylation in controlling cell death or survival.

Here, we show that the Pim-1 kinase can physically interact with Bad and phosphorylate it on Ser¹¹² both under in vitro and in vivo conditions. Pim-1 can also protect cytokine-deprived FDCP1 cells from Bad-promoted apoptosis, suggesting that phosphorylation of Bad by Pim-1 is important, although probably not sufficient for its ability to enhance cell survival after cytokine withdrawal.

2. Materials and methods

2.1. Plasmids

Prokaryotic glutathione *S*-transferase (GST)-Pim-1 fusion vectors expressing the wild-type murine protein or the kinase-deficient K67M mutant have been described previously [5] as also pcDNA3-*pim-1*-FLAG [4]. SV40 promoter-driven constructs encoding full-length Pim-1 (pSV-*pim-1*) or an N-terminally truncated mutant (pSV-*NT81*) were prepared by PCR from pLTR-*pim-1* [5] into the pSV-poly vector [20]. Prokaryotic GST-Bad constructs were generated by transferring full-length murine *bad* cDNA from pEBG-mBad (New England Biolabs) into the pGEX-2T vector (Amersham Biosciences). Mutagenesis of Ser¹¹² and Ser¹³⁶ to alanine residues in both prokaryotic and eukaryotic GST-Bad vectors were introduced by PCR using the QuikChange kit (Stratagene).

2.2. Cell culture and transfections

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Roswell Park Medical Institute medium 1640 with equal supplements was used to grow murine myeloid FDCP1 cell lines expressing neomycin

* Corresponding author. Fax: +358-2-333-8000.

E-mail address: paivi.koskinen@btk.utu.fi (P.J. Koskinen).

¹ Present address: Department of Physiology, University of Turku, Finland.

Abbreviations: IL, interleukin; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Neo, neomycin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

(FDCP1/Neo), the murine 44 kD Pim-1 protein (FDCP1/Pim44) or an N-terminally truncated mutant of Pim-1 (FDCP1/NT81) [10]. 10% WEHI-conditioned medium was used as a source of IL-3 for the IL-3-dependent FDCP1 cells. For transient transfections, cells were electroporated with GenePulser II (Bio-Rad).

2.3. Immunoblotting

100 µg aliquots of protein samples were mixed with buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄ and 1 mM PMSF). GST-Bad was precipitated with glutathione-Sepharose beads (Amersham Biosciences) at 4 °C. The precipitates were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF-membrane (Millipore). For Western blotting, the membranes were blocked, incubated first with Bad or Phospho-Bad (Ser¹¹², Ser¹³⁶ or Ser¹⁵⁵) primary antisera and then with secondary horse-radish peroxidase-linked antibodies (all from Cell Signaling Technology). Proteins were visualized using the ECL+plus kit (Amersham Biosciences). Pim-1 proteins were similarly recognized with the 19F7 anti-Pim-1 antibody (Santa Cruz) and endogenous Bad with anti-Bad antibody (BD Biosciences Pharmingen).

2.4. Phosphorylation assays

In vitro kinase assays were carried out as described [5]. For two-dimensional tryptic phosphopeptide mapping, protein gels were fixed in 50% MeOH, dried on cellophane and exposed to film. Proteins of interest were cut out, digested overnight with 10 µg/ml TPCK-treated trypsin (Sigma–Aldrich), and the resulting phosphopeptides separated on two dimensions on thin layer cellulose plates [21]. For in vivo assays, cells were metabolically labeled with 1 mCi/ml of ³²P-labeled orthophosphate (Amersham Biosciences) in phosphate-free DMEM at 37 °C for 2 h. GST-Bad precipitates prepared as above were fractionated by SDS-PAGE and ³²P-labeled proteins were detected by autoradiography. Parallel samples were prepared without ³²P-labeled orthophosphate and analysed by Western blotting.

2.5. Protein interaction assays

For in vitro binding assays, purified GST or GST-Bad proteins bound to glutathione-Sepharose beads were incubated first with 1% BSA to block non-specific binding and then with in vitro translated, ³⁵S-labeled Pim-1 protein in binding buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 2 mM CaCl₂, 0.5% Triton X-100, 1 mM PMSF, 1 mM DTT, 1 mM Na₃VO₄ and 2 µg/ml aprotinin). After washes, bound proteins were separated by SDS-PAGE and visualized by Coomassie staining followed by autoradiography. For coprecipitation assays, FLAG-tagged proteins from transfected COS-7 cells were immunoprecipitated with anti-FLAG antibody (Kodak) as described [5], separated by SDS-PAGE and immobilized onto PVDF membrane. Coprecipitating GST-Bad proteins were analysed by immunoblotting with anti-Bad antiserum and the intensities of GST-Bad versus IgG protein bands were determined by Imaging Research MCID M5+ image analysis software.

2.6. Immunofluorescence assays

COS-7 cells were transfected with Pim-1 and GST-Bad expression vectors and plated on coverslips. 48 h after transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Nonidet P-40. Subcellular distributions of Pim-1 and GST-Bad proteins were visualized by staining with anti-Pim-1 antibody or anti-Bad antiserum followed by TRITC-conjugated anti-mouse or FITC-conjugated anti-rabbit antibodies (Zymed), respectively. Images were captured with Zeiss LSM510 confocal microscope and scattergram plots were acquired with Zeiss LSM image analyzer program.

2.7. Apoptosis assays

FDCP1/Neo and FDCP/Pim44 cells, transiently transfected with GST-Bad constructs, were centrifuged through 2 ml of FCS to remove cells that had died during electroporation. 24 h after transfection, cells were washed once with PBS and IL-3 was withdrawn from the medium. For 72 h, cell samples were collected at multiple time-points with 8 h intervals. Dead cells were stained with propidium iodide (PI), counted with the FACScan flow cytometer (Becton Dickinson) and further analysed with the CellQuest software (Becton Dickinson).

3. Results and discussion

3.1. Pim-1 phosphorylates Bad on Ser¹¹² in vitro

Sequences surrounding Ser¹¹² (L–R–R–M–S) and Ser¹³⁶ (P–R–P–K–S) of Bad slightly resemble the Pim-1 consensus target site (K/R–K/R–R–K/R–L–S–X) identified by the use of synthetic peptides [22]. To find out whether the Pim-1 kinase can phosphorylate Bad and thereby inactivate it, we performed in vitro kinase assays with bacterially expressed GST-fusion proteins of Bad and the 33 kDa isoform of Pim-1. Wild-type GST-Pim-1 phosphorylated both itself and GST-Bad, but not the GST only control, whereas no phosphate was incorporated in the presence of the kinase-deficient K67M mutant of Pim-1 (Fig. 1A). To be able to identify the phosphorylation target site(s) for Pim-1 in Bad, we mutated Ser¹¹² and/or Ser¹³⁶ of GST-Bad to alanine residues and used the mutants as sub-

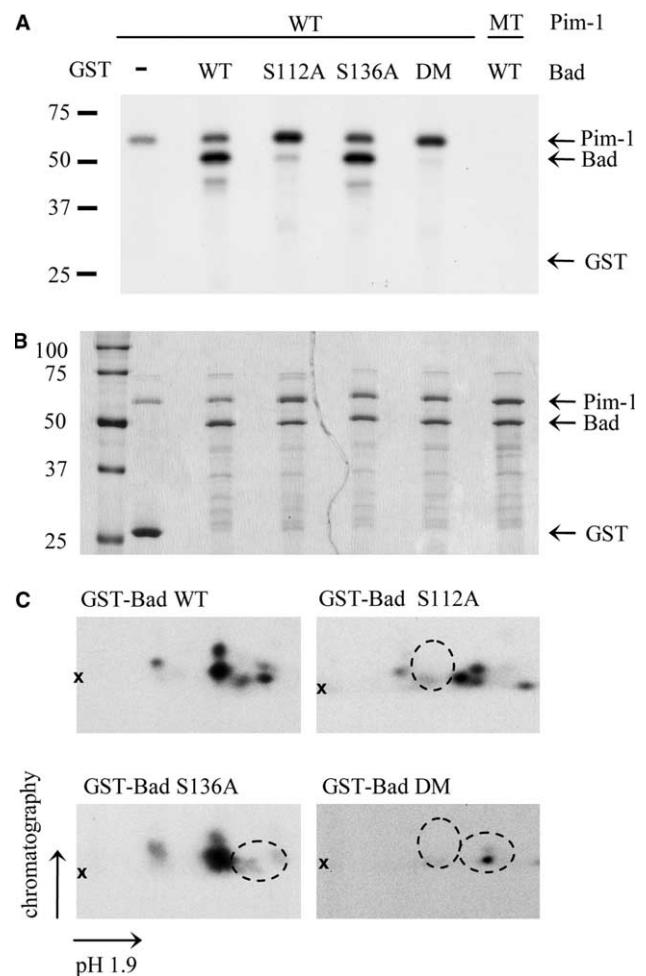


Fig. 1. Pim-1 phosphorylates Bad on Ser¹¹² in vitro. (A) Bacterially produced wild-type (WT), S112A or S136A single mutants or S112A/S136A double mutant (DM) of GST-Bad were incubated with wild-type (WT) or kinase-deficient K67M mutant (MT) of GST-Pim-1 in an in vitro kinase assay. The phosphorylation products were analysed by SDS-PAGE followed by autoradiography. GST alone (-) was used as a negative control. (B) The amounts of proteins loaded on the gel were visualized by silver staining. (C) Comparison of two-dimensional tryptic phosphopeptide maps from the indicated samples of in vitro phosphorylated wild-type or mutant GST-Bad proteins. The point of application is marked with an x and the spots missing from mutant samples with dotted circles.

strates for GST-Pim-1. The Ser¹³⁶ mutation did not have any major effect, whereas phosphorylation of Bad by Pim-1 was strongly prohibited by the Ser¹¹² mutation and almost completely abolished by a double mutation. Silver staining of the gel confirmed that all the Pim and Bad fusion proteins were expressed to an equivalent extent (Fig. 1B).

Very similar results were obtained, when the in vitro phosphorylated GST-Bad proteins were cut out of the gel, subjected to trypsin digestion and analysed by two-dimensional phosphopeptide mapping (Fig. 1C). Several Pim-1-induced phosphopeptide spots were observed for the wild-type GST-Bad, the major ones of which disappeared with the S112A mutation and the minor ones with the S136A mutation. Hardly any spots were detectable anymore when both sites had been mutated. Taken together, our in vitro results indicate that Bad is a direct substrate for Pim-1, and that Pim-1 primarily phosphorylates Bad on Ser¹¹².

3.2. Pim-1 enhances phosphorylation of Bad on Ser¹¹² also within cells

To examine the ability of Pim-1 to phosphorylate Bad also under in vivo conditions, COS-7 cells were transiently transfected with expression vectors for GST-Bad and the 33 kDa isoform of Pim-1. 24 h later, serum was withdrawn from half of the transfected cells for 18 h to downregulate serum-induced kinase activities. Cells grown in the presence or absence of serum were then metabolically labeled with ³²P-labeled orthophosphate for 2 h. In the presence of serum, equivalent amounts of phosphate were incorporated into GST-Bad whether or not cells coexpressed Pim-1 (Fig. 2A, left lanes). However, when serum had been withdrawn prior to labeling, fresh phosphorylation of GST-Bad was diminished more effi-

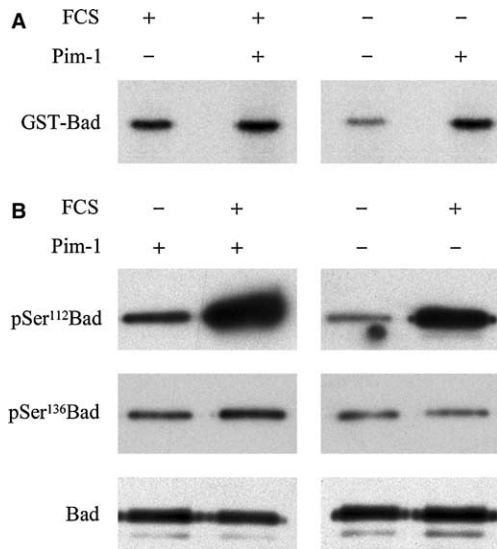


Fig. 2. Pim-1 maintains Bad Ser¹¹² phosphorylation in serum-starved COS-7 cells. (A) GST-Bad (pEBG-mBad, 10 µg) was transiently expressed in COS-7 cells without or with 33 kD Pim-1 (pSV-poly or pSV-pim-1, 4 µg). 24 h after transfection, serum was withdrawn from the cells for 18 h and cells labeled with ³²P-labeled orthophosphate for 2 h. GST-Bad was then precipitated from cell lysates and its phosphorylation status visualized by autoradiography. (B) Steady-state phosphorylation of GST-Bad was visualized by immunoblotting with phospho-Bad (Ser¹¹² or Ser¹³⁶) antisera from parallel unlabeled samples. Equal loading was confirmed with anti-Bad antiserum.

ciently from control cells than from Pim-1-expressing cells (Fig. 2A, right lanes). To determine whether this was due to phosphorylation of Bad by Pim-1 on Ser¹¹² and/or Ser¹³⁶, the steady-state phosphorylation levels of these sites were analysed from parallel samples by Western blotting with phosphorylation site-specific antibodies against Bad (Fig. 2B). Results from this analysis indicated that Ser¹¹² phosphorylation was much more pronounced in Pim-1-expressing cells than in the control cells, both in the absence and presence of serum (Fig. 2B). By contrast, Pim-1 did not significantly affect Ser¹³⁶ phosphorylation or GST-Bad expression levels. Thus, these results implicate that Pim-1 is actively involved in intracellular phosphorylation of Bad and that Ser¹¹² of Bad is the preferential target site for Pim-1 also under in vivo conditions.

To further investigate phosphorylation of Bad by Pim-1 under more physiological conditions, we carried out Western blot analyses with IL-3-dependent FDCP1 cell lines expressing Neo (FDCP1/Neo) or the 44 kDa isoform of Pim-1 (FDCP1/

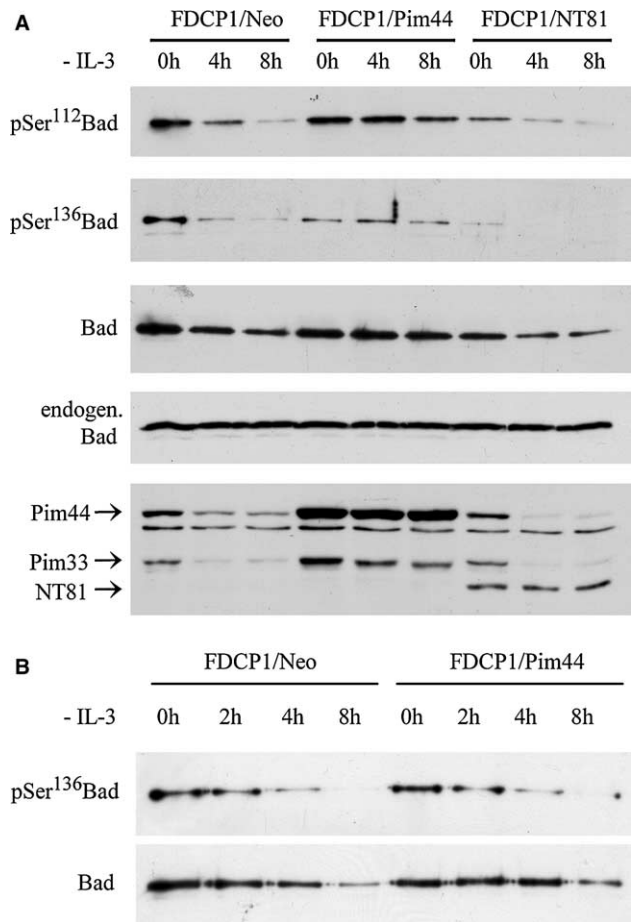


Fig. 3. Constitutive expression of Pim-1 enhances Bad Ser¹¹² phosphorylation in FDCP1 cells. (A) FDCP1/Neo, FDCP1/Pim44 or FDCP1/NT81 cells were transiently transfected with the GST-Bad expression vector (pEBG-mBad, 10 µg). 24 h after transfection, IL-3 was withdrawn and samples collected at indicated time-points. GST-Bad was precipitated from cell lysates and its phosphorylation status analysed with Phospho-Bad (Ser¹¹² or Ser¹³⁶) antisera. Ectopic or endogenous Bad or Pim-1 expression levels were measured by anti-Bad and anti-Pim-1 antisera. (B) FDCP1/Neo and FDCP1/Pim44 cells were transfected with S112A mutant expression vector for GST-Bad (pEBG-mBad S112A, 10 µg), and samples collected and analysed as above.

Pim44). Phosphospecific Western blot analyses of cells transiently transfected with the GST-Bad expression vector revealed that in the presence of IL-3, both Ser¹¹² and Ser¹³⁶ of GST-Bad were phosphorylated to a similar extent in FDCP1/Pim44 cells as in the FDCP1/Neo control cells (Fig. 3A and data not shown). However, these two sites remained persistently phosphorylated in FDCP1/Pim44 cells even after IL-3 withdrawal, while both of them were rapidly dephosphorylated

in IL-3-deprived FDCP1/Neo cells. By contrast, a third regulatory serine of Bad, Ser¹⁵⁵ was phosphorylated to an equivalent extent in all samples, irrespective of the presence or absence of Pim-1 or IL-3 (data not shown). The ectopic or endogenous expression levels of Bad were also not affected (Fig. 3A). Interestingly, when FDCP1/NT81 cells expressing an N-terminally truncated, kinase-deficient mutant of Pim-1 were analysed in parallel, phosphorylation of Bad on both

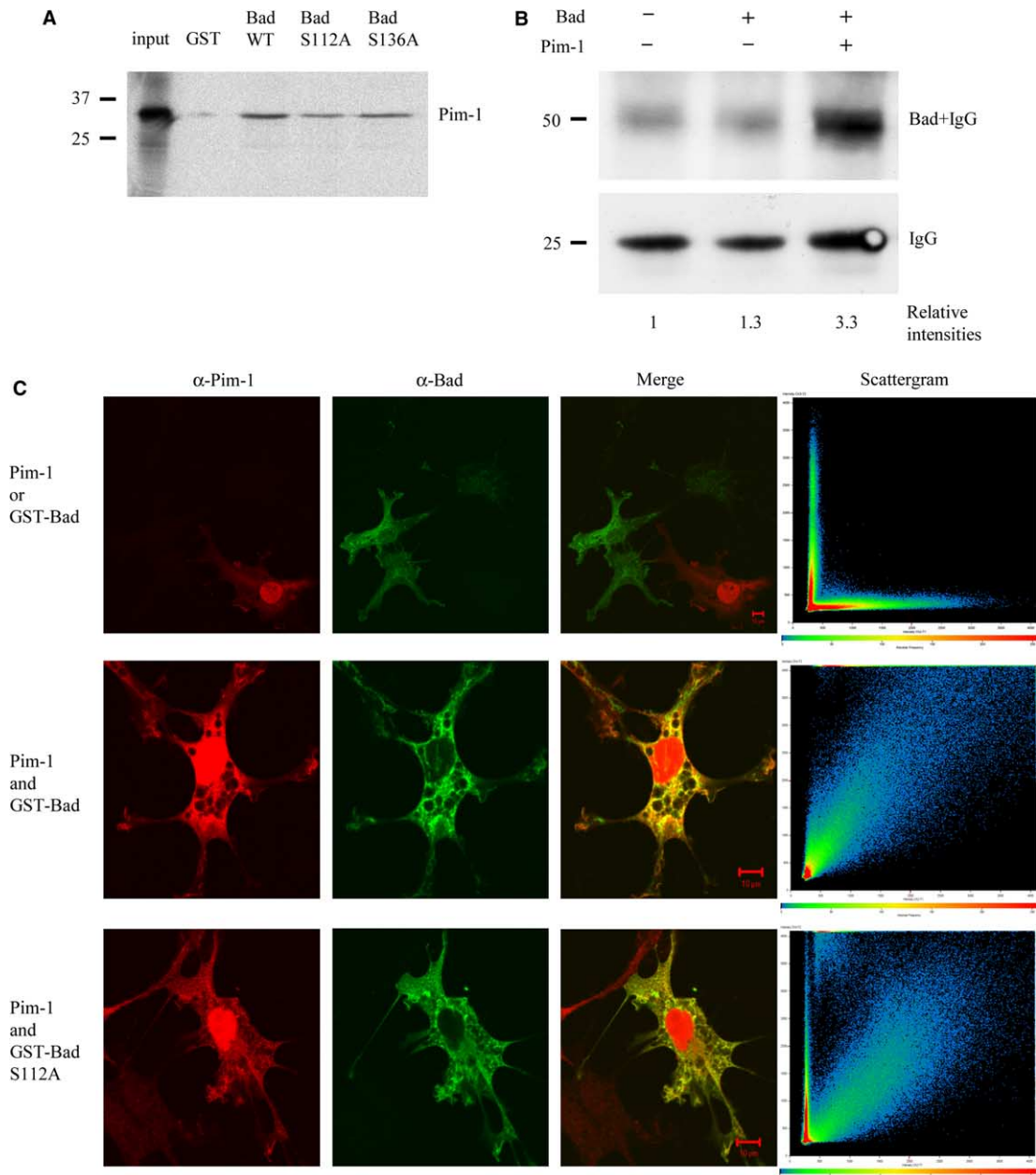


Fig. 4. Pim-1 interacts with Bad in vitro and in vivo. (A) Wild-type Pim-1 protein was ³⁵S-labeled by in vitro translation and incubated with bacterially expressed GST or wild-type or mutant GST-Bad proteins. Pim-1 proteins bound to glutathione-Sepharose beads via GST-Bad were analysed by SDS-PAGE followed by autoradiography. Ten percent of the in vitro translated Pim protein sample was included as a loading control. (B) GST-Bad (pEBG-mBad, 6 μg) was expressed in COS-7 cells without or with FLAG-tagged Pim-1 (pcDNA3-*pim-1*-FLAG, 1 μg). GST-Bad proteins co-precipitating with FLAG-tagged proteins were analysed by immunoblotting. Shown are also the 25 kD and 50 kD IgG proteins, the latter one of which comigrated with GST-Bad. The relative intensities of the upper versus lower bands are indicated under the figure. (C) Pim-1 (pSV-*pim-1*, 2 μg) was coexpressed with wild-type GST-Bad (pEBG-mBad, 5 μg) or the corresponding S112A mutant in COS-7 cells and the subcellular localization of these proteins was visualized by indirect immunofluorescence. Shown are single- or double-positive cells stained with anti-Pim-1 (red) or anti-Bad (green) antisera. Colocalization (yellow) observed in merged images was confirmed by scattergrams, where green and red fluorescence are shown in the X- and Y-axis, respectively. Bar represents 10 μm.

Ser¹¹² and Ser¹³⁶ was significantly reduced even in the presence of IL-3. Since we have previously demonstrated that the NT81 mutant sequesters wild-type Pim-1 protein into inactive oligomers [5] and even promotes apoptotic cell death after cytokine withdrawal [10], our current results suggest that the NT81 mutant had again acted in a dominant-negative fashion to inhibit phosphorylation of Bad by the endogenously expressed Pim-1 kinase. Very similar results were obtained, when FDCP1/Neo cells were transiently cotransfected with expression vectors for GST-Bad and the 33 kDa isoform of Pim-1 or the kinase-deficient NT81 mutant (data not shown).

In the presence of IL-3, the ectopic expression levels for the wild-type or mutant Pim-1 protein were not vastly higher than those for the endogenously expressed Pim-1 isoforms (Fig. 3A). As expected, the endogenous Pim44 and Pim33 proteins were downregulated upon IL-3 withdrawal, while the levels for the ectopically expressed Pim44 or NT81 proteins remained constant (Fig. 3A). However, it should be noted that the endogenous 44 kDa isoform of Pim-1 is more stable than the 33 kDa isoform [1] and therefore disappears more slowly.

While our data clearly indicated that both the 44 and 33 kDa isoforms of Pim-1 are able to phosphorylate Bad on Ser¹¹² under both in vitro and in vivo conditions, it remained unclear why also Ser¹³⁶ phosphorylation of Bad was enhanced by wild-type Pim-1 in FDCP1 cells and reduced by the Pim-1 mutant. Therefore, we investigated whether Pim-1 could affect Ser¹³⁶ phosphorylation also when Ser¹¹² was mutated. We expressed S112A or S136A mutants of GST-Bad in FDCP1/Neo and FDCP1/Pim44 cells and carried out phosphospecific Western blot analyses with IL-3-deprived samples. When Ser¹¹² was replaced with an alanine residue, Ser¹³⁶ was similarly dephosphorylated in both cell lines (Fig. 3B), indicating that Pim-1 did not directly target Ser¹³⁶ under in vivo conditions. Thus, the observed enhancement of Ser¹³⁶ phosphorylation on wild-type Bad was dependent on prior phosphorylation of Ser¹¹² by Pim-1 and was most likely due to activities of other kinases targeting Ser¹³⁶ such as Akt. More surprisingly, when phosphorylation status of the S136A mutant was analysed, no phosphate had been incorporated into Ser¹¹² even in the

presence of Pim-1 (data not shown), suggesting that there is cross-talk to both directions between the two regulatory phosphorylation sites. Our conclusions are further supported by the recent finding that Ser¹¹² acts as a gatekeeper site that needs to be dephosphorylated first to allow phosphatases to access the other regulatory sites of Bad [23]. Thus, if dephosphorylation of Ser¹¹² is prevented by either inhibitors against Ser¹¹² phosphatases such as PP2A or by constitutive activation of Ser¹¹² kinases such as Pim-1, Ser¹³⁶ cannot be properly dephosphorylated. Phosphorylation of Ser¹³⁶ in turn is essential for the ability of 14–3–3 to bind Bad and to protect also the other sites of Bad from dephosphorylation [15,23].

3.3. Pim-1 physically interacts and colocalizes with Bad

To examine the ability of Pim-1 to interact with wild-type or mutant Bad proteins, we carried out in vitro binding assays with bacterially expressed GST or GST-Bad proteins and ³⁵S-labeled, in vitro translated 33 kDa isoform of Pim-1 (Fig. 4A). Hardly any binding was observed with GST alone, while both wild-type GST-Bad and the S112A or S136A mutant proteins bound Pim-1 at equivalent levels. These results indicate that Pim-1 and Bad are able to directly interact with each other and that the interaction is not dependent on the ability of Pim-1 to phosphorylate Bad.

We next analysed intracellular interactions within COS-7 cells transiently coexpressing GST-Bad and FLAG-tagged 33 kDa isoform of Pim-1. Immunoprecipitation with FLAG antibody followed by Western blotting with Bad antibody revealed that GST-Bad could be coprecipitated from cells coexpressing Pim-FLAG, but not from control cells where only GST-Bad, Pim-FLAG or corresponding empty vectors were expressed (Fig. 4B and data not shown).

To determine whether Pim-1 and GST-Bad proteins can colocalize within transfected COS-7 cells, their subcellular distribution was analysed by indirect immunofluorescence assays. As expected, Pim-1 protein was expressed throughout the cells, while GST-Bad localized in a punctate pattern in mitochondria-like organelles or in a more diffuse pattern in the cytosol (Fig. 4C). Furthermore, merging of the images from double-positive cells followed by scattergram analysis revealed

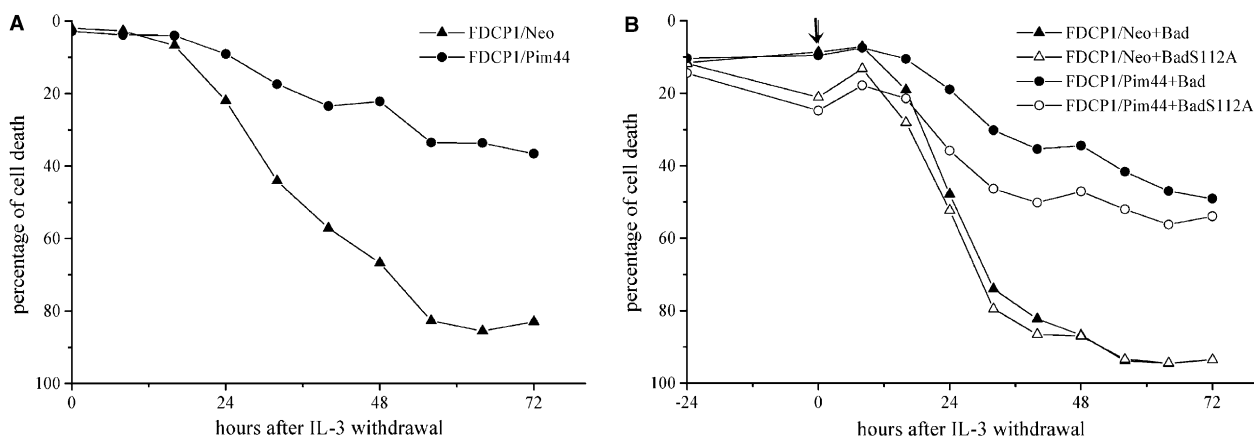


Fig. 5. Pim-1 can prolong survival of IL-3-deprived FDCP1 cells also in the presence of wild-type Bad or the S112A mutant. (A) IL-3 was withdrawn from the growth medium of FDCP1/Neo and FDCP1/Pim44 cells and samples were collected at indicated time-points to determine the percentage of dead cells by propidium iodide staining. (B) FDCP1/Neo and FDCP1/Pim44 cells were transiently transfected with wild-type GST-Bad or the S112A mutant (pEBG-mBad or pEBG-mBadS112A, 5 μ g). 24 h after transfection, cells were treated and analysed as above. The start-point of starvation is indicated by an arrow.

that Pim-1 and GST-Bad partially colocalized within the cytoplasmic compartment. Again, this colocalization was independent of the ability of Pim-1 to phosphorylate Bad, since similar results were obtained when the S112A mutant of GST-Bad was coexpressed with Pim-1. Altogether, our *in vitro* and *in vivo* results confirmed that Pim-1 can colocalize and physically interact with Bad and should thereby also be able to directly phosphorylate it.

3.4. Pim-1 can protect cytokine-deprived FDCP1 cells from Bad-promoted apoptosis

Next we wanted to examine whether Pim-1 could protect FDCP1 cells from the pro-apoptotic effects of Bad, and whether the anti-apoptotic effects of Pim-1 were in turn dependent on its ability to phosphorylate Bad on Ser¹¹². For this purpose, we first compared the survival properties of FDCP1/Neo and FDCP1/Pim44 cell lines at several time-points after IL-3 withdrawal. Consistently with our previous observations [23], most FDCP1/Neo cells died within three days, while more than 60% of FDCP1/Pim44 cells were still alive at that time-point (Fig. 5A). We then transiently expressed wild-type GST-Bad or the S112A mutant in both cell lines. As shown in Fig. 5B, the presence of either GST-Bad protein accelerated the death rate of cytokine-deprived FDCP1/Neo cells to a similar extent. By contrast, the FDCP1/Pim44 cells remained relatively resistant to Bad-promoted apoptosis. Furthermore, these cells survived slightly better in the presence of wild-type Bad as compared to the mutant. However, in both cell lines expressing the S112A mutant, there was an initial decrease in survival within the first 24 h after transfection. This suggests that despite the presence of IL-3, expression of the mutant had inhibited the ability of the transfected cells to fully recover from electroporation and that Pim-1 could not protect cells at this step, most likely due to its inability to phosphorylate Bad on Ser¹¹². While our results indicate that Pim-1 can efficiently antagonize Bad-promoted cell death, and suggest that phosphorylation of Ser¹¹² of Bad plays a role in Pim-dependent cell survival, it should be noted that the results were strongly diluted by the fact that only about 10% of the cells had been successfully transfected with either of the GST-Bad-expressing plasmids (data not shown). Therefore, we attempted to select in the presence of IL-3 for puromycin-resistant FDCP1/Neo or FDCP1/Pim44 cell clones expressing wild-type GST-Bad or the S112A mutant. However, no stable cell clones with full-length S112A protein could be obtained for further analyses (data not shown), most likely due to strong counterselection against this potent pro-apoptotic mutant, as also observed by others [23,24].

We have recently shown that also the Pim-2 kinase can enhance phosphorylation of Bad, again primarily on Ser¹¹² [24]. Furthermore, coexpression of Pim-2 in Jurkat T-cells can rescue part of the pro-apoptotic effects of transiently overexpressed wild-type Bad, but less of the S112A mutant, which is in line with the data reported here. Together these results suggest that the Pim family kinases can share similar anti-apoptotic mechanisms, including inactivation of Bad. The ability of Pim kinases to promote cell survival can in turn explain, why both of them can efficiently cooperate with Myc family transcription factors to transform hematopoietic cells [25–27], since even though Myc-overexpressing cells have a greater proliferation potential, they are more susceptible to apoptotic

death, as shown also for myeloid cells [28]. Another pro-apoptotic protein that was recently shown to be phosphorylated by Pim-2 is the translational repressor 4E-BP1 [29]. However, it remains to be determined whether 4E-BP1 is a substrate also for Pim-1 and whether its phosphorylation contributes to the anti-apoptotic effects of Pim kinases.

Yet it is clear that Pim kinases alone cannot fully protect cells from apoptosis, since FDCP1/Pim44 cells eventually die five days after IL-3 withdrawal [30] and FDCP1 cells stably expressing Pim-2 isoforms do not survive any better (M. Lilly, unpublished observation). Additional factors may thus be required in a cell type-dependent fashion. However, even if the downstream effects of Pim-1 were only transient, they may give cells enough time to adjust to apoptotic conditions and to gather additional mutations to allow full transformation of cells towards apoptosis-resistant tumor cells. Interestingly, the phenotype of Bad-deficient mice resembles those of Pim-1 or Pim-2 transgenic mice in that they develop lymphomas with quite a long latency [31]. While Bad seems to be required to suppress lymphoid tumorigenesis, phosphorylation of Bad may be only one of the several mechanisms for Pim kinases to promote cell survival and thereby also lymphomagenesis.

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