

Cutting Edge: Transcriptional Activity of NFATc1 Is Enhanced by the Pim-1 Kinase¹

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Pim-1 is an oncogenic serine/threonine kinase implicated in cytokine-induced signal transduction and in development of lymphoid malignancies. However, its precise function as well as physiological substrates have remained unknown. In this study we demonstrate that Pim-1 can physically interact with the NFATc1 transcription factor and phosphorylate it in vitro on several serine residues. In contrast to previously recognized NFATc kinases, wild-type Pim-1 enhances NFATc-dependent transactivation and IL-2 production in Jurkat T cells, while kinase-deficient Pim-1 mutants inhibit them in a dominant negative fashion. Our results reveal a novel, phosphorylation-dependent regulatory mechanism targeting NFATc1 through which Pim-1 acts as a downstream effector of Ras to facilitate IL-2-dependent proliferation and/or survival of lymphoid cells. *The Journal of Immunology*, 2002, 168: 1524–1527.

The *pim* family of proto-oncogenes consists of at least three members encoding highly related serine/threonine-specific protein kinases (1–4). When overexpressed, *pim* genes can efficiently cooperate with *myc* or *bcl-2* oncogenes in lymphomagenesis (2, 5–7). During embryonal development, *pim* genes are expressed in partially overlapping fashions in cells of both the immune and the central nervous systems as well as in epithelia (4). In hematopoietic cells, expression of *pim-1* can be induced by several cytokines (8, 9), suggesting that the Pim-1 kinase may mediate signal transduction initiated by cytokine receptors. Pim-1 has been reported to protect thymocytes against glucocorticoid-induced apoptosis (10) and to promote cell proliferation or survival in several IL-3- or IL-6-dependent hematopoietic cell lines (11–13). Expression of *pim-1* has also been shown to be up-regulated during T cell activation in a protein kinase C-dependent fashion (14), but its function there has remained unclear.

When TCRs become activated, several signaling pathways are initiated that later converge in NFAT activation (15, 16). Pathways dependent on protein kinase C and Ras induce AP-1 expression, while calcium-dependent pathways lead to dephosphorylation of the NFATc transcription factor and its translocation to the nucleus. AP-1 and NFATc then cooperatively activate genes such as IL-2, IL-4, GM-CSF, and TNF- α . Nuclear import of NFATc family members has been reported to be opposed by phosphorylation of critical serine residues by several kinases, including glycogen synthase kinase-3, protein kinase A, casein kinases, and several mitogen-activated protein kinases (17–20). In this study we demonstrate that Pim-1 can also phosphorylate NFATc1, but, unlike all the other known NFATc kinases, it does not prevent the nuclear entry of NFATc1. By contrast, Pim-1 acts downstream of Ras to enhance NFAT-mediated transactivation as well as IL-2 production in activated Jurkat T cells.

Materials and Methods

Plasmid constructs

Murine *pim-1* cDNA (kindly provided by A. Berns, Netherlands Cancer Institute, Amsterdam, The Netherlands) was amplified by PCR and cloned into pLTRpoly vector (21). PCR was also used to mutate the AP-1 binding sites in the NFAT-luciferase (LUC)³ reporter from TGTTTCA to CTG-GAAT, to introduce the K67M mutation in Pim-1 that abolished its kinase activity, and to transfer the protein coding regions of wild-type and mutant Pim-1 into the pGEX-2T (Amersham Pharmacia Biotech, Uppsala, Sweden) or pECFP-C1 (Clontech Laboratories, Palo Alto, CA) fusion vectors. pEF-*ras* plasmid and LUC reporters containing either NFAT binding sites derived from the IL-2 promoter or AP-1 binding sites from the metallothionein promoter were provided by G. R. Crabtree (Stanford University, Stanford, CA), pGEX-3X-NFATc1 (1–418 aa) and pBJ5-NFATc1-FLAG by S. N. Ho (Stanford University) and pLXSN*pim*NT81 by M. Lilly (Loma Linda University, Loma Linda, CA). The pM plasmid encoding the Gal4 DNA binding domain and the VP16 activation domain fused to it were obtained from Clontech Laboratories and the pSV- β -galactosidase reporter from Promega (Madison, WI).

Transactivation assays and IL-2 measurements

Jurkat T cells or their derivatives, JTAG cells, expressing the SV40 T Ag (22) were transfected by electroporation with 2 μ g of reporter plasmids and indicated amounts of Pim-1 and other expression vectors. Two days after transfection, cells were left unstimulated or stimulated for 6–9 h with 15 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and/or 1 μ M ionomycin (Calbiochem, La Jolla, CA), collected and analyzed for LUC activity using Luminoskan luminometer (Labsystems, Helsinki, Finland). The transfection efficiencies were normalized against β -galactosidase activities. Shown are means and mean deviations of representative experiments with duplicate or triplicate samples. For measuring IL-2 production, cells had been cotransfected with 2 μ g of pEGFP (Clontech Laboratories). Twenty-four hours after transfection enhanced green fluorescent protein (EGFP)-positive cells were sorted out with FACStar^{plus} (BD Biosciences, San Jose, CA)

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³ Abbreviations used in this paper: LUC, luciferase; GFP, green fluorescent protein; EGFP, enhanced GFP; ECFP, enhanced cyan fluorescent protein.

and allowed to recover for another 24 h before stimulation. Concentrations of IL-2 in the growth medium were determined with OptEIA Human IL-2 Set (BD Pharmingen, San Diego, CA). Data from both assays were analyzed for significant differences by Student's *t* test ($p \leq 0.05$).

Coprecipitation assays

COS-7 cells were transfected by electroporation with 1 or 2 μg of indicated plasmids. Two days later cells were collected, lysed by freeze-thawing in 20 mM PIPES (pH 7), 30 mM NaCl, and 5 mM MgCl_2 plus protease inhibitors and immunoprecipitated with M2 anti-FLAG mAb (Kodak, Rochester, NY) in 50 mM Tris (pH 7.4), 150 mM NaCl, and 1% Tween 20. Precipitated proteins were then separated by SDS-PAGE, immobilized onto polyvinylidene difluoride plus membrane (Micron Separations, Westboro, MA), stained with anti-green fluorescent protein (GFP) antiserum (Clontech Laboratories) and anti-rabbit-HRP, and visualized by ECL plus reagents (Amersham Pharmacia Biotech). To determine protein expression levels, part of the lysates were directly analyzed by Western blotting with anti-FLAG or anti-GFP Abs.

In vitro phosphorylation assays

GST-Pim-1 and GST-NFATc1 (1–418 aa) fusion proteins grown in *Escherichia coli* and attached to glutathione Sepharose beads (Amersham Pharmacia Biotech) were mixed with each other in kinase buffer (20 mM PIPES (pH 7), 5 mM MnCl_2 , 7 mM 2-ME, 0.25 mM β -glycerophosphate, 0.4 mM spermine, 10 μM rATP) supplemented with 10 μCi of [γ - ^{32}P]ATP and incubated at 30°C for 30 min. Part of the samples were washed with dephosphorylation buffer (50 mM Tris-HCl (pH 7.4), 1 mM NiCl_2 , 0.5 mg/ml BSA), resuspended in the buffer with 1 U of calcineurin (Promega) and 1 U of calmodulin (Sigma-Aldrich), and incubated at 30°C for 15 min. Activity of calcineurin was confirmed according to the manufacturer's instructions by using *p*-nitrophenyl phosphate (Sigma-Aldrich) as a substrate. All samples were further analyzed by SDS-PAGE followed by autoradiography. For phosphoamino acid analysis, phosphorylated proteins from the in vitro kinase assay were transferred from gel to Immobilon-P membrane (Millipore, Bedford, MA). Phosphoamino acids were released by acid hydrolysis and separated on two dimensions under different pH conditions on thin layer plates (23). The migration patterns of radioactive spots revealed by autoradiography were then compared with those of nonradioactive phosphoamino acid standards visualized by ninhydrin staining.

Results

Pim-1 enhances NFATc activity and IL-2 production

To determine whether the Pim-1 kinase could be involved in signaling pathways that lead to NFAT activation, we transfected Jurkat T cells with a *pim-1* expression vector together with a LUC reporter construct containing multiple NFAT binding sites. To mimic TCR activation, cells were stimulated with the phorbol ester PMA and the calcium ionophore ionomycin. Ectopic expression of *pim-1* enhanced drug-induced NFAT-LUC activity in a dose-dependent fashion but had no effects in unstimulated cells (Fig. 1A and data not shown). Very similar results were also obtained when TCRs of transfected cells were activated with anti-CD3 Abs (data not shown). The effects of Pim-1 were reproducible and statistically highly significant ($p < 0.001$). Pim-1-induced enhancement of NFAT-LUC activity was mediated by endogenous AP-1 and NFATc family members and depended on the presence of intact NFAT binding sites, because reporter constructs with mutated or no binding sites remained unaffected (Fig. 1B and data not shown). Furthermore, Pim-1 enhanced AP-1-LUC activity only slightly ($p > 0.05$; Fig. 1B), suggesting that the major effects of Pim-1 are targeted toward the NFATc component of the NFAT-binding protein complex.

To find out whether Pim-1 is able to affect physiological targets of NFATc, we decided to measure levels of IL-2 secreted into the growth medium of stimulated Jurkat T cells. Because nontransfected cells were also expected to produce IL-2, we cotransfected cells with expression vectors for both Pim-1 and EGFP and used EGFP as a marker to sort out positively transfected cells by FACS. After stimulation with PMA and ionomycin, we could indeed observe a statistically significant increase in IL-2 production in EGFP-positive cells ($p < 0.05$; Fig. 1C, right panel), most of

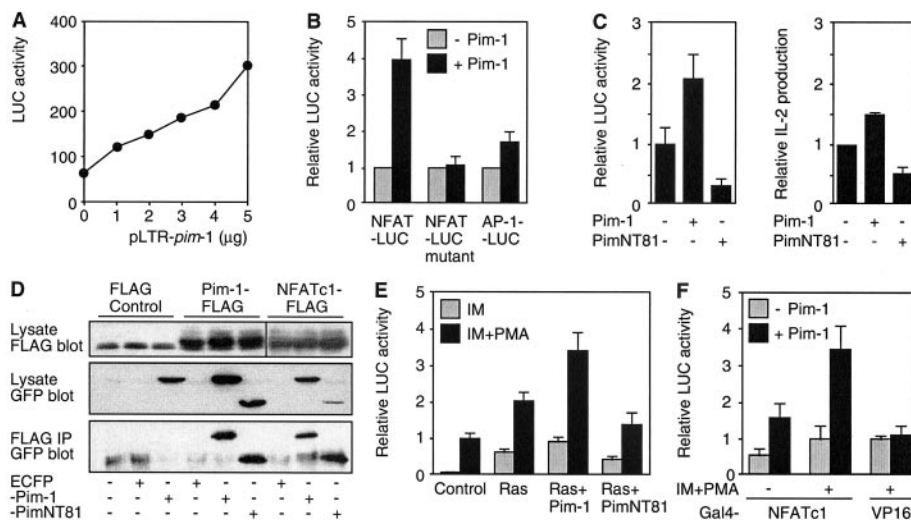


FIGURE 1. Pim-1 enhances endogenous NFAT activity and IL-2 production in a dose-dependent fashion. *A*, NFAT-LUC activity in Jurkat T cells transfected with indicated amounts of pLTR-*pim-1* and stimulated with PMA and ionomycin. Shown are relative light units. *B*, Comparison of the effects of Pim-1 on activities of indicated reporters in stimulated cells transfected with 5 μg of pLTR-*pim-1*. Shown are LUC activities relative to those obtained with an empty vector. *C*, Comparison of the effects of wild-type Pim-1 and the PimNT81 mutant on NFAT-LUC activity (left panel) and on IL-2 production (right panel) in stimulated cells cotransfected with 3 μg of pLTR-*pim-1* or 6 μg of pLXSN-*pimNT81*. *D*, Interactions between FLAG- or ECFP-tagged Pim-1, PimNT81, and NFATc1 proteins in transfected COS-7 cells. Shown above are expression levels of each protein in cell lysates as determined by Western blotting with anti-FLAG or anti-GFP Abs. Note that Pim-1-FLAG and NFATc1-FLAG samples are derived from different parts of the same gel. Shown below are ECFP-tagged proteins coprecipitated with anti-FLAG and blotted with anti-GFP Abs. *E*, Comparison of the effects of Pim-1 and PimNT81 on Ras-induced NFAT-LUC activity in cells cotransfected with 3 μg of pEF-*ras* and stimulated with ionomycin (IM) without or with PMA. *F*, Pim-1 enhances transactivation by Gal4-NFATc1 but not by Gal4-VP16 in cells cotransfected with 3 μg of Gal4-NFATc1 or Gal4-VP16. Shown are Gal4-LUC activities relative to those obtained with an empty vector in stimulated cells.

which also coexpressed Pim-1 (data not shown). This result correlated well with Pim-1-enhanced NFAT-LUC activity (Fig. 1C, compare *left* and *right* panels). As partly shown in our previous study (11), the effects of Pim-1 were strictly dependent on its kinase activity, because a kinase-deficient K67M mutant as well as an N-terminal NT81 deletion mutant were unable to enhance NFAT activity or IL-2 production but rather inhibited them in a dominant negative fashion (Fig. 1C and data not shown). This inhibition was most likely due to formation of inactive oligomers between wild-type and mutant Pim-1 proteins, because from transiently transfected COS-7 cells we were able to coprecipitate FLAG-tagged Pim-1 with both types of proteins tagged with ECFP (enhanced cyan fluorescent protein; Fig. 1D). In addition, we could observe coprecipitation of both wild-type and mutant Pim-1 with NFATc1, suggesting that all these proteins can physically interact with each other within cells. Wild-type and mutant Pim-1 proteins were expressed at equivalent levels and their interactions were specific, because we were unable to coprecipitate them with vectors expressing just the tags or some irrelevant tagged proteins (Fig. 1D and data not shown).

Pim-1 acts in a novel pathway downstream of Ras

Pim-1 was unable to replace the requirements for either PMA or ionomycin (data not shown), suggesting that its effects on endogenous NFAT activity in Jurkat T cells are dependent on convergence of the drug-induced pathways. As expected, a constitutively active Ras mutant was able to cooperate with the ionomycin-induced pathway to induce NFAT activity also in the absence of PMA (Fig. 1E). Interestingly, coexpression of Ras and Pim-1 in ionomycin-stimulated cells resulted in NFAT activity comparable to that in control cells stimulated with both ionomycin and PMA. In addition, the enhancing effects of Ras in double-stimulated cells were further potentiated by coexpression of Pim-1. By contrast, the NT81 mutant of Pim-1 inhibited the effects of Ras irrespective of which way the cells were treated. These statistically significant results ($p < 0.05$) suggest that Pim-1 and its kinase activity are at least partially responsible for the Ras-induced downstream signaling leading to NFAT activation in Jurkat T cells.

To test whether the effects of Pim-1 on NFAT activity were directly mediated through the transcriptional regulatory domain of NFATc1, we fused the N-terminal half (aa 1–418) of NFATc1 to the Gal4 DNA binding domain and measured Gal4-dependent LUC activity in transiently transfected Jurkat T cells. In accordance with the results obtained from NFAT-LUC assays, coexpression of Pim-1 augmented transcriptional activity of NFATc1 but did not significantly affect the activity of the VP16 activation domain fused to Gal4 (Fig. 1F). Because the Gal4-NFATc1 fusion protein was able to enter the nucleus via the Gal4 domain and activate transcription independently of AP-1, stimulation of cells with PMA and ionomycin was not required. Without stimulation the overall activity of Gal4-NFATc1 remained lower than in stimulated cells, but in both cases Pim-1 enhanced it to a very similar extent.

Pim-1 phosphorylates NFATc1 in vitro on serine residues

To examine whether NFATc1 could be a direct substrate for Pim-1, we conducted in vitro kinase assays with GST-fusion proteins of either wild-type or mutant Pim-1 and the N-terminal regulatory domain of NFATc1 (aa 1–418). Hardly any phosphorylation was detected with the kinase-deficient K67M mutant of Pim-1, whereas the wild-type kinase was able to phosphorylate both NFATc1 and itself but not GST-derived sequences (Fig. 2A). To find out whether Pim-1 phosphorylates NFATc1 on the same sites that the calcium-dependent phosphatase calcineurin dephosphory-

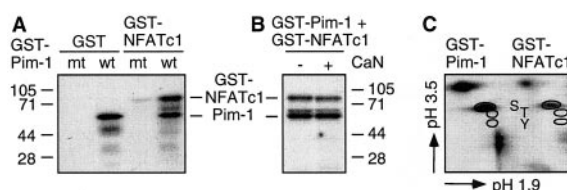


FIGURE 2. Pim-1 phosphorylates NFATc1 in vitro on serines that are not dephosphorylated by calcineurin. *A*, GST or GST-NFATc1 (1–418 aa) was subjected to in vitro phosphorylation in the presence of mutant (mt) or wild-type (wt) GST-Pim-1. *B*, Part of the samples prephosphorylated by Pim-1 were further incubated with calcineurin (CaN). *C*, Phosphoamino acid analyses of in vitro phosphorylated GST-Pim-1 and GST-NFATc1 samples derived from the gel shown in Fig. 1A. Locations of ninhydrin-stained phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standard residues have been circled.

lates, part of the samples prephosphorylated by Pim-1 were further incubated in the presence of calcineurin. However, calcineurin had no effects (Fig. 2B), even though under the same experimental conditions it properly dephosphorylated its known substrates such as *p*-nitrophenyl phosphate as well as endogenously phosphorylated, FLAG-tagged NFATc1 protein immunoprecipitated from transfected COS-7 cells (data not shown). These results indicated that the target sites of Pim-1 in NFATc1 are distinct from those recognized by calcineurin. Phosphoamino acid analyses of proteins phosphorylated by Pim-1 showed that significant amounts of phosphate were incorporated only on serine residues in both Pim-1 and NFATc1 (Fig. 2C). These results were of interest because all in vivo phosphate in NFATc1 has been reported to be attached to serines (24).

Discussion

The data presented in this study provide the first piece of evidence for positive regulation of NFAT activity by a kinase capable of phosphorylating NFATc1. We demonstrate that the Pim-1 kinase can enhance NFAT-dependent transactivation and thereby also IL-2 production in Jurkat T cells. The effects of Pim-1 are statistically significant and specific for NFATc, because Pim-1 does not markedly increase activities of other transcription factors like AP-1 or VP16. Moreover, the effects of Pim-1 are dependent on its kinase activity, because kinase-deficient Pim-1 mutants not only are unable to stimulate NFAT activity or IL-2 production but rather inhibit them in a dominant negative fashion. These mutants may compete with both endogenously and ectopically expressed wild-type Pim-1 by sequestering them into inactive oligomers. Finally, Pim-1 can physically interact with NFATc1 within cells and is therefore likely to be able to phosphorylate it not only in vitro but also in vivo. Using immunofluorescence assays, we have observed that Pim-1 and NFATc1 colocalize and that their colocalization does not interfere with nuclear entry of NFATc1 upon T cell activation (J. Sandholm, K. Heiskanen, and P. J. Koskinen, manuscript in preparation). While all our evidence suggests that Pim-1 enhances activity of NFATc1 directly in a phosphorylation-dependent manner, the sites phosphorylated by Pim-1 and their physiological significance remain the goal of our future studies.

It has previously been shown that nuclear NFATc is not completely dephosphorylated by calcineurin (25). Furthermore, a detailed analysis of NFATc2 has revealed that in addition to 13 serine residues that are dephosphorylated upon T cell activation there is also one site that is inducibly phosphorylated (26). These results fit well with our observations that calcineurin cannot dephosphorylate the in vitro target sites of Pim-1 in NFATc1 and that both calcineurin and Pim-1 enhance NFAT activity despite their

opposing enzymatic activities. Moreover, our results with the constitutively nuclear Gal4-NFATc1 fusion protein indicate that activation of calcineurin is dispensable for enhancement of NFAT activity by Pim-1.

The kinetics of *pim-1* expression during T cell activation (14) suggests that Pim-1 is not needed for the initial induction of NFAT activity but may be necessary at later points to allow continuous IL-2 production. Because Pim-1 can enhance IL-2 production and because IL-2 in turn can up-regulate Pim-1 production (8, 9), this may even lead to a positive feedback loop. This conclusion is further supported by the observation that cytokines that induce Pim-1 expression also enhance cell responsiveness to IL-2 (27). Thus, even a slight but constitutive overexpression of Pim-1, as is often observed in human patients with lymphoid or myeloid malignancies (28), could thereby result in increased proliferation and/or cell survival.

Phosphorylation of Pim-1 target sites in NFATc1 may result in conformational changes and/or recruitment of additional coactivators such as CREB-binding protein or p300 which have been reported to bind to NFATc1 and enhance its activity (29). This would fit well with our previous observation that, in cooperation with the p100 transcriptional coactivator, Pim-1 can stimulate the activity of another hematopoietic cell transcription factor, c-Myb (30). Even more intriguingly, our current and previous data indicate that kinase-deficient mutants of Pim-1 can inhibit the positive effects of Ras on both NFATc and c-Myb activities, suggesting that Pim-1 acts as one of the downstream effectors of activated Ras. This conclusion is further supported by the ability of wild-type Pim-1 to partially rescue inhibition of both NFATc and c-Myb activities by dominant negative versions of Ras (Ref. 30 and our unpublished observations). Taken together, our results may explain the ability of overexpressed Pim-1 to enhance lymphoproliferation and even lymphomagenesis, especially in collaboration with Myc or Bcl-2 oncoproteins.

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