The oncogenic serine/threonine kinase Pim-1 phosphorylates and inhibits the activity of C-TAK1: a novel role for Pim-1 at the G2/M cell cycle checkpoint

Malte Bachmann, Hanjo Hennemann\textsuperscript{1}, Pei Xiang Xing\textsuperscript{2}, Ingrid Hoffmann\textsuperscript{3} and Tarik Möröy\textsuperscript{*}

Institut für Zellbiologie (Tumorforschung), IFZ, Universitätsklinikum Essen, Virchowstrasse 173, D-45122 Essen, \textsuperscript{2}Austin Research Institute, Cancer Immunotherapy Laboratory, Heidelberg, Victoria 3084, Australia. \textsuperscript{3}Deutsches Krebsforschungszentrum, DKFZ, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
\textsuperscript{1}present address: Caesar, Centre of Advanced European Studies and Research, Ludwig-Erhard-Allee 2, D-53175 Bonn,

*corresponding author:
Institut für Zellbiologie (Tumorforschung), IFZ
Virchowstrasse 173, D-45122 Essen, Germany,
Tel.: 49 (201) 723 – 3380, Fax: 49 (201) 723 – 5904
Email: moeroey@uni-essen.de
Abstract

The Pim-1 oncogene encodes a serine-threonine kinase which relays signals from cytokine receptors and contributes to the formation of lymphoid tumors when expressed at high levels. Here we show that the protein kinase C-TAK1 (Cdc twenty-five C associated kinase 1) is a binding partner and a substrate of Pim-1. A physical interaction of Pim-1 and C-TAK1 could be shown biochemically and in yeast two hybrid assays. Immuno-fluorescence experiments suggested that Pim-1/C-TAK1 complexes are predominantly cytoplasmic. When transiently transfected, Pim-1 is also found in the nucleus and can recruit C-TAK1 to this compartment. Both Pim-1 and C-TAK1 undergo auto-phosphorylation but only Pim-1 is able to phosphorylate C-TAK1 but not vice versa. Mass spectrometry analysis of C-TAK1 suggested that the sites of auto-phosphorylation and Pim-1-mediated phosphorylation are distinct and not overlapping. Phosphorylation by Pim-1 decreases C-TAK1 kinase activity significantly, in particular its ability to phosphorylate and inactivate Cdc25C, a protein which actively promotes cell cycle progression at the G2/M phase. Hence, our findings directly suggest a novel role for Pim-1 as a positive regulator at the G2/M transition of the cell cycle.

Keywords: Pim-1, C-TAK1, cell cycle, G2/M checkpoint

Running title: Pim-1 phosphorylates C-TAK1
Introduction

The pim-1 gene encodes a 33kD cytoplasmic serine/threonine kinase (1, 2) and was first discovered as a locus frequently activated by proviral insertion in Moloney Murine Leukemia Virus (MoMuLV) induced T-cell lymphomas (3-5). The expression pattern of Pim-1 is widespread and the protein is found in a series of tumors and tissues but highest expression levels are found in cells of the hematopoietic and lymphoid system. Evidence that pim-1 is directly implicated in the tumorigenic process was provided by the analysis of Eµ pim-1 transgenic animals (6).

Mice carrying a homozygous deletion of pim-1 generated by gene targeting show a very subtle phenotype (7, 8) probably because Pim-1 is active in several redundant signaling pathways or because other Pim family members such as Pim-2 or Pim-3 can rescue a loss of Pim-1 (9-11). Experiments with IL-3 dependent cells suggested that Pim-1 is mediating gp130 mediated cell proliferation (12) and that the pim-1 gene is a direct target of the latent STAT transcription factors (13) in particular STAT3 but also STAT5. Therefore, Pim-1 is considered to be an effector of many cytokine signaling pathways in particular of those that initiate signaling through STAT3 and STAT5 as for instance interleukin (IL)-2, -3, -6, -7 and prolactin (12, 14-19). More recent data implicated Pim-1 in the regulation of Socs-1 which is a negative regulator of the Jak/STAT pathway and suggested that Pim-1 can also modulate cytokine signaling pathways in addition to its role as a direct effector kinase (20).

A putative substrate target sequence of Pim-1 has been identified by using a chemically synthesized peptide library (21). However, since Pim-1 is able to phosphorylate itself but does not contain such a recognition sequence (22, 23) it is very likely that other sites exist that can be phosphorylated by Pim-1. Efforts to shed more light on the function of Pim-1 have resulted in the identification of several interaction partners and substrates including
p100, which is an activator of the c-Myb transcription factor (24) and NFAT (25) suggesting that Pim-1 can affect the regulation of transcription in the nucleus. Furthermore, the G1 specific phosphatase Cdc25A was found to be a substrate of Pim-1 and it could be demonstrated that it can be activated through phosphorylation by Pim-1 (26). Cdc25A activates the kinase activity of G1 specific cyclin/CDK complexes by removing inhibitory phosphate groups and is a positive regulator of cell cycle progression in the G1 phase. The identification of Cdc25A as a Pim-1 substrate was therefore the first direct proof that Pim-1 activity is linked to cell cycle progression. Later, other experiments indicated that the CKI (cycline kinase inhibitor) p21Waf is inactivated through Pim-1 phosphorylation (27) or suggested a synergistic role for Pim-1 and Myc in cell cycle progression dependent of STAT3 (12).

In addition to a role in promoting cell cycle progression, Pim-1 also has been linked to the regulation of programmed cell death and an anti-apoptotic effect of Pim-1 has been demonstrated in several independent experimental systems (12, 28-33). A direct effect of Pim-1 on particular constituents of the known apoptotic signaling pathways could however not be shown and the question how Pim-1 regulates apoptosis remains open. A number of other substrates of Pim-1 have been found among them are HP-1, a heterochromatin binding protein with a role in gene silencing (34) and PAP1, a novel protein with a putative function in transcription repression and the regulation of mRNA splicing (35). Other Pim-1 interacting proteins such as TRAF2/SNX6 or Socs-1 belong to the group of adapter proteins and are involved in STAT or TNF Receptor signal transduction pathways (20, 36).

We wished to further elucidate how the Pim-1 kinase connects signal transduction pathways initiated by cytokines and the Jak/STAT pathway to the cell cycle machinery and to describe how Pim-1 translates this signal into a proliferative response. To this end, we aimed to find so far unknown substrates of Pim-1 that have a direct role in the regulation of cell cycle
progression. Using a novel yeast interaction cloning system we identify the kinase C-TAK1 (Cdc twenty-five C associated kinase 1) as a Pim-1 interaction partner and substrate, and also demonstrate that phosphorylation by Pim-1 significantly decreases C-TAK1 activity suggesting that Pim-1 is involved in the regulation of cell cycle progression at the G2/M transition by affecting the activity of Cdc25C through C-TAK1 in vitro and in vivo (37).
Material and Methods

**Ras Recruitment Screening (RRS) and mutational analysis in yeast**

All yeast plasmids used in this study were derived from the galactose-inducible Yes2 (Invitrogen) and the constitutive ADNS vector. DNA-fragments for Pim-1-K67M and c-Jun fused in frame to a Ha-Ras sequence (constitutively active form) were generated by polymerase chain reaction (PCR). Yes2 derived plasmids: human c-Fos fused to Src myristoylation signals (M-Fos). ADNS derived plasmids: Human Pim-1-K67M (amino acids 1-313; ADNS-Pim-1-K67M-5'Ras), and human c-Jun leucine zipper (amino acids 249-331; Jun-Z-Ras) were fused with Ha-Ras sequences. The cDNA library used in this study has been described before (38, 39). Ras Recruitment System (RRS) library screening with Pim-1-K67M bait was performed essentially as described (38, 40). For the β-galactosidase assay the Pim-1-K67M cDNA was subcloned into a BamHI/ EcoRI cut pLexA vector in frame with the LexA binding domain (41). The sequence encoding the C-TAK1-Y6131 fragment was amplified by PCR and inserted in frame with the VP16 transactivation domain into pVP16 (42). The VP16-Gfi-VII plasmid was used as a negative control (41). The LexA-Pim-1-K67M and either the VP16-C-TAK1-Y6131 or VP16-Gfi-VII plasmids were introduced into the yeast strain L40 (42). The assay was performed as described previously (41). Expression of proteins was tested by western blotting using either anti VP16 (Santa Cruz, 1-21) or anti LexA (Santa Cruz, 2-12) antibodies.

**Antibodies**

The following primary antibodies were used for western blotting: anti Cdc25C, Biodiagnostics (BM-025C-100A) or Santa Cruz (C-20; H6); anti C-TAK1; anti Pim-1, Santa Cruz (19F7) and anti FlagM2 (Sigma). As secondary antibodies, peroxidase-conjugated donkey anti-rabbit IgG or peroxidase-conjugated donkey anti-mouse IgG (Dianova) were used. For immuno-precipitations a LexA (Santa Cruz; 2-12) and an anti
Pim-1 monoclonal antibody P9 (unpublished data) were used. Anti C-TAK1 antibodies was produced by immunization of rabbit using C-TAK1-1-165, which was purified on GST-C-TAK1-1_165 affinity column after expressed in bacteria and removed from GST by thrombin digestion.

**Kinase assay**

To assay Pim-1 and C-TAK1 kinase activities, the respective GST-proteins were purified, mixed (40 µl bead-slurry) and resuspended in 50 µl kinase buffer (Pim-1: 20 mM PIPES, pH=7.0, 5 mM MnCl2, 7 mM β-mercaptoethanol; C-TAK1: 50 mM Tris-HCl, pH=7.4, 10 mM MgCl2; both: 10 µm ATP and 10 µCi of [α32P]ATP. Flag-tagged Pim-1 or C-TAK1 proteins immuno-precipitated from transfected Cos7 cells also served as kinases. Reactions were incubated at 30 °C for 30 min. (Pim-1) or at 20 °C for 30 min. (C-TAK1), boiled in SDS-sample buffer, resolved on a SDS-gel and subsequently analyzed by x-ray film exposure.

**Mass spectrometry**

GST-C-TAK1-wt, C-TAK1-N183A and Pim-1wt proteins were purified. Kinase assays were performed as described but were done with non-radioactively labeled ATP. Phosphorylated or non-phosphorylated proteins were cut out of the gel and digested either with the proteases trypsin, chymotrypsin or glu-C. To detect phosphorylation fragments were analyzed by a mass spectrometer.

**C-TAK1 inactivation assay**

Two consecutive kinase assays were performed. In the first kinase assay, purified GST-Pim-1-wt or GST-Pim-1-K67M proteins were eluted from the GSH-agarose beads and used as kinases. The purified substrate (C-TAK1) remained coupled to the GSH-agarose beads. After the first kinase assay C-TAK1-GSH-agarose beads were precipitated, washed once with C-TAK1 kinase buffer to eliminate soluble Pim-1 protein and were used
in the second kinase assay this time as a kinase. Purified GST-Cdc25C protein served as a substrate in the second kinase assay. The samples were boiled in SDS-sample buffer, resolved on a SDS-gel and subsequently analyzed by x-ray film exposure.

**Cell cycle analysis**

The cell cycle phase distribution of 293 cells was examined by flow cytometry using FACScan and Cell Quest software (Beckton Dickinson). 1 x 10⁶ cells were transfected with pBB14 (GFP; 43, 44) and Pim-1 or C-TAK1 constructs. 24 h after transfection 293 cells were treated with 10 µg/ml bleomycin for 24 h and harvested. The cells were washed with PBS and fixed in PBS/ethanol for 1 h. After centrifugation cells were stained with propidium iodide (20 µg/ml) for 30 min and analyzed.
Results

Pim-1 interacts with C-TAK1 in yeast

To identify proteins that interact with the Pim-1 kinase, we used a yeast interaction cloning strategy based on the "Ras Recruitment System" (RRS, 40) We constructed a "bait" plasmid able to express the kinase-inactive 33 kD form of Pim-1 (Pim-1-K67M) in fusion with Ha-Ras in the vector pADNS-Ras(mut) (40) and introduced this along with a mGAP expression plasmid (45) into the yeast mutant strain cdc25-2 which contains a temperature sensitive allele of the GTPase exchange factor (GEF) CDC25. This GEF activates the endogenous yeast Ras pathway under permissive conditions (25 °C), but is inactivated by a shift to the restrictive temperature of 36 °C. After introduction of DNA from the GC library (38) 417 initial clones were obtained, 13 of which showed library plasmid dependency on galactose/glucose medium. Four of 11 clones that passed a bait specificity test (Fig. 1a) contained DNA sequences coding for C-TAK1 (37). One of the clones (Y6131, see Fig. 1) which contained the longest C-TAK1 sequence covering amino acid positions 1-263 was used in the subsequent experiments.

To verify a potential interaction between Pim-1 and C-TAK1, we fused the obtained C-TAK1 sequence to sequences encoding the herpes simplex virus VP16 transactivation domain in a galactose-inducible vector. This construct was cotransfected with plasmids encoding fusion proteins between the DNA binding domain of LexA and the kinase inactive mutant of Pim-1. As a control, we used a construct encoding a fusion protein between LexA and a stretch of the zinc finger transcription factor Gfi1 (Fig. 1b, 41). Western blot analysis of extracts from transformed yeast cells demonstrated that the expression constructs were functional (Fig. 1b). In the presence of galactose, a high α-galactosidase activity was obtained only with the constructs expressing LexA-Pim-1 and C-TAK1-VP16 fusion proteins (Fig. 1b) supporting an interaction between Pim-1 and C-TAK1. To obtain the human full length C-TAK1 clone, we screened a human spleen
cDNA library by PCR and isolated besides the wt C-TAK1 cDNA two novel variants of C-TAK1 with additional coding sequences that were termed C-TAK1-α (two additional coding regions: X, 9 aa; Y, 15 aa) and C-TAK1-α (one additional coding region: Y, 15 aa; Fig. 1c).

**Pim-1 binds C-TAK1 *in vitro* and *in vivo***

To test whether Pim-1 and C-TAK1 can form complexes *in vitro*, we generated fusion proteins between glutathione S-transferase (GST) and the C-TAK1 fragment initially isolated during the RRS screen (C-TAK1-Y6131), the full length C-TAK1-wt, the variant C-TAK1-α and -α forms and different mutants of C-TAK1. Radioactively labeled Pim-1 and C-TAK1-wt proteins produced in an *in vitro* transcription-translation system were retained by GST-C-TAK1-wt, the N-terminal C-TAK1 fragments spanning aa 1-263 (Y-6131) and aa 1-165 as well as by the C-TAK1 variants α and α (Fig. 2a) indicating that the N-terminal part of C-TAK1 is sufficient to bind Pim-1 and that C-TAK1 can interact with itself (Fig. 2a). Next, radio-labeled C-TAK1-wt protein was mixed with extracts from cells transfected with expression constructs for the Flag-tagged C-TAK1 fragment Y6131, Pim-1-wt, the kinase inactive Pim-1 mutant (K67M) or the irrelevant proteins Evi5 or PIAS3. Complexes were precipitated with anti-Flag antibodies and were analyzed by SDS-page and auto- radiography. Clearly, radioactively labeled C-TAK1 protein was precipitated only from lysates containing C-TAK1 itself, or the Pim-1 proteins but a precipitation from lysates containing Evi5 or PIAS3 was not readily detected confirming the specificity of the C-TAK1/Pim-1 interaction (Fig. 2b). Similar results were obtained when C-TAK1 splice variants were used (data not shown).

Using the RRS and the original yeast clone with the prey plasmid Y6131 that encodes the C-TAK1 fragment spanning aa 1-263, we selected a C-TAK1 point mutant by error-prone PCR that was unable to interact with Pim-1. The selected mutants carried a proline residue instead of a leucine at position 128 (C-TAK-Y6131-L128P). The wt C-TAK1-Y6131 and
the mutant C-TAK1-Y6131-L128P fragments were transiently expressed as Flag-tagged versions along with Pim-1-wt or Pim-1-K67M in Cos7 cells (Fig. 2c). Immuno-precipitations with anti Flag antibodies from these cells revealed that only C-TAK1-Y6131 but not the mutant containing the L128P “loss of interaction” substitution formed complexes with Pim-1-wt or Pim-1-K67M (Fig. 2c) indicating that the aa 128 in C-TAK1 is critical for Pim-1 binding. A GST-pull down experiment with lysates from cells transfected with a Pim-1 expression construct confirmed that a bona fide “loss of interaction” mutant had been created (Fig. 2d). The previously described kinase-inactive C-TAK1 mutants (37), the two novel splice variants of C-TAK1 and the amino terminal deletion mutant C-TAK1-81-729 were still able to bind Pim-1 (Fig. 2d) suggesting that the domain in the C-TAK1 protein that is responsible for Pim-1 binding is localized in an amino terminal region spanning aa 81-165.

Pim-1 co-localizes with C-TAK1 in myeloid and epithelial cells

The C-TAK1-wt protein but also the C-TAK1-N183A mutant and the mutant that lacks interaction with Pim-1 were localized exclusively in the cytoplasm when expressed transiently in NIH 3T3 cells (Fig. 3a) regardless whether non-tagged or Flag-tagged versions or fusions between C-TAK1-wt or both splice variants and the green fluorescent protein (GFP) were expressed (Fig. 3a and data not shown). In contrast, Pim-1-wt and the kinase inactive mutant K67M both resided in the nucleus as well as in the cytoplasm of NIH 3T3 cells upon transient transfection of the respective expression constructs, regardless whether anti Pim-1 or anti Flag antibodies were used (Fig. 3a, data not shown). When co-expressed in fibroblasts, both the wt C-TAK1 and the C-TAK1-N183A mutant co-localized in the nucleus with Pim-1 (Fig. 3b, upper and lower panels) or with Pim-1-K67M (not shown) in all transfected cells whereas the loss of interaction mutant C-TAK1-L128P and Pim-1 did not show such a nuclear interaction (Fig. 3b middle panel).
Next, we stained HeLa cells with antibodies against Pim-1 or C-TAK1 and found a predominantly cytoplasmic and to a minor degree nuclear localization of the endogenous proteins (Fig. 4a). Here, the staining for both endogenous C-TAK1 and Pim-1 appeared to overlap suggesting co-localization of both proteins (Fig. 4b, upper panels). As expected a co-localization and complete overlap of C-TAK1 and Cdc25C was observed and served as a positive control (Fig 4b, lower panels). To verify that Pim-1 forms complexes with C-TAK1 at the endogenous expression level, we chose HeLa cells, U937 promyelocytes stimulated with phorbol ester (TPA) and cells from the myeloid tumor line K562 which all express endogenous Pim-1 and C-TAK1 (Fig. 4c). We were able to precipitate endogenous Pim-1 from U937 and K562 lysates with GST-C-TAK1 fusion protein coupled to agarose beads but not with the irrelevant Evi5-GST fusion protein or GST alone (Fig. 4d). In addition, C-TAK1 could be immuno-precipitated with anti Pim-1 antibodies but not with irrelevant LexA antibodies from all three cell lines (Fig. 4e, and data not shown).

**Pim-1 phosphorylates C-TAK1**

Whereas both GST-C-TAK1 and GST-Pim-1 fusion proteins showed auto-phosphorylation, the inactive GST-Pim-1-K67M mutant was unable to phosphorylate itself, as expected (Fig. 5a). Neither Pim-1 nor C-TAK1 phosphorylated purified GST protein indicating the specificity of the assay (Fig. 5a). The GST-C-TAK1 but also the GST-Cdc25C fusion proteins were each detected as two signals in gels: as a slower migrating form representing the full length fusion protein and as a faster migrating form representing a specific degradation product that still contains part of the C-TAK1 or Cdc25C proteins and the GST portion (see Fig. 5a, b and data not shown). C-TAK1-wt and the two new splice variants C-TAK1-α and -α could auto-phosphorylate themselves and phosphorylated Cdc25C either as a GST-fusion protein or as a recombinant purified protein as expected (Fig. 5b). When GST-C-TAK1 fusion proteins were mixed with GST-Pim-1-wt, both auto-phosphorylated proteins were detected, when GST-C-TAK1
fusion proteins and the inactive Pim-1 kinase mutant K67M were mixed only the auto-
phosphorylated GST-C-TAK1 proteins were detected suggesting that C-TAK1 does not
phosphorylate Pim-1 (Fig. 5c). The kinase inactive C-TAK1 mutants N183A and D196N
did not show auto-phosphorylation and remained un-phosphorylated in the presence of
the inactive Pim-1-K67M mutant (Fig. 5d). However, when GST-Pim-1-wt was added,
both C-TAK1 mutants were phosphorylated (Fig. 5d). In addition, the phosphorylation of
C-TAK1-wt was significantly stronger in the presence of GST-Pim-1-wt than in the
presence of the inactive Pim-1-K67M mutant (Fig. 5d) suggesting that Pim-1
phosphorylates C-TAK1. A similar result was obtained when a Flag-tagged Pim-1 or a
Flag-tagged kinase inactive Pim-1-K67M mutant were expressed in Cos7 cells, immuno-
precipitated and used as a kinase to phosphorylate C-TAK1 (data not shown). As an
additional control the "loss of interaction" mutant C-TAK1-L128P was used in this assay
and was not phosphorylated by Pim-1 wt, as expected (Fig. 5d). Furthermore, Pim-1 was
able to phosphorylate a C-TAK1 mutant covering aa positions 81 to 729 and a truncated
C-TAK1 mutant spanning the amino terminal end from aa positions 1-165, suggesting that
the region between aa 81 and 165 is important for Pim-1 binding and may represent the
Pim-1 interaction domain (Fig. 5e-f).

To map more precisely the sites in the C-TAK1 protein that can be phosphorylated by
Pim-1, we purified GST-C-TAK1-N183A that lacks auto-phosphorylation either
phosphorylated by Pim-1 in vitro or non-phosphorylated as a control or GST-C-TAK1-wt
non-phosphorylated by Pim-1. All proteins were digested with trypsin, chymotrypsin and
the endoproteinase glu-C and analyzed by mass spectrometry with a sequence coverage
between 60 - 75 %. The analysis of the trypsin digestion products of GST-C-TAK1 alone
suggested a site of auto-phosphorylation in the region of aa 598-607 (SRGSTNLFSK)
(Fig. 6). The analysis of the Pim-1 phosphorylated GST-C-TAK1-N183A protein showed
a mass shift of 160 Dalton for the tryptic peptide TQLNPTSLQK localized within the
kinase domain and putative Pim-1 interaction domain of C-TAK1 between aa 90 and 99
and suggested the existence of at least two phosphorylation sites at threonine 90 or 95 or at serine 96 (Fig. 6a). Several C-TAK mutants were generated where the serine and both threonine residues were replaced by alanin or glycine in the inactive C-TAK1-N183A form. All mutants were found to interact with Pim-1 (data not shown) and to be phosphorylated by Pim-1 (Fig. 6b), suggesting that C-TAK1 contains additional Pim-1 phosphorylation sites that were undetectable by mass spectrometry.

**Pim-1 phosphorylation inhibits C-TAK1 kinase activity**

One of the main functions of C-TAK1 is the phosphorylation of Cdc25C at serine 216 which enables 14-3-3 proteins to bind Cdc25C and to sequester it in the cytoplasm. To test whether phosphorylation by Pim-1 could affect this activity of C-TAK1, we performed two consecutive kinase assays (“KA” in Fig. 7a) with either the GST-Pim-1 fusion protein or the GST-Pim-1-K67M inactive mutant as kinases. Both proteins were affinity purified and freed from agarose beads (termed “eluate”, Fig. 7a). Substrates were either GST alone and a GST-C-TAK1 fusion protein coupled to agarose beads. After the first reaction, either GST or GST-C-TAK1 beads were collected, separated from the soluble Pim-1 proteins by washing and used for the second kinase assay with Cdc25C as a substrate. Soluble GST-Pim-1-wt or -K67M proteins were efficiently removed from GST-C-TAK1 beads or GST-beads and did not bind to the beads again, since we were unable to detect any signal of Pim-1-wt auto-phosphorylation in the second kinase assay. In reactions where GST-C-TAK1 beads had been incubated previously with the active Pim-1 kinase, Cdc25C phosphorylation was significantly decreased compared to reactions where C-TAK1 was used that had seen the kinase inactive Pim-1 mutant before (Fig. 7a). This suggested that phosphorylation of C-TAK1 by Pim-1 lowers the activity of C-TAK1 to phosphorylate Cdc25C.
Next, we wished to test whether Pim-1 is able to promote enhanced G2/M progression of cells by setting off the activity of C-TAK1. To this end, we transiently transfected 293 cells blocked in G2/M phase by bleomycin treatment with a GFP expression vector or with vectors allowing the expression of C-TAK1, the C-TAK1 kinase inactive mutant N183A, Pim-1 and the kinase inactive mutant Pim-1 K67M. After transfection, cells were stained with propidium iodide and percentages of GFP positive cells in G1 phase were measured by FACS. We observed that expression of either Pim-1 or the dominant negative C-TAK1 mutant (C-TAK1-N183A) enhanced the fraction of 293 cells in G1 after bleomycin treatment significantly compared to similarly treated cells transfected with the active C-TAK1 (Fig. 7b). This indicated that the G2/M arrest maintained by C-TAK1 expression and bleomycin can be overcome by inhibiting C-TAK either by expressing a dominant negative C-TAK1 mutant or by expressing Pim-1 (Fig. 7b). The kinase inactive Pim-1 mutant (K67M) is clearly less active but still maintains a residual ability to inactivate C-TAK1 in this assay (Fig. 7b) probably due to the physical interaction of both proteins.
Discussion

Pim-1 plays a prominent role in transmitting signals from cytokine receptors and in the malignant transformation of lymphoid cells and it has been demonstrated that Pim-1 is associated with enhanced cell proliferation (12). We have identified C-TAK1 - a serine/threonine kinase and a cell cycle regulator active at the G2/M transition as a novel Pim-1 substrate. *In vitro* synthesized proteins as well as endogenous C-TAK1 and Pim-1 were shown to bind to each other by and to co-localize in different cell types supporting a physical interaction between Pim-1 and C-TAK1 proteins. As a consequence of this interaction C-TAK1 is phosphorylated by Pim-1 but, vice versa, Pim-1 is not a substrate for the C-TAK1 kinase and the phosphorylation of C-TAK1 by Pim-1 leads to a significant decrease of C-TAK1 kinase activity.

**Pim-1 is an upstream negative regulator of C-TAK1**

C-TAK1 is constitutively expressed in all cell cycle phases (37) in contrast to the DNA damage activated kinases Chk1 or Chk2/Cds1 which - similar to C-TAK1 - inactivate Cdc25C by phosphorylation at serine 216 which leads to 14-3-3 dependent sequestering of the protein to the cytoplasm (46-48). Recently, the phosphatase PP1 was identified as an antagonist of C-TAK1 since it is able to de-phosphorylate Cdc25C on serine 216 resulting in a de-repression of its activity (49). Our experimental findings described here suggest that Pim-1 is a regulator of C-TAK1 activity since it phosphorylates C-TAK1 which causes a significantly reduced activity of C-TAK1 with regard to its ability to phosphorylate Cdc25C at serine 216. Our studies with C-TAK1 mutants and mass spectrometry data suggested that phosphorylation by Pim-1 occurs at several sites within the C-TAK1 protein. It is therefore difficult to assign the loss of C-TAK1 activity after Pim-1 phosphorylation to a specific single amino acid. Nevertheless, since the activity of C-TAK1 appears to depend directly on Pim-1 phosphorylation, Pim-1 can be considered as an upstream negative regulator of C-TAK1.
The physical interaction between Pim-1 and C-TAK1 is independent of phosphorylation of C-TAK1 by Pim-1 since the kinase inactive mutant of Pim-1 (K67M) is still able to bind C-TAK1 and to recruit it into the nucleus like the wt Pim-1 protein. Recent observations in normal lymphoid cells suggest that Pim-1 can reside in the nucleus as well as in the cytoplasm and that a nuclear localization is required for the protein to exert its biological function (50). Other reports also indicate that endogenous Pim-1 can reside in both nuclear and cytoplasmic compartments (27, 33, 51) and the interaction of Pim-1 with the transcription factors or co-factors NF-AT, p100 or c-Myb that have been reported have to occur in the nucleus. It is therefore likely that Pim-1 resides in both cellular compartments. We had indeed found a predominantly cytoplasmic localization of endogenous Pim-1 in HeLa cells or in cells of the lines K 562 or U 937, but upon transient transfection, we observed that Pim-1 is both cytoplasmic and nuclear. Considering that Pim-1 has both nuclear and cytoplasmic interaction partners it is conceivable that Pim-1 is active and finds substrates in both cellular compartments or can shuttle between cytoplasm and nucleus depending on the type or status of the cells or on the actual expression level. Evidence for such a behavior comes from experiments with U 937 cells where Pim-1 becomes nuclear after treatment and activation of the cells with phorbol ester (27). Still, the significance of a recruitment of C-TAK1 to the nucleus by Pim-1 as observed in transfected cells remains to be clarified.

**Regulation of the G2/M transition of the cell cycle**

Phosphorylation at serine 216 by DNA damage kinases Chk1/Cds1 or C-TAK1 inactivates Cdc25C and removes it as an active player from the stage of the G2/M transition since it is no longer available for the polo like kinase 1 (Plk1) that phosphorylates Cdc25C at the N-terminus to render it fully active as a phosphatase (52, 53). Inactivation of Cdc25C by serine 216 phosphorylation then leads to an arrest at the G2/M transition. The aa serine 216 is located in the neighborhood of the NES-sequence (nuclear export sequences, aa 190-199) and the NLS (nuclear localization signal; aa
After phosphorylation of Cdc25C at serine 216 by DNA damage kinases or C-TAK1 and the subsequent binding of 14-3-3 proteins, the NLS-sequence is no longer accessible and Cdc25C remains in the cytoplasm. In its first activation step, Cdc25C is de-phosphorylated at serine 216 by PP1 (49). Moreover, it is known that Cdc25C is phosphorylated during prophase by Plks at the N-terminus and is then transported to the nucleus or is retained there (55, 56). It is conceivable that Pim-1 acts to ensure full activation of Cdc25C and prevents its re-phosphorylation by C-TAK1 by phosphorylating and inactivating C-TAK1. The inactivation of C-TAK1 would confer the role to Pim-1 as a C-TAK1 antagonist and indirect activator of Cdc25C to Pim-1; a role similar to Plks. Such a role of Pim-1 is further supported by our findings with transfected cells that are blocked in G2/M phase. Here, Pim-1 was able to set off the effect of C-TAK1 and enhanced the percentages of cells in G1 phase. Since the kinase inactive mutant of Pim-1 (K67M) was noticeably less active in this assay, this result is consistent with a role of Pim-1 in promoting cell cycle progression by inactivating C-TAK1 via direct phosphorylation. However, the kinase inactive Pim-1 mutant clearly had a residual activity and could still increase the amount of the G1-phase cells albeit at a lower degree than active Pim-1. This indicates that the inhibitory effect of Pim-1 on C-TAK1 activity is not only attributable to the phosphorylation of C-TAK1 by Pim-1 but may also be caused by the mere physical interaction of both proteins since the kinase inactive Pim-1 K67M mutant is still able to bind C-TAK1.

**Pim-1 as a positive regulator of cell cycle progression at the G2/M transition**

After N-terminal hyper-phosphorylation by Plk-1, the active Cdc25C phosphatase is able to activate the MPF-complex through de-phosphorylation of one of its components, Cdk1. Subsequently, the active MPF complex translocates into the nucleus after phosphorylation of its other component, cyclin B1, and initiates the transcription of M-Phase specific genes leading to the start of prophase. Our data presented here suggest that the Pim-1 kinase may play a role in this process since it can modulate the activity of C-TAK1 in this
signaling pathway through binding and phosphorylation. Pim-1 decreases the kinase activity of C-TAK1 which could relieve newly synthesized Cdc25C from the restriction represented by a phosphorylated serine 216 or inactivated Cdc25C is de-phosphorylated by PP1. Thus, Cdc25C would be available for Piks to be phosphorylated at the N-terminal region which enables it to stimulate the activity of MPF. In this model, Pim-1 would act at a critical point of the G2/M transition phase by blocking a reaction that is inhibitory for the activity of Cdc25C, namely its phosphorylation on serine 216 by C-TAK1 (Fig. 8). According to our findings it is not unlikely that this may occur in the cytoplasm as well as in the nucleus.

A first indication that Pim-1 may indeed have a regulatory role at the G2/M transition was obtained when NuMA (Nuclear mitotic apparatus protein) was identified as a Pim-1 substrate (51). NuMA is located in the spindle poles during mitosis and it was shown that Pim-1 has a role in promoting the formation of a complex between NuMA, HP-1α, a heterochromatin binding protein, dynein and dynactin which is necessary for progression through mitosis. These findings also support a role of Pim-1 as a positive cell cycle regulatory kinase active at the G2/M phase transition. Such a role of Pim-1 could explain how signals from cytokines that initiate the activation of STAT3/STAT5 and the up regulation of their target genes, among them the gene for Pim-1, result in the support of cell proliferation but also offers an explanation for the oncogenic activity of Pim-1 and its potential to malignantly transform lymphoid cells.
Acknowledgements

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References

Figure Legends

Fig. 1

Yeast two hybrid screen (RRS) identifies C-TAK1 as a partner protein for Pim-1
(a) Patched colonies of double transformed cdc25-2 yeast cells carrying either the library plasmid (GCBY6131) and one of the bait plasmids Pim-1-K67M-Ras or pADNS (empty bait plasmid) or containing the bait plasmid with empty library vector (pYesMdpA). As a positive control, c-Fos (M-Fos) and Jun-Leucine Zipper-Ras (Jun-Z-Ras) expressing plasmids were used. From each yeast transformation four independent colonies were patched. Glucose, 25°C (Column 1): All tested yeast clones show the same viability at the permissive temperature of 25°C. From this master plate the galactose and glucose plates shown in Galactose, 36 C (Column 2) and Glucose, 36 C (Column 3) were replica plated and incubated at 36°C. Galactose, 36°C: At the restrictive temperature, full complementation of the cdc25 phenotype occurs in the positive control (Jun/Fos) and with the library plasmid (GCBY6131) only in combination with the Pim-1-K67M-Ras bait used for the screening. Glucose, 36°C: Repression of the library plasmid promoter on glucose plates inhibits growth at the restrictive temperature for the positive control as well as for Pim-1-K67M-Ras/ GCBY6131 transfected cells. (b) Quantification of the Pim-1/ C-TAK1-Y6131 interaction by a α-galactosidase assay in the yeast strain L40. LexA-Gfi1-VII served as a negative control. The same galactose inducible samples were used for a liquid β-galactosidase assay and western blotting (right panel) to show that all constructs were functional. (c) Schematic representation of the isolated clone (C-TAK1-Y6131) and the three different human C-TAK1 splice variants. C-TAK1-α contains two (exon X and Y) and C-TAK1-α one additional exon (exon Y). C-TAK1 contains a kinase domain (amino acids (aa) 55-307), a ubiquitin binding domain (aa 328-364, UBA) and a kinase associated domain (aa 680-729 in the C-TAK1-wt protein). Mutants at aa 128 (inactive and loss of interaction regarding to Pim-1), 183 and 196 (inactive C-TAK1-mutants). The known Cdc25C binding region is indicated.
Fig. 2

Pim-1 interacts with C-TAK1 in vitro

(a) Various C-TAK1 fragments were expressed as GST fusion proteins in bacteria. 
$^{35}$S-labeled Pim-1-wt or $^{35}$S-labeled C-TAK-1-wt proteins were produced by in vitro transcription/translation reactions and were added to the GST fusion proteins bound to glutathione agarose beads and their interaction was analyzed by auto-radiography. (b) Upper panel: Cos7 cells were transfected with indicated expression constructs and harvested after 24 h. Lysates of transfectants were mixed with $^{35}$S-labeled C-TAK1-wt protein, immuno-precipitated with a monoclonal anti-Flag antibody and analyzed by SDS-PAGE and auto-radiography. Lower panel: The same samples were probed with an anti Flag-antibody as a control. Arrows indicate the immuno-precipitated Flag-proteins. (c) Cos7 cells were transfected with indicated expression constructs (Flag-tagged versions) and harvested after 24 h. Lysates of transfectants were immuno-precipitated (IP) with a monoclonal anti-Flag antibody and analyzed by SDS-PAGE and western blotting (We). (d) Various fragments of C-TAK1 were expressed as GST fusion proteins in bacteria. Whole cell lysates of Cos7 cells transfected with a Pim-1 wt expression construct were added to the GST fusion proteins bound to glutathione agarose beads and the retained material was analyzed for interaction by immuno-blot with an anti Pim-1 antibody.
Fig. 3

C-TAK1 and Pim-1 co-localize in NIH 3T3 cells

(a) NIH 3T3 cells were transfected with the indicated constructs, fixed in methanol 24 h later and stained with DAPI. To detect Pim-1-Flag proteins cells were treated with anti-Flag antibodies and Rhodamin labeled secondary antibodies. (b) NIH 3T3 cells were cotransfected with Pim-1-wt-Flag and C-TAK1-GFP (wt, L128P and N183A mutants). To detect Pim-1-wt protein, cells were treated with anti-Pim-1 antibodies and Rhodamin labeled secondary antibodies. All pictures were analyzed by laser scanning confocal microscopy.
**Fig. 4**

Endogenous sub-cellular localization and expression of Pim-1 and C-TAK1 and immuno-precipitation of endogenous proteins

(a) Hela cells were immuno-stained with anti-Pim-1 or anti-C-TAK1 antibodies and specific Rhodamin-labeled secondary antibodies. Both endogenous proteins are localized in the cytoplasm. (b) Hela cells were immuno-stained with the indicated antibodies and specific Rhodamin- or FITC-labeled secondary antibodies. Secondary antibodies alone were used as negative controls and produced no readily detectable staining (data not shown). (c) U 937 (U; + phorbol ester (TPA), 2 days), Hela (H) and K 562 (K) cells were harvested and lysates were analyzed by western blotting (We) to detect endogenous C-TAK1 and Pim-1. Lysates of Cos7 cells (lane C+) transfected with C-TAK1-Flag and Pim-1-Flag or untransfected Cos7 cells (lane C-) were used as a control. Expression of endogenous C-TAK1 and Pim-1 could be detected in all three cell lines albeit at different levels. (d) The indicated GST-fusion proteins bound to glutathione agarose beads were mixed with lysates of U 937 or K 562 cells and the retained proteins were analyzed by immuno-blotting. (e) Endogenous Pim-1/C-TAK1 complexes could be precipitated from TPA treated U 937 cells. A whole cell extract was prepared after stimulation with TPA for two days and immuno-precipitations were performed with anti-Pim-1 antibodies or the irrelevant anti-LexA antibodies to control for the specificity of the anti Pim-1 antibody. To detect C-TAK1 the precipitates were analyzed by western blotting (We) using anti C-TAK1 antibodies. Anti-Pim-1 immuno-precipitates were also developed with the anti Pim-1 antibody to show that the immuno-precipitation was successful. 1/10 of the input was used as a control.
**Fig. 5**

**Pim-1 phosphorylates C-TAK1**

Purified GST fusion proteins bound to glutathione agarose beads or recombinant Cdc25C protein were mixed for *in vitro* kinase assays and the resulting reactions were analyzed by SDS-PAGE and autoradiography. (a) Both GST-Pim-1 and GST-C-TAK1-wt were mixed with GST protein to show that both Pim-1-wt and C-TAK1-wt have the ability to auto-phosphorylate themselves but do not phosphorylate GST. The Pim-1-K67M mutant lacked kinase activity demonstrated by the absence of auto-phosphorylation. (b) Wild type C-TAK1 and the two splice variants (−wt, −α, −α) were able to phosphorylate GST-Cdc25C, recombinant Cdc25C protein or themselves. (c) Pim-1-wt, C-TAK1 wt and C-TAK-1 splice variants showed auto-phosphorylation. The kinase inactive Pim-1-K67M was not phosphorylated by C-TAK1. (d) Pim-1-wt phosphorylated all C-TAK1 mutants but not the "loss of interaction" mutant C-TAK1-L128P. C-TAK1-wt showed auto-phosphorylation but not the N183A and D196N inactive single amino acid exchange mutants. GST-C-TAK1-N183A and -D196N were phosphorylated when Pim-1-wt kinase was used. (e) Pim-1-wt phosphorylates a C-TAK1-deletion mutant which covers the amino acids 1-165 and the C-TAK-81-729 mutant where the N-terminal part of the protein is deleted. Both deletion mutants are functionally inactive. Either purified GST-Pim-1-wt or Flag-Pim-1-wt protein immuno-precipitated from transfected Cos7 cells were used as kinases. (f) Schematic representation of the GST fusion proteins used for interaction studies and kinase assays. A putative Pim-1 interaction domain in C-TAK1 could be mapped to a region within aa positions 81-165. The known Cdc25C interaction domain on C-TAK1 is also indicated.
Fig. 6

Potential phosphorylation sites in C-TAK1

(a) Schematic representation of the C-TAK1-wt protein. Two putative phosphorylation sites were found by mass spectrometry (arrows). Pim-1 phosphorylation occurs at the peptide aa 90-99 in the kinase domain. In contrast, auto-phosphorylation was detectable in the c-terminus of the protein (aa 598-607). (b) Pim-1 was able to phosphorylate C-TAK1-N183A mutants in which the aa Threonine 90 or Threonine 95 and the Serine 96 were replaced by Alanine or Glycine residues one by one or in combination. GST-C-TAK1-N183A alone was used as positive control for Pim-1 phosphorylation.
Fig. 7

**Regulation of C-TAK1 activity by Pim-1**

(a) C-TAK1 inactivation assay: The indicated GST fusion proteins were expressed in bacteria and purified. The GST-Pim-1 fusion proteins were eluted from the agarose bead matrix whereas GST-C-TAK1 remained coupled to beads. Two consecutive kinase assays (KA) were performed and the reactions were analyzed by SDS-PAGE and autoradiography. In the first kinase assay, GST-Pim-1-wt or GST-Pim-1-K67M eluates were allowed to react with GST beads or with GST-C-TAK1 beads. Then the beads were washed and thereby freed from the Pim-1 eluates and were used for the second kinase assay with GST-Cdc25C as a substrate. When inactive Pim-1-K67M eluate was used, C-TAK1 was able to phosphorylate Cdc25C which was detected by two prominent signals (lane 4) representing the GST-Cdc25C proteins. When Pim-1-wt was used in the first kinase assay, C-TAK1 activity appeared significantly reduced, resulting in a much weaker Cdc25C phosphorylation in the second kinase assay (lane 3).

(b) Distribution of 293 cells transfected with a GFP expression vector and the indicated constructs among different cell cycle phases. 293 cells were transfected, treated with bleomycin (10 µg/ml) for 24 h, stained with propidium iodide and then subjected to the cell cycle analysis by FACS. Only GFP expressing cells were analyzed. Percentages of cells in G1 phase of the cell cycle are shown. Data representative for five independent experiments that yielded similar results are shown.
**Fig. 8**

Schematic representation of the known and published interactions between C-TAK1 and Cdc25C and the ensuing phosphorylation reactions (37) and a model of Pim-1 function within this pathway. For discussion see text.
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Fig. 1
Fig. 2

(a) GST-C-TAK1-Y6131

(b) C-TAK1-wt

(c) C-TAK1-Y6131

(d) Pim1-wt

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**Fig. 3**

(a) C-TAK1-wt-GFP Pim-1-wt-Flag overlay

(b) C-TAK1-L128P-GFP Pim-1-wt-Flag overlay

C-TAK1-N183A-GFP Pim-1-wt-Flag overlay

C-TAK1-N183A-GFP Pim-1-wt-Flag overlay

Pim-1-wt-Flag

Pim-1-K67M-Flag

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Fig. 4
**Fig. 5**

**a**
- **Kinases:**
  - GST-Pim-1-wt
  - GST-Pim-1-K67M
  - GST-C-TAK1-wt

- **Substrates:**
  - GST-C-TAK1-auto-P

- **Substrate:** GST

**b**
- **Kinases:**
  - GST-C-TAK1-wt
  - GST-C-TAK1-K67M
  - GST-C-TAK1-L128P
  - GST-C-TAK1-N183A
  - GST-C-TAK1-D196N

- **Substrates:**
  - GST-Cdc25C
  - Cdc25C-rec.protein

**c**
- **Kinases:**
  - GST-C-TAK1-wt
  - GST-C-TAK1-K67M

- **Substrates:**
  - GST-Cdc25C
  - Cdc25C-rec.protein

**d**
- **Kinases:**
  - GST-Pim-1-wt
  - GST-Pim-1-K67M

- **Substrates:**
  - GST-C-TAK1
  - GST-Pim-1

**e**
- **Kinases:**
  - GST-Pim-1-wt
  - Flag-Pim-1-wt

- **Substrates:**
  - GST-C-TAK1-1-165
  - GST-C-TAK1-81-729
  - GST-C-TAK1-N183A

**f**
- **Kinases:**
  - Flag-Pim-1-wt

- **Substrates:**
  - GST-Cdc25C

**Interaction with Pim-1**

- only wt
- C-TAK1 auto-P

- **Pim-1 interaction domain (81-165)**
- Cdc25C interaction domain (308-412)
**Fig. 6**

**a**

C-TAK1-wt (human)

- Kinase domain: 1-55
- UBA: 307
- KA1 domain: 614-680-729

Peptide: aa 90-99
TQLNPTSLQK

Peptide: aa 598-607
SRGSTNLFSK

**b**

Kinase: Pim-1-wt

Substrates:
- C-TAK1-N183A
- T90A
- T95A
- S96G
- T95A/S96G
- T90A/T95A
- T90A/S96G
- T90A/T95A/S96G
- N183A

Peptide: aa 90-99
TQLNPTSLQK

Peptide: aa 598-607
SRGSTNLFSK

Peptide: aa 90-99
TQLNPTSLQK

Peptide: aa 598-607
SRGSTNLFSK

GST-C-TAK1

auto-P

GST-Pim-1-wt

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Fig. 6
### Fig. 7

**a**

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2nd KA

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**b**

- Bleomycin

- C-TAK1-wt vector: 58%
- C-TAK1-N183A vector: 21.2%
- C-TAK1-wt Pim-1-wt: 17.8%
- C-TAK1-wt Pim-1-K67M: 14.6%

+ Bleomycin

- C-TAK1-wt vector: 9.2%
- C-TAK1-N183A vector: 21.2%
- C-TAK1-wt Pim-1-wt: 17.8%
- C-TAK1-wt Pim-1-K67M: 14.6%

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**Fig. 7**
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Fig. 8

MPF

G2

M

Cdk1

Cdc25C

Pim-1

C-TAK1

Cdc25C
The oncogenic serine/threonine kinase Pim-1 phosphorylates and inhibits the activity of C-TAK1: a novel role for Pim-1 at the G2/M cell cycle checkpoint
Malte Bachmann, Hanjo Hennemann, Pei Xiang Xing, Ingrid Hoffmann and Tarik Moroy
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