A NOVEL ROLE FOR Pim-1 AT THE G₂/M CELL CYCLE CHECKPOINT

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 46, Issue of November 12, pp. 48319–48328, 2004

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The Oncogenic Serine/Threonine Kinase Pim-1 Phosphorylates and Inhibits the Activity of Cdc25C-associated Kinase 1 (C-TAK1)

Received for publication, April 22, 2004, and in revised form, August 19, 2004
Published, JBC Papers in Press, August 19, 2004, DOI 10.1074/jbc.M404440200

The Pim-1 oncogene encodes a serine-threonine kinase that 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 Cdc25C-associated kinase 1 (C-TAK1) 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. Immunofluorescence experiments suggested that Pim-1-C-TAK1 complexes are predominantly cytoplasmic. When transiently transfected, Pim-1 was also found in the nucleus and could recruit C-TAK1 to this compartment. Both Pim-1 and C-TAK1 underwent autophosphorylation, but only Pim-1 was able to phosphorylate C-TAK1 but not vice versa. Mass spectrometry analysis of C-TAK1 suggested that the sites of autophosphorylation and Pim-1-mediated phosphorylation are distinct and not overlapping. Phosphorylation by Pim-1 decreased C-TAK1 mediated phosphorylation are distinct and not overlapping. Phosphorylation by Pim-1 decreased C-TAK1 kinase activity significantly, in particular its ability to phosphorylate and inactivate Cdc25C, a protein that 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.

The pim-1 gene encodes a 33-kDa cytoplasmic serine/threonine kinase (1, 2) and was first discovered as a locus frequently activated by proviral insertion in Moloney murine leukemia virus 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 interleukin-3-dependent cells suggest that Pim-1 is mediating gp130-mediated cell proliferation (12) and that the pim-1 gene is a direct target of the latent STAT1 transcription factors (13) in particular STAT3 but also STAT5. Therefore, Pim-1 is considered to be an effector of many cytokine signaling pathways, particularly of those that initiate signaling through STAT3 and STAT5 as for instance interleukin-2, -3, -6, and -7 and prolactin (12, 14–19). More recent data has implicated Pim-1 in the regulation of Sox-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 nuclear factor of activated T-cells (25) suggesting that Pim-1 can affect the regulation of transcription in the nucleus. Furthermore the G₂-specific phosphatase Cdc25A was found to be a substrate of Pim-1, and it has been demonstrated that it can be activated through phosphorylation by Pim-1 (26). Cdc25A activates the kinase activity of G₂-specific cyclin-cyclin-dependent kinase complexes by removing inhibitory phosphate groups and is a positive regulator of cell cycle progression in the G₂ 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. Other experiments have indicated that the cyclin kinase inhibitor p21Waf is inactivated through Pim-1 phosphorylation (27) or have 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 antiapoptotic effect of Pim-1 has been dem-
onstrated in several independent experimental systems (12, 28–33). A direct effect of Pim-1 on particular constituents of the known apoptotic signaling pathways, however, could 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 tumor necrosis factor receptor-associated factor 4-associated factor 2/sorting nexin 6 or Socs-1 belong to the group of adapter proteins and are involved in STAT or tumor necrosis factor 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 identified the kinase Cdc twenty-five C-associated kinase 1 (C-TAK1) as a Pim-1 interaction partner and substrate and also demonstrated 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).

MATERIALS AND METHODS

Ros Assay 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 PCR. For Yes2-derived plasmids, human c-Fos was fused to Src myristoylation signals (M-Fos). For ADNS-derived plasmids, human Pim-1-K67M (amino acids 1–313, ADNS-Pim-1-K67M/S/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). RRS library screening with Pim-1-K67M bait was performed essentially as described previously (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 library plasmid containing the C-TAK1 bait fragment was amplified by PCR and inserted in frame with the VP16 transactivation domain into pVP16 (42). The VP16-GFP VII plasmid was used as a negative control (41). The Lex-A-Pim-1-K67M and either the VP16-C-TAK1-Y6131 or VP16-G6-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 Biotechnology, 1–21) or anti-LexA (Santa Cruz Biotechnology, 2–12) antibodies.

Antibodies—The following primary antibodies were used for Western blotting: anti-Cdc25C (Biodiagnostics (BM-025C-100A) or Santa Cruz Biotechnology, C-20, H6), anti-C-TAK1, anti-Pim-1 (Santa Cruz Biotechnology, 19F7), and anti-FLAG M2 (Sigma). As secondary antibodies, peroxidase-conjugated donkey anti-rabbit IgG or peroxidase-conjugated donkey anti-mouse IgG (Dianova) were used. For immunoprecipitations anti-LexA (Santa Cruz Biotechnology, 2–12) and anti-Pim-1 monoclonal antibody P97 were used. Anti-C-TAK1 antibody was produced by immunization of a rabbit using C-TAK1-(1–165), which was purified on a GST-C-TAK1-(1–165) affinity column after expression in bacteria and removed from glutathione S-transferase (GST) by thrombin digestion.

Kinase Assay—To assay Pim-1 and C-TAK1 kinase activities, the respective GST proteins were purified, mixed (40 µM of bead slurry), and resuspended in 50 µl of kinase buffer (Pim-1: 20 mM Pipes, pH 7.0, 5 mM MnCl2, 5 mM β-mercaptoethanol; C-TAK1: 50 mM Tris-HCl, pH = 7.4, 10 mM MgCl2, both: 10 µM ATP and 10 µM of γ-32PATP). FLAG-tagged Pim-1 or C-TAK1 proteins immunoprecipitated from transfected COS7 cell cultures were used 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 an SDS gel, and subsequently analyzed by x-ray film exposure.

Mass Spectrometry—GST-C-TAK1-wt, C-TAK1-N183A, and Pim-1-wt proteins were purified. Kinase assays were performed as described above but were done with non-radioactively labeled ATP. Phosphorylated or non-phosphorylated proteins were cut out of the gel and digested 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 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 an 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 (BD-Fluorobetes). 1 × 106 cells were transfected with pEFB4 (green fluorescent protein (GFP); Refs. 43 and 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 phosphate-buffered saline and fixed in phosphate-buffered saline/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 RRS (40). We constructed a “bait” plasmid able to express the kinase-inactive 33-kDa form of Pim-1 (Pim-1-K67M) in fusion with Ha-Ras in the vector pADNS-Ras(mut) (40) and introduced this along with an mammalian RASactivating protein expression plasmid (45) into the yeast mutant strain cdc25-2, which contains a temperature-sensitive allele of the GTPase exchange factor CDC25. This RASactivating protein factor 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 dependence 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 used the obtained C-TAK1 sequence to sequence encoding the herpes simplex virus VP16 transactivation domain of 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 strech of the zinc finger transcription factor Gfi1 (Fig. 1b and Ref. 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 β-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 sequenced a human spleen cDNA library by PCR and isolated, in addition to 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 amino acids).
acids (aa); Y, 15 aa) and C-TAK1-β (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 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 -β 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 amino-terminal C-TAK1 fragments spanning aa 1–263 (Y6131) and aa 1–165, and the C-TAK1-α and -β forms (Fig. 2a) indicating that the amino-terminal part of C-TAK1 is sufficient to bind Pim-1 and that C-TAK1 can interact with itself (Fig. 2a). Next radiolabeled 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 autoradiography. 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). Immunoprecipitations 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 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 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.

**FIG. 2. Pim-1 interacts with C-TAK1 in vitro.** a, various C-TAK1 fragments were expressed as GST fusion proteins in bacteria. 35S-Labeled Pim-1-wt or 35S-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 autoradiography. b, upper panel, COS7 cells were transfected with the indicated expression constructs and harvested after 24 h. Lysates of transfected cells were mixed with [35S]labeled C-TAK1-wt protein, immunoprecipitated with a monoclonal anti-FLAG antibody, and analyzed by SDS-PAGE and autoradiography. Lower panel, the same samples were probed with an anti-FLAG antibody as a control. Arrows indicate the immunoprecipitated FLAG proteins. c, COS7 cells were transfected with the indicated expression constructs (FLAG-tagged versions) and harvested after 24 h. Lysates of transfected cells were immunoprecipitated (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 immunoblot with an anti-Pim-1 antibody.
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. 5, a and b, and data not shown). C-TAK1-wt and the two new splice variants C-TAK1-α and -β could autophosphorylate 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 autophosphorylated proteins were detected; when GST-C-TAK1 fusion proteins and the inactive Pim-1 kinase mutant K67M were mixed only the autophosphorylated 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 autophosphorylation and remained unphosphorylated 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 was expressed in COS7 cells, immunoprecipitated, and used as a kinase to phosphorylate C-TAK1 (data not shown). As an additional control the loss
Pim-1 Phosphorylation Inhibits C-TAK1 Kinase Activity—One of the main functions of C-TAK1 is the phosphorylation of Cdc25C at serine 216, enabling 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 (Fig. 7a, b) 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 (Fig. 7a, eluate). Substrates were either GST alone and a GST-C-TAK1 fusion protein coupled to agarose beads (Fig. 7a, eluate). 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 autophosphorylation in the second kinase assay. In reactions in which GST-C-TAK1 beads had been incubated previously with the active Pim-1 kinase, Cdc25C phosphorylation was significantly decreased compared with reactions in which C-TAK1 was used that had been exposed to 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 fluorescence-activated cell sorting. We observed that expression of either Pim-1 or the dominant negative C-TAK1 mutant (C-
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 autophosphorylate themselves but do not phosphorylate GST. The Pim-1-K67M mutant lacked kinase activity as demonstrated by the absence of autophosphorylation. b, wild type C-TAK1 and the two splice variants (-wt, -α, and -β) were able to phosphorylate GST-Cdc25C, recombinant Cdc25C protein, or themselves. c, Pim-1-wt, C-TAK1-wt, and C-TAK1 splice variants showed autophosphorylation. 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 autophosphorylation, but the N183A and D196N inactive single amino acid exchange mutants did not. 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 that covers the amino acids 1–165 and the C-TAK-(81–729) mutant where the amino-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 immunoprecipitated from transfected COS7 cells was 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. auto-P, autophosphorylation; Asterisks, mutants were at aa positions 128, 183, and 196.

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, autophosphorylation was detectable in the carboxyl 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. auto-P, autophosphorylation.
enhanced the fraction of 293 cells in G1 after bleomycin treatment significantly compared with 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) was clearly less active but still maintained a residual ability to inactivate C-TAK1 in this assay (Fig. 7b) probably due to the physical interaction of both proteins.

Pim-1 phosphatase PP1 was identified as an antagonist of C-TAK1 since it is able to dephosphorylate Cdc25C on serine 216 resulting in a derepression 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, causing 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. Nonetheless, 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 cytoplas-
mic compartments (27, 33, 51), and the interaction of Pim-1 with the transcription factors or cofactors NFAT, p100, or c-Myc that have been reported to 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 amino 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 nuclear export sequence (aa 190–199) and the nuclear localization signal (aa 240–244; Ref. 54) of Cdc25C. After phosphorylation of Cdc25C at serine 216 by DNA damage kinases or C-TAK1 and the subsequent binding of 14-3-3 proteins, the nuclear localization signal sequence is no longer accessible and Cdc25C remains in the cytoplasm. In its first activation step, Cdc25C is dephosphorylated at serine 216 by PP1 (49). Moreover it is known that Cdc25C is phosphorylated during prophase by Plks at the amino 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 rephosphorylation 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 that of 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 kinase-inactive Pim-1-K67M mutant clearly had a residual activity inactivating C-TAK1 via direct phosphorylation. However, the kinase-inactive mutant of Pim-1 (K67M) was noticeably less active in this assay, this result is consistent with the transcription factors or cofactors NFAT, p100, or c-Myc that initiate the activation of STAT3/STAT5 and the 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.

**Acknowledgments—**We are indebted to M. Karin for the GST-Jun-(1–233) plasmid, L. W. Enquist for the pBB14 plasmid, and Denise Furgmann for the Env plasmids. We thank Angelika Warda for technical assistance.

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