The *pim* family of proto-oncogenes encodes three serine-threonine kinases that have been implicated in the development of malignancies in mice and in humans. Expression of the Pim protein kinases is tightly regulated at the transcriptional, post-transcriptional, and translational levels. Dysregulation of *pim* transcription and *pim* mRNA stability have been implicated in Pim-mediated transformation. The data presented herein demonstrate that expression of the Pim kinases is additionally regulated at the post-translational level, by the serine-threonine phosphatase protein phosphatase 2A (PP2A). The catalytic subunit of PP2A associates with the Pim kinases in *vivo*, and the Pim kinases are substrates of PP2A phosphatase activity in *vitro*. Furthermore, overexpression of PP2A reduces the levels of the Pim proteins, whereas inhibition of PP2A activity by the protein phosphatase inhibitor okadaic acid stabilizes the Pim proteins. Finally, the effects of PP2A on the expression of the Pim proteins can affect Pim function. Taken together, these data suggest that PP2A activity is important for the regulation of the stability and function of the Pim kinases.

The *pim* family of proto-oncogenes encodes three serine-threonine kinases, Pim-1, -2, and -3 (1–3). Little is known about the functional role of the Pim kinases in vivo, although a number of observations have pointed to a potential role for Pim-1 in signal transduction. Pim-1 has been shown to phosphorylate the transcriptional co-activator p100 and activate the transcription factor c-Myb downstream of Ras (4). Moreover, Pim-1 has been implicated in glycoprotein 130-mediated induction of cell proliferation and protection from apoptosis downstream of signal transducer and activator of transcription 3 (5). In addition, pim-1-deficient mast cells exhibit decreased responsiveness to interleukin-3 in *vitro* (6). Finally, the Pim kinases have recently been shown to associate with and stabilize the suppressor of cytokine signaling protein, SOCS-1 (7). SOCS-1 has been shown to be a potent inhibitor of JAK kinase activity and to play an important role in regulating the responsiveness of cells to cytokine stimulation (8–11). These observations suggest that the Pim kinases may play an important role in cytokine signaling.

Expression of the Pim kinases in normal cells is highly regulated, and several mechanisms by which Pim-1 expression is controlled have been characterized. *Pim-1* mRNA levels are regulated by transcriptional attenuation of *pim-1* (12) as well as by induction of transcription of *pim-1* upon mitogenic stimulation of cells (13). Pim-1 expression is rapidly induced by a number of cytokines including interferon-γ, interleukin-2, and interleukin-3 in hematopoietic cells, and by synaptic stimulation in neurons (3, 13, 14). Pim-2 transcription, like that of Pim-1, is cytokine-inducible, whereas Pim-3 and Pim-1 transcription are induced by synthetic activity (3, 15). Levels of *pim-1* mRNA are also regulated post-transcriptionally by modulation of mRNA stability (13, 16). Expression of the Pim kinases is therefore highly regulated by multiple mechanisms.

Dysregulation of Pim expression has been implicated in the pathogenesis of several different forms of leukemia and lymphoma. *Pim-1* was originally identified as a common proviral insertion site of Moloney murine leukemia virus in T cell lymphomas in mice (1), and viral activation of *pim-2* (2) and transgenic overexpression of *pim-1* and *pim-2* (15, 17) also result in T cell lymphoma development. The Pim kinases have been implicated in the development of human hematopoietic malignancies as well. High levels of Pim-1 protein have been seen in acute myeloid, lymphoid, and erythroid leukemia (18–20).

The observation that transgenic mice overexpressing wild-type Pim-1 or Pim-2 develop tumors demonstrates that Pim-mediated transformation can occur as a consequence of dysregulated Pim expression (15, 17). Diverse mechanisms have been identified in spontaneous tumors that lead to increased Pim-1 expression. Enhanced Pim-1 mRNA stability has been observed in numerous transformed cell lines (21) and the half-life of the Pim-1 protein has been shown to be prolonged in several tumor cell lines (22, 23). Therefore disruption of the regulation of Pim-1 transcript levels and Pim-1 protein stability have been shown to be associated with Pim-mediated transformation.

Protein phosphatase 2A (PP2A) is a highly conserved serine-threonine phosphatase expressed in all eukaryotic cells that is involved in a multitude of cellular functions including transcription and translation, cell cycle progression, and cytokine signaling (reviewed in Ref. 24). In the present study, PP2A is demonstrated to be a binding partner and regulator of the protein stability of the Pim kinases. PP2A associates with Pim-1 and Pim-3 in *vivo* and dephosphorylates Pim-3 in *vitro*. Furthermore, overexpression of PP2A results in a decrease in the expression levels of Pim-1 and Pim-3 protein, and inhibition of PP2A activity by okadaic acid results in stabilization of...
the Pim-1 protein. Finally, PP2A inhibits the effects of the Pim kinases on the SOCS-1 protein. These data demonstrate a novel mechanism for the regulation of Pim protein expression and function and suggest a role for PP2A in Pim-mediated transformation.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The 293T human embryonic kidney cell line is a gift from Dr. Chris Schindler, Columbia University, New York, and was maintained in Isocove’s modification of DMEM containing 10% fetal calf serum unless otherwise noted. BALB/c mice were purchased from Charles River, Wilmington, MA, and splenocytes and thymocytes isolated from the mice were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 μM β-mercaptoethanol, 1 mM sodium pyruvate, 2 mM l-glutamine, 10 mM HEPES, and nonessential amino acids. Cycloheximide, okadaic acid, PMA, and ionomycin were purchased from Sigma. Recombinant PP2A was purchased from Upstate Biotechnology, Lake Placid, NY.

Plasmids—The SOCS-1, Pim-1, and Pim-2 expression vectors have previously been described (7). The human Pim-3 expression vector was a gift from Dr. Vicki Cohan of Pfizer Inc., Groton, CT. Kinase inactive Pim-3 was generated by site-directed mutagenesis of lysine 69 of human Pim-3 to methionine using a PCR-based approach. Murine PP2A was subcloned into the mammalian expression vector pcDNA3.1 His-C, purchased from Invitrogen, San Diego, CA.

Antibodies—The anti-Pim-1 and anti-Pim-3 antibodies were a gift from Dr. Vicki Cohan, Pfizer Inc., Groton, CT. The anti-His antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The anti-HA antibody was purchased from Roche Molecular Biochemicals. The anti-PP2A antibody used in immunoprecipitation was purchased from Upstate Biotechnology, Lake Placid, NY. The anti-PP2A antibody used in Western blotting was purchased from Transduction Laboratories, Lexington, KY. The anti-β-actin and anti-Flag antibodies were purchased from Sigma.

Whole Cell Extracts, Transfections, and Immunoprecipitation—Calcium-phosphate transient transfections of 293T cells were performed as previously described (7). For cycloheximide time course experiments, cycloheximide was used at a concentration of 10 μg/ml. For okadaic acid treatments of cells, okadaic acid was used at a concentration of 25 μM. For LnL treatment of cells, LnL was used at a concentration of 10 μM. Whole cell extracts were prepared and immunoprecipitations were performed also as previously described (7).

In Vitro Kinase and in Vitro Phosphatase Assays—In vitro kinase assays were performed as previously described (6, 22). In vitro phosphatase reactions were performed as above except that, after washing twice in buffer containing 20 mM PIPES and 14 mM β-mercaptoethanol, the immunoprecipitates were washed one time in phosphatase buffer containing 50 mM HEPES, pH 7.5, 0.5% bovine serum albumin, and 1 mM dithiothreitol, and then resuspended in 40 μl of phosphatase buffer (25). Recombinant PP2A was then added, with or without 5 μM okadaic acid, and reactions were incubated at room temperature for 45 min with agitation. Reactions were terminated by washing twice in 50 mM HEPES, pH 8.0.

Pulse-Chase—293T cells grown in DMEM supplemented with 10% fetal calf serum (cDMEM) were transfected by calcium-phosphate transfection in 6-cm dishes. After 24 h, cells were washed once with phosphate-buffered saline and cultured in cDMEM for 24 h. Cells were then washed twice with phosphate-buffered saline and cultured in methionine-free DMEM supplemented with 10% dialyzed fetal calf serum for 1 h. 0.5 μCi per plate of [35S]-labeled methionine was then added to the transfections for 30 min. Cells were then washed twice with phosphate-buffered saline and cultured in cDMEM for the indicated amounts of time, after which cells were harvested and lysed. Whole cell extracts were immunoprecipitated with anti-Pim-3 antibody, immunoprecipitates were run on SDS-PAGE, and the gels were dried and subjected to autoradiography.

Northern Blotting—Total cellular RNA was isolated using the RNeasy MiniKit purchased from Qiagen, Valencia, CA, and Northern blots were prepared using standard techniques. Pim-1 was detected with a full-length cDNA probe radiolabeled by the NEBlot Kit purchased from New England Biolabs, Beverly, MA.

RESULTS

Pim Protein Kinases Associate with the Serine-threonine Phosphatase PP2A in Vivo—The three Pim kinases and the catalytic subunit of PP2A have previously been identified as proteins that can bind the regulator of cytokine signaling, SOCS-1 (7). To determine whether PP2A and the Pim kinases can directly associate, His-tagged PP2A and His-tagged Pim-1 were expressed alone or together in 293T cells, and lysates were subjected to immunoprecipitation with anti-PP2A antibody. His-tagged Pim-1 was detected in PP2A immunoprecipitates of extracts of cells expressing both Pim-1 and PP2A, but not in extracts of cells expressing Pim-1 alone (Fig. 1A). Similar results were observed when Pim-3 and PP2A were co-expressed in 293T cells (data not shown). SOCS-2 was used as a negative control for the immunoprecipitations. In extracts of cells co-expressing His-tagged SOCS-2 and His-tagged PP2A, SOCS-2 did not co-immunoprecipitate with PP2A (Fig. 1A). Thus, in an overexpression system, the Pim protein kinases and PP2A are capable of interacting in vivo.

To confirm that endogenous Pim kinases and PP2A can associate, the ability of Pim-1 to co-immunoprecipitate with PP2A in lysates from primary mouse cells was determined. High levels of Pim-1 are induced by culturing of either mouse spleen or thymus with PMA and ionomycin (26). Therefore, extracts from primary splenocytes and thymocytes, unstimu-
Previous work has demonstrated that the Pim kinases are capable of phosphorylating and stabilizing the SOCS-1 protein (7). The observation that overexpression of PP2A decreases the steady-state levels of expression of the Pim kinases therefore implies that PP2A can modulate the function of the Pim kinases. Co-expression of SOCS-1 and Pim-1 resulted in an increase in the levels of expression of SOCS-1 and in the appearance of multiple hyperphosphorylated forms of SOCS-1 of slower mobility (Fig. 3A, lanes 1 and 2). Upon co-expression of PP2A, however, the levels of SOCS-1, and the presence of the slower mobility forms of SOCS-1, decreased with increasing PP2A expression (Fig. 3A, lanes 3–5). Similar results were obtained when SOCS-1, Pim-3, and increasing amounts of PP2A were co-expressed in 293T cells (Fig. 3B). Therefore overexpression of PP2A in 293T cells modulates the increase in the steady-state levels of SOCS-1 and the appearance of hyperphosphorylated forms of SOCS-1 induced by co-expression of SOCS-1 with the Pim kinases.

To confirm that the effect of overexpression of PP2A on SOCS-1 mobility is not a result of direct dephosphorylation of SOCS-1 by PP2A, the ability of PP2A to dephosphorylate SOCS-1 was determined. SOCS-1 was co-expressed with Pim-3, immunoprecipitated, and incubated in in vitro kinase assays, and SOCS-1 phosphorylated by Pim-3 was then used as a substrate in in vitro phosphate assays with recombinant PP2A dephosphorylates Pim-3 in vitro. 293T cells were transfected with 15 μg of wild-type or kinase-inactive Pim-3, and lysates were immunoprecipitated with αPim-3 antibody. Wild-type Pim-3 immunoprecipitates were incubated with 10 μCi of γ-32P/ATP in in vitro kinase reactions, and then incubated with 0.5, 0.0, and 2 units of recombinant PP2A (rPP2A) in in vitro phosphatase reactions. Autophosphorylated Pim-3 was detected by autoradiography (upper panel), and immunoprecipitates were immuno-blotted with αPim-3 antibody (lower panel). Wild-type but not kinase-inactive Pim-3 underwent autophosphorylation (lanes 1 and 3), and incubation of wild-type Pim-3 immunoprecipitates with rPP2A decreased the intensity of labeling of Pim-3 (compare lane 3 to lanes 4 and 5). Co-incubation of wild-type Pim-3 immunoprecipitates with rPP2A and okadaic acid inhibited the dephosphorylation of Pim-3 by rPP2A (lanes 7 and 8).

Fig. 2. Recombinant PP2A dephosphorylates Pim-3 in vitro. 293T cells were transfected with 15 μg of wild-type or kinase-inactive Pim-3, and lysates were immunoprecipitated with αPim-3 antibody. Wild-type Pim-3 immunoprecipitates were incubated with 10 μCi of γ-32P/ATP in in vitro kinase reactions, and then incubated with 0, 0.5, and 2 units of recombinant PP2A (rPP2A) in in vitro phosphatase reactions. Autophosphorylated Pim-3 was detected by autoradiography (upper panel), and immunoprecipitates were immuno-blotted with αPim-3 antibody (lower panel). Wild-type but not kinase-inactive Pim-3 underwent autophosphorylation (lanes 1 and 3), and incubation of wild-type Pim-3 immunoprecipitates with rPP2A decreased the intensity of labeling of Pim-3 (compare lane 3 to lanes 4 and 5). Co-incubation of wild-type Pim-3 immunoprecipitates with rPP2A and okadaic acid inhibited the dephosphorylation of Pim-3 by rPP2A (lanes 7 and 8).

PP2A regulates Pim protein stability

Fig. 3. Overexpression of PP2A decreases the steady-state levels of the Pim proteins, and SOCS-1, in 293T cells. 5 μg of His-tagged lacZ and 3 μl of Flag-tagged SOCS-1 were transfected into 293T cells alone or together with 10 μg of HA-tagged Pim-1 and 0, 0.1, 0.5 or 1.0 μg of His-tagged PP2A (A) or 3 μg of untagged Pim-3 and 0, 0.1, 0.5, 1.0, 2.5, or 5.0 μg of His-tagged PP2A (B), and lysates were immuno-blotted with αHA (upper panel, A) and αPim-3 (upper panel, B) antibodies, αHis (second and fourth panels, A and B), and αFlag (third panels, A and B). Co-expression of SOCS-1 and Pim-1 (A), and of SOCS-1 and Pim-3 (B), resulted in increased expression of SOCS-1 and the appearance of slower mobility forms of SOCS-1 (A and B, lane 2). Co-expression of SOCS-1, Pim-1, and increasing amounts of PP2A (A), and of SOCS-1, Pim-3, and increasing amounts of PP2A (B), resulted in a decrease in the steady-state levels of the Pim kinases, and in a decrease in the steady-state levels and appearance of the slower mobility forms of SOCS-1 (A, lanes 3–5; B, lanes 3–6). LacZ served as a control for transfection efficiency, protein loading, and nonspecific effects of overexpression of Pim kinases and PP2A.
PP2A. Incubation of the labeled SOCS-1 immunoprecipitates with recombinant PP2A did not result in the dephosphorylation of SOCS-1 (data not shown). Thus SOCS-1 is not a direct substrate of PP2A, and the effects of overexpression of PP2A on SOCS-1 mobility are therefore likely an indirect effect of the decreased levels of Pim protein expressed upon overexpression of PP2A.

Inhibition of Proteasome Function Results in Stabilization of the Pim Kinases—SOCS-1 has been shown to be involved in the recruitment of signaling molecules to the proteasome (28–31). Interestingly, the decrease in the expression levels of the Pim proteins upon overexpression of PP2A requires the presence of SOCS-1. When 293T cells were transfected with Pim-3 and increasing amounts of PP2A in the absence of overexpressed SOCS-1, no decrease in the steady-state levels of Pim-3 were observed (data not shown). The observation that down-regulation of expression of the Pim kinases by PP2A requires the expression of SOCS-1 raises the possibility that the Pim kinases undergo proteasomal degradation and that they are targeted for degradation by SOCS-1. To determine whether the Pim kinases are degraded by the proteasomal pathway, 293T cells were transfected with Pim-3, the cells were treated with the proteasomal inhibitor LLnL, and pulse-chase assays were performed to measure the effect of LLnL on the stability of the Pim-3 protein. LLnL treatment resulted in enhancement of stability of the Pim-3 protein (Fig. 4). These data, combined with the observation that altering cellular levels of PP2A affects the levels of expression of the Pim protein kinases, suggest that the Pim kinases are targeted for proteasomal degradation in a SOCS-1-dependent mechanism.

Inhibition of PP2A Activity in Primary Cells Results in an Increase in the Stability of Endogenous Pim-1 Protein—To determine whether PP2A activity can regulate the stability of endogenous Pim proteins, the effect of inhibition of PP2A on the half-life of Pim-1 in primary cells was determined. Primary mouse splenocytes and thymocytes (B) were untreated, treated with okadaic acid (25 nM), PMA (50 ng/ml), and ionomycin (500 ng/ml), or PMA, ionomycin, and okadaic acid, and then treated with cycloheximide (100 μg/ml) for the indicated times. Cell lysates were then immunoblotted with αPim-1 antibody (A and B, upper panels). Pim-1 levels were barely detectable in untreated cells (A and B, lane 1) and okadaic acid treatment alone did not increase the expression of Pim-1 (A and B, lane 2). PMA and ionomycin dramatically increased Pim-1 levels (A and B, lane 3), and Pim-1 was rapidly degraded in the presence of the protein synthesis inhibitor cycloheximide (A and B, lanes 4–8). Pretreatment of PMA and ionomycin-cultured cells with okadaic acid resulted in sustained expression of Pim-1 upon cycloheximide treatment (A and B, compare lanes 4–8 to 10–14). β-Actin (A and B, lower panels) served as a control for protein loading and for nonspecific effects of okadaic acid.

As PMA and ionomycin also induce Pim-1 expression in the thymus, the effect of okadaic acid on Pim-1 protein stability was confirmed in thymocytes. In thymocytes, okadaic acid treatment had a similar effect on the stability of the 35-kDa isoform of Pim-1 (Fig. 5B). In cells not pretreated with okadaic acid, the levels of the 35-kDa isoform of Pim-1 were significantly decreased after 15 min of cycloheximide treatment, whereas in cells pretreated with okadaic acid the levels of the 35-kDa isoform were still detectable after 45 min of cycloheximide treatment (Fig. 5A, lanes 6 and 13). Okadaic acid alone, however, did not induce expression of Pim-1 in the splenocytes (Fig. 5A, lane 2).

Pim-1 in primary cells. Primary mouse splenocytes (A) and thymocytes (B) were untreated, treated with okadaic acid (25 nM), PMA (50 ng/ml), and ionomycin (500 ng/ml), or PMA, ionomycin, and okadaic acid, and then treated with cycloheximide (100 μg/ml) for the indicated times. Cell lysates were then immunoblotted with αPim-1 antibody (A and B, upper panels). Pim-1 levels were barely detectable in untreated cells (A and B, lane 1) and okadaic acid treatment alone did not increase the expression of Pim-1 (A and B, lane 2). PMA and ionomycin dramatically increased Pim-1 levels (A and B, lane 3), and Pim-1 was rapidly degraded in the presence of the protein synthesis inhibitor cycloheximide (A and B, lanes 4–8). Pretreatment of PMA and ionomycin-cultured cells with okadaic acid resulted in sustained expression of Pim-1 upon cycloheximide treatment (A and B, compare lanes 4–8 to 10–14). β-Actin (A and B, lower panels) served as a control for protein loading and for nonspecific effects of okadaic acid.

Inhibition of PP2A Activity in Primary Cells Results in an Increase in the Stability of Endogenous Pim-1 Protein—To determine whether PP2A activity can regulate the stability of endogenous Pim proteins, the effect of inhibition of PP2A on the half-life of Pim-1 in primary cells was determined. Primary mouse splenocytes and thymocytes (B) were untreated, treated with okadaic acid (25 nM), PMA (50 ng/ml), and ionomycin (500 ng/ml), or PMA, ionomycin, and okadaic acid, and then treated with cycloheximide (100 μg/ml) for the indicated times. Cell lysates were then immunoblotted with αPim-1 antibody (A and B, upper panels). Pim-1 levels were barely detectable in untreated cells (A and B, lane 1) and okadaic acid treatment alone did not increase the expression of Pim-1 (A and B, lane 2). PMA and ionomycin dramatically increased Pim-1 levels (A and B, lane 3), and Pim-1 was rapidly degraded in the presence of the protein synthesis inhibitor cycloheximide (A and B, lanes 4–8). Pretreatment of PMA and ionomycin-cultured cells with okadaic acid resulted in sustained expression of Pim-1 upon cycloheximide treatment (A and B, compare lanes 4–8 to 10–14). β-Actin (A and B, lower panels) served as a control for protein loading and for nonspecific effects of okadaic acid.

Inhibition of PP2A Activity in Primary Cells Results in an Increase in the Stability of Endogenous Pim-1 Protein—To determine whether PP2A activity can regulate the stability of endogenous Pim proteins, the effect of inhibition of PP2A on the half-life of Pim-1 in primary cells was determined. Primary mouse splenocytes and thymocytes, untreated or pretreated with 25 nM okadaic acid were stimulated with PMA and ionomycin and subsequently treated with 100 μg/ml cycloheximide for various times, and the levels of Pim-1 protein expression were determined by Western blotting (Fig. 5). The concentration of okadaic acid used in these experiments has previously been shown to potently inhibit PP2A but not the other major known serine-threonine phosphatases PP-1, PP2B/calcineurin, and PP2C (32). Stimulation of splenocytes with PMA and ionomycin resulted in induction of expression of two, 44 and 35 kDa, isoforms of Pim-1, and both isoforms displayed half-lives of less than 5 min (Fig. 5A, lanes 1 and 3–8). Upon pretreatment of the splenocytes with okadaic acid, however, the half-lives of both isoforms of Pim-1 increased. In cells pretreated with okadaic acid, the levels of the 44-kDa isoform of Pim-1 did not begin to decrease until after 30 min of cycloheximide treatment, and the protein was still clearly detectable at 60 min (Fig. 5A, lanes 12–14). Furthermore, in untreated cells, the levels of the 35-kDa isoform of Pim-1 were barely detectable after 30 min of cycloheximide treatment, whereas in cells pretreated with okadaic acid the levels of the 35-kDa isoform were still detectable after 45 min of cycloheximide treatment (Fig. 5A, lanes 6 and 13). Okadaic acid alone, however, did not induce expression of Pim-1 in the splenocytes (Fig. 5A, lane 2).

As PMA and ionomycin also induce Pim-1 expression in the thymus, the effect of okadaic acid on Pim-1 protein stability was confirmed in thymocytes. In thymocytes, okadaic acid treatment had a similar effect on the stability of the 35-kDa isoform of Pim-1 (Fig. 5B). In cells not pretreated with okadaic acid, the levels of the 35-kDa isoform of Pim-1 were significantly decreased after 15 min of cycloheximide treatment (Fig. 5B, lane 4). In cells pretreated with okadaic acid, however, the levels of the 35-kDa isoform of Pim-1 did not begin to decrease until after 60 min of cycloheximide treatment (Fig. 5B, lane 10). The 44-kDa isoform of Pim-1 has a longer half-life in thymocytes than in splenocytes (Fig. 5B, lanes 3–6), and the levels of the 44-kDa isoform of Pim-1 did not begin to decrease until after 60 min of treatment of the cells with cycloheximide (Fig. 5B, lane 6). Okadaic acid treatment did not significantly affect the half-life of the 44-kDa isoform of Pim-1 expressed in thymocytes (Fig. 5B, lanes 7–10). Therefore inhibition of PP2A activity by okadaic acid preferentially enhances the protein...
Inhibition of PP2A Activity Does Not Alter the Steady-state Levels of pim-1 mRNA—To confirm that the effects of okadaic acid on the levels of expression of Pim-1 are not the result of increased steady-state levels of pim-1 mRNA, the effect of okadaic acid on the levels of pim-1 mRNA was determined. Splenocytes untreated or pretreated with 25 nM okadaic acid were stimulated with PMA and ionomycin and subsequently treated with cycloheximide for various times, RNA was isolated from the cells, and the level of pim-1 mRNA expressed under the different conditions was determined by Northern blotting (Fig. 6). Low levels of pim-1 mRNA were expressed in untreated cells, and these levels were not increased by treatment with okadaic acid alone (Fig. 6, lanes 1 and 2). PMA and ionomycin, however, induced the expression of pim-1 mRNA, and the levels of pim-1 mRNA induced by PMA and ionomycin, and by PMA, ionomycin, and okadaic acid, were equivalent (Fig. 6, compare lanes 3 and 7). Inhibition of PP2A results in an increase in the stability of the Pim-1 protein, but no change in the steady-state levels of pim-1 mRNA, in primary cells.

**DISCUSSION**

In this study, the role PP2A plays in the regulation of the Pim kinases was examined. The Pim kinases were found to associate with PP2A in vivo, and were found to be substrates of PP2A in vitro. Moreover, PP2A was found to regulate the expression of the Pim proteins. Overexpression of PP2A in 293T cells resulted in a decrease in the expression levels of the Pim-1 and Pim-3 proteins, and inhibition of PP2A activity in primary mouse splenocytes and thymocytes by okadaic acid resulted in the stabilization of endogenous Pim-1 protein. Finally, overexpression of PP2A resulted in the inhibition of Pim kinase function in 293T cells. The lower mobility forms of SOCS-1 induced by co-expression of SOCS-1 and the Pim kinases disappeared upon co-expression of PP2A. Thus, regulation of Pim protein stability by PP2A represents an additional mechanism by which Pim kinase expression and function are regulated.

D Dephosphorylation of the Pim kinases by PP2A may directly affect the stability of the Pim kinases. Phosphorylation has been shown to regulate the stability of many cellular proteins by modulating the targeting of the proteins to degradation. Whereas phosphorylation has most often been identified as an event that enhances protein degradation, several proteins, including the antiapoptotic protein Bcl-2, have recently been found to be protected from degradation by phosphorylation (33). Dephosphorylation of the Pim kinases by PP2A may therefore target the Pim proteins to proteasomal degradation. To determine whether the phosphorylation status of the Pim kinases has a direct effect on their stability, the half-lives of wild-type and kinase-inactive Pim-3 protein, which has very weak autophosphorylation activity in vitro (Fig. 2, lane 1), were examined. In cycloheximide time course experiments, wild-type and kinase-inactive Pim-3 protein had similar half-lives (data not shown). This implies that phosphorylation of the Pim kinases and, by extension, dephosphorylation of the Pim kinases by PP2A, does not have a direct effect on the stability of the Pim kinases. However, the Pim kinases could be stabilized in vivo by phosphorylation at the same or other sites than those that undergo autophosphorylation, and these phosphorylation events could affect the stability of endogenous Pim kinases.

Alternatively, the regulation of the stability of the Pim kinases by PP2A may not be a direct consequence of Pim dephosphorylation by PP2A but may instead be an indirect result of PP2A regulating the phosphorylation state and/or function of other cellular proteins that regulate Pim protein stability. One gene that may be involved in the PP2A-mediated targeting of the Pim kinases for degradation is SOCS-1. As well as being a JAK kinase inhibitor, SOCS-1 has also been identified as a potential tumor suppressor gene. SOCS-1 targets TEL-JAK2, an oncogenic form of JAK (29–31)—to JAK-KAK2, thereby inhibiting TEL-JAK2-mediated transformation. This targeting is thought to involve the association of the SOCS box with elongin B and elongin C, homologues of components of the ubiquitination pathway (34, 35). Binding of SOCS-1 to elongin B and elongin C is thought to recruit SOCS-1-binding proteins to proteosomal degradation. The observations that SOCS-1 binds the Pim kinases (7) and that SOCS-1 is required for the PP2A-mediated decrease in steady-state levels of Pim-1 and Pim-3 in 293T cells, suggest that SOCS-1 acts as an adapter between the Pim kinases and the ubiquitination pathway.

Inhibition of the proteasome by LlLnL enhances the stability of the Pim kinases in 293T cells (Fig. 4). Furthermore, the proteasome has been found to be phosphorylated, and phosphorylation of subunits of the proteasome is thought to be involved in the regulation of the assembly of proteasome complexes and the function of the proteasome (36–38). PP2A may therefore regulate phosphorylation of the proteasome, or of proteasome-associated factors, thereby modulating the activity of the complex. As the stability of Pim-1 is known to be enhanced in several Pim-1-expressing tumor lines (22, 23), this raises the possibility that dysregulation of PP2A contributes to pim-mediated transformation.

The observation that inhibition of PP2A activity by okadaic acid results in the stabilization of endogenous Pim-1 protein in primary cells suggests that PP2A plays a role in vivo in the regulation of Pim protein stability. Okadaic acid has been shown to preferentially inhibit PP2A at the low concentration used in these experiments, and at this concentration, okadaic acid does not inhibit the other major serine-threonine phosphatases P1, P2B/calcineurin, and P2C (32). This does not rule out, however, that other less well characterized serine-threonine phosphatases, some of which share greater sequence similarities with PP2A than do P1, P2B, and P2C, may also be inhibited under these conditions. The stabilization of Pim-1 in primary cells by okadaic acid treatment may therefore result from the inhibition of other cellular phosphatases than just PP2A. However, the observation that increasing cellular PP2A activity by overexpression of PP2A decreases the levels of expression of Pim-1 and Pim-3 in 293T cells, whereas decreased cellular PP2A activity, as a result of okadaic acid treatment,
stabilizes the Pim-1 protein in primary cells, strongly implicates PPA2 in the modulation of the stability and expression of the Pim kinases.

In conclusion, regulation of Pim protein stability is an important site of regulation of Pim kinase function, and disruption of that regulation may be an important mechanism by which the Pim kinases lead to transformation. Mutations in the various genes involved in the PP2A-mediated regulation of Pim protein stability may potentially contribute to Pim-induced tumor formation.

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