Protein Phosphatase-1 Modulates the Function of Pax-6, a Transcription Factor Controlling Brain and Eye Development*

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Pax-6 is an evolutionarily conserved transcription factor and acts high up in the regulatory hierarchy controlling eye and brain development in humans, mice, zebrafish, and Drosophila. Previous studies have shown that Pax-6 is a phosphoprotein, and its phosphorylation by ERK, p38, and homeodomain-interacting protein kinase 2 greatly enhances its transactivation activity. However, the protein phosphatases responsible for the dephosphorylation of Pax-6 remain unknown. Here, we present both in vitro and in vivo evidence to show that protein serine/threonine phosphatase-1 is a major phosphatase that directly dephosphorylates Pax-6. Western blot revealed that both protein phosphatase-1 and protein phosphatase-1/H9251 play important roles in regulating gene expression through modulation of various transcription factors. In this regard, here we present first evidence to show that PP-1 acts as a survival promoter of lens epithelial cells. Inhibition of PP-1 activity leads to apoptosis of rabbit, rat, and human lens epithelial cells. Part of the mechanism by which PP-1 promotes survival is derived from its negative regulation of p53, a master regulator of apoptosis. PP-1 can directly dephosphorylate p53 at Ser-15 and Ser-37 to attenuate its transcriptional and apoptotic activities. PP-1 regulates gene expression through modulation of various transcription factors. In this regard, here we present first evidence to show that PP-1 acts as a major phosphatase to dephosphorylate Pax-6, an important transcription factor in the eye.

Pax-6 is an evolutionarily conserved transcription factor that controls eye and brain development in humans, mice, zebrafish, and Drosophila (14–29). Mutations of the Pax-6 gene result in the absence of eyes in humans (30). On the other hand, targeted expression of Pax-6 induces ectopic eye formation in Drosophila (31). Previous studies have shown that various forms of Pax-6 with different molecular weights exist, and at least four variants of Pax-6 (p46, p48, p43, and p32) were detected in cellular extracts (Fig. 1A) (32, 33). All forms of Pax-6 bear a conserved C-terminal transactivation domain, which contains relatively rich proline (P), serine (S) and threonine (T) residues, and is thus named the PST domain. Several phosphorylation sites have been identified in this region of the human and zebrafish Pax-6 (Fig. 1B). It has been shown that phosphorylation of these sites in Pax-6 is carried out by p38, ERK (34), and homeodomain-interacting protein kinase 2 (35). On the other hand, dephosphorylation of Pax-6 remains largely unknown. Here, we present both in vitro and in vivo evidence to show that PP-1 is involved in the dephosphorylation of Pax-6 to modulate its function. In normal physiological conditions, most cellular Pax-6 protein is in an inactivate status, probably due to its dephosphorylation by PP-1. Dephosphorylation of Pax-6 by

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3 The abbreviations used are: PP, protein phosphatase; HLE, human lens epithelial; HLEC, human lens epithelial cell; GST, glutathione S-transferase; RT, reverse transcription; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase.
the C-terminal fragment above. The human Pax-6 gene was purchased from ATCC. The hPax-6 coding sequence was isolated and inserted into the NotI/Sall sites of pCI-neo vector to construct pCI-neo-hPax-6. The human PP-1α catalytic subunit was purchased from ATCC. The coding sequence was isolated and inserted into the EcoRI/XbaI sites of pCI-neo vector. The αB-crystallin promoter (−426 to +44)/chloramphenicol acetyltransferase construct was kindly provided by Dr. Joram Piatigorsky (NEI, National Institutes of Health). A pair of primers with additional restriction enzyme sites were designed as follows: 5′-ATCTGTCCTGACGTCAGCTCCTGTCGCGCAG-3′ (forward) and 5′-ATCTGTAGCTAGAAGGAGGCGCCGGATCC-3′ (reverse). The two primers were used to amplify the αB-crystallin promoter (−426 to +44). The luciferase assay construct pGL3 Basic vector was purchased from Promega (catalog number E1751). The amplified αB-crystallin promoter fragment was isolated and inserted into the XhoI/HindIII sites of pGL3 Basic vector to create pAβ-Luc.

Stable and Transient Transfection—The pCI-neo, pCI-neo-hPax-6, and pCI-neo-PP1α constructs were amplified in DH-5α and purified by Qiagen kits, according to the instruction manual. After treatment with RNAse and extraction by phenol/chloroform, the plasmids were transfected into HLE using Lipofectamine 2000 from Invitrogen, according to the company instruction manual. The transfected cells were then subjected to G418 (400 µg/ml) selection for 4–6 weeks, and then the individual clones for the stable transfected cell lines were established and confirmed with Western blot and RT-PCR analysis.

Treatment by Calyculin A—Parental or various transfected HLE cells were grown to 100% confluence. Then 10 ml of Dulbecco’s modified Eagle’s minimal essential medium containing 0.01% Me3SO (control) or different concentrations of calyculin A were used to replace the culture medium, and the treatment was continued for 1 h.

RNA Interference to Silence Expression of PP-1—The PP-1α, PP-1β, and PP-2A siRNA oligonucleotides as well as the control oligonucleotides were purchased from Santa Cruz Biotechnology. Transfection was conducted as previously described (11).

Protein Preparation and Western Blot Analysis—Protein preparation from both treated and nontreated cells was conducted as previously described (38). Western blot analysis was performed as described before (38).

Immunoprecipitation—Irradiation of HLECs was conducted as previously described (39). Immunoprecipitation of total proteins from normal or UVA-irradiated cells was conducted as previously described (38, 39).

In Vitro Phosphorylation of GST and GST-PST—The fusion proteins of GST-PST and GST protein were prepared as described before (40). To prepare active p38 kinase and parallel control, 10 µg of mouse anti-p38 (pT180/pY182) or 10 µg of mouse anti-β-actin antibody (control) was added into 1 mg of HLE total proteins, and after vortexing for 10 s the mixture was incubated on ice for 1 h. Then 100 µl of protein A/G plus agarose beads were added into the protein solution, and the protein solution was subjected to rotation at 4°C for overnight. The pellet was collected by centrifugation and washed four
times with washing buffer as previously described (11). After washing, the pellet was left in 50 μl of 1X kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35, 0.02% bovine serum albumin) and used for dephosphorylation substrate.

**In Vitro Dephosphorylation Assays**—The in vitro dephosphorylation assays were conducted as previously described (11).

**In Vivo Dephosphorylation Assays**—The in vivo dephosphorylation assays were conducted as described before (11, 41). Briefly, HLECs transfected with pCl-neo-hPax-6 were labeled with [32P]orthophosphate (200 μCi/ml) in phosphatase-free Dulbecco’s modified Eagle’s minimal essential medium. Then total proteins were extracted for immunoprecipitation with an antibody against Pax-6. The precipitated protein pellets were dissolved in 100 μl of 1X protein phosphatase assay buffer. Then, 10 μl of the precipitated protein solution was mixed with 2 μl 0.01% Me₃SO, or 10 nm specific PP-1 inhibitor, PP1-I₂ (from Calbiochem, catalog number 539516, IC₅₀ = 2 nM), or 90 nM specific PP-2A inhibitor, PP2A-I₂ (from Calbiochem, catalog number 539620, IC₅₀ = 30 nM), or 5 nM calyculin A (Invitrogen). After incubation on ice for 30 min, the immunoprecipitated protein complex was incubated at 30°C for 10 min. After trichloroacetic acid precipitation, the supernatant fraction was recovered for counting the release of free 32P in a scintillation counter.

**RNA Extraction and RT-PCR**—Various transfected HLECs were grown to 100% confluence and then harvested for RNA extraction. Briefly, after phosphate-buffered saline washing twice, the pelleted cells were resuspended in RNA extraction buffer, Trizol. The total RNAs were extracted according to the instruction manual. For reverse transcription, 500 ng of total RNAs, 500 ng of oligo(dT)15, and diethyl pyrocarbonate-H₂O were mixed in a total reaction volume of 11 μl. After brief centrifugation, the mixture was heated at 65°C for 5 min followed by immediate incubation on ice for 5 min. After brief centrifugation, the reverse transcription reaction was carried out in a 20-μl system with 1 nM dNTP, 0.01 μM dithiothreitol, 2 units/μl RNase inhibitor, and 15 units of avian myeloblastosis virus at 42°C for 60 min. Then the reaction was stopped at 85°C for 5 min. The following primers were used for PCR analysis: Pax-6, 5’-AGCCAAAATAGATCTACCTGAAG-3’ (forward) and 5’-ACACCAGGGGCAATGAGTCCT-3’ (reverse); β-actin, 5’-GTGGGGCGGCCGGGACCCA-3’ (forward) and 5’-CTCCTTAAGTGTCGCACGATTTCC-3’ (reverse); and αβ-crystallin, 5’-TACCTCAGATGGACATCGGCATCCAC-3’ (forward) and 5’-CAACCCGGGTGTCAAGAAAGGGCCATCTAC-3’ (reverse). PCR was carried out as previously reported (38).

**Analysis of Transient Gene Expression**—The Promega dual luciferase reporter assay system was used in our analysis. In brief, 2.5 μg of luciferase reporter gene (pGL3-Basic), together with 3.5 μg of pCl-neo-Pax-6 plasmid plus 1.5 μg of pCl-neo-PP1α, or 1.5 μg of pCl-neo-PP2A and 0.2 μg of control plasmid Renilla luciferase pRL-SV40-Luc (catalog number E2231) were co-transfected into mouse lens epithelial cell line αTN4-1 or rabbit lens cell line N/N1003A by using the Lipofectamine 2000 kit from Invitrogen. At 24 h after transfection, the cells were harvested for luciferase assay according to the company instruction manual. And the luciferase activity was determined by using the Luminoskan RS microplate reader from Thermo Labsystems Corp.

**Quantitation of the Total Phospho-Pax-6 and Nonphospho-Pax-6**—The Western blot gel and RT-PCR gel were analyzed with UN-SCAN-IT software from Silk Scientific Corp. Total pixel data were averaged from three or more different groups of samples of each species after normalization against the background.

**Statistical Analysis**—In the present study, all of the data presented are derived from at least three experiments. During data analysis, statistical analysis was conducted for all sets of data when necessary. Both average and S.D. value were calculated and included in the figures.

**RESULTS**

**PP-1 and PP-2A Can Dephosphorylate Pax-6 in Vitro**—To explore what phosphatases may dephosphorylate Pax-6, we first tested the serine/threonine phosphatase-1 and -2A with the in vitro dephosphorylation assays. Our previous studies have shown that both PP-1 and PP-2A are present in the lens epithelial cells (8). To do so, the full-length Pax-6 protein or its PST domain was fused into the pGEX 4T-1 vector to make fusion proteins (Fig. 2A). Both Coomassie Blue staining and Western blot analysis were applied to test their expression in Escherichia coli BL-21. Both full-length GST Pax-6 fusion protein (GST-mPax-6) (Fig. 2B, right lane) and the GST PST domain fused protein (GST-PST) (Fig. 2B, middle lane) were obtained. Since the full-length GST-mPax-6 only accounted for a small portion of the expressed GST-mPax-6 (the majority of the GST-mPax-6 full-length fusion proteins were truncated by bacterial enzymes and thus appeared in multiple bands with molecular mass less than 74 kDa), the purified GST-PST protein was used for in vitro dephosphorylation assays, and GST was used for the parallel control (Fig. 2B). Both GST-PST and GST substrates were labeled with [γ-32P]ATP and the immunoprecipitated p38 kinase or the immunoprecipitated β-actin (for mock). The labeled GST or GST-PST was then subjected to dephosphorylation by purified catalytic subunits of PP-1 or PP-2A obtained from Calbiochem. After dephosphorylation, the released free 32P was determined. As shown in Fig. 2, C and D, respectively, both PP-1 and PP2A were able to dephosphorylate Pax-6. In contrast, the GST did not generate a significant amount of free 32P, indicating that both PP-1 and PP2A specifically dephosphorylated Pax-6 but not GST (Fig. 2, C and D). Moreover, the labeling reaction with immunoprecipitated β-actin did not yield significant free 32P from the reactions with either PP-1 or PP-2A (data not shown), further demonstrating that the specificity of dephosphorylation of the serine/threonine residues in Pax-6 by PP-1 and PP-2A. Under the present assay conditions, dephosphorylation of Pax-6 by PP-2C was not detected (data not shown). Together, these results suggest that PP-1 and PP-2A are able to dephosphorylate Pax-6 in vitro.

**The Catalytic Subunits for PP-1 but Not for PP-2A Form a Complex with Pax-6 in Vivo**—To demonstrate that PP-1 and PP-2A are able to dephosphorylate Pax-6 in vivo, we first examined whether the catalytic subunits for PP-1 or PP-2A could form interacting complexes. To do so, total proteins extracted from HLECs with or without UVA irradiation were subjected to...
not PP-2A is involved in direct dephosphorylation of Pax-6 in normal physiological conditions. UVA irradiation enhances the interactions of both PP-1α and PP-1β with Pax-6 (panels 1–4 of Fig. 3B). To further explore the interactions between PP-1α and Pax-6, as well as PP-1β and Pax-6, we treated human lens epithelial cells with calcyclin A, an inhibitor for both PP-1 and PP-2A. Inhibition of PP-1α or PP-1β with calcyclin A attenuates the interaction between PP-1 and Pax-6 (Fig. 3F). Thus, both PP-1α and PP-1β are likely to participate in dephosphorylation of Pax-6.

PP-1α Can Dephosphorylate Pax-6 in Vivo, and Overexpression of PP-1α Leads to Dephosphorylation of Pax-6—To demonstrate that PP-1 actually dephosphorylates Pax-6 in vivo, we conducted the in vivo dephosphorylation assay as previously described (11, 41). HLECs overexpressing hPax-6 were labeled with [32P]orthophosphate, and the labeled proteins were immunoprecipitated with rabbit anti-Pax-6 antibody. The immunoprecipitated proteins were resuspended in dephosphorylation buffer and then incubated at 30°C 10 min for the dephosphorylation reaction. After the reaction, the released free 32P was determined. As shown in Fig. 4A, a significant amount of free 32P was released, and this release was mostly blocked when a specific PP-1 inhibitor, PP1-I2 (IC50 = 2 nM), or 5 nM calcyclin A was present in the dephosphorylation reaction (Fig. 4A). However, a specific inhibitor for PP-2A, PP2A-I2 (IC50 = 30 nM), did not block the dephosphorylation reaction. This result clearly showed that PP-1 binds to Pax-6 and dephosphorylates it in normal human lens epithelial cells. To further explore this in vivo dephosphorylation, we examined the phosphorylation status of the 32- and 46-kDa Pax-6 in normal HLE cells, vector-transfected HLE cells, or PP-1α overexpression HLE cells. As shown in Fig. 4B, whereas 46-kDa Pax-6 appears in hypophosphorylation status in all three types of cells, the 32-kDa Pax-6 exists in both hyper- and hypophosphorylation status. Moreover, in the PP-1α overexpression cells, the ratio of hyperphospho-Pax-6 to hypophospho-Pax-6 is much lower than that in the vector-transfected or nontransfected HLE cells (Fig. 4C). Together, these results show that two different forms of Pax-6 with different phosphorylation status are present in human lens epithelial cells. Overexpression of PP-1α leads to enhanced dephosphorylation of the hyperphosphorylated 32-kDa Pax-6.

Inhibition of PP-1 by Calyculin A Enhances the Phosphorylation of Pax-6—Since overexpression of PP-1α did not change the phosphorylation status of the 46-kDa Pax-6, we next...
explored whether inhibition of PP-1 activity would affect the phosphorilation status of the 46-kDa Pax-6 as well as 32-kDa Pax-6. As shown in Fig. 5A, inhibition of PP-1 with 5 nM calyculin A led to the presence of multiple forms of hyperphosphorylated Pax-6, either 32 or 46 kDa in both vector and PP-1α transfected human lens epithelial cells. Analysis of the ratio between hyper- and hypophosphorylated Pax-6 through the density scanning of the Western blots revealed that the hyperphosphorylation of the 46-kDa Pax-6 observed in the vector-transfected clone due to inhibition of PP-1 by calyculin A was
almost reversed by overexpression of PP-1α in the pCI-PP-1α-transfected clone (Fig. 5B). For the 32-kDa Pax-6, inhibition of PP-1α by calcin A caused more than 30% hyperphosphorylation in vector-transfected cells. In the pCI-PP-1α transfected clone, however, much reduced hyperphosphorylation in p32 Pax-6 was observed due to the action of the overexpression of PP-1α either without or with treatment by calcin A (Fig. 5C). Together, these results indicate a counteraction of the overexpressed PP-1α to the inhibition function by calcin A.

A further comparison of the phosphorylation status of the 46- and 32-kDa Pax-6 under treatments of different concentrations of calcin A revealed that with the increase in its concentration, calcin A resulted in gradual hyperphosphorylation status of both the 46-kDa Pax-6 (Fig. 5, D and E) and the 32-kDa Pax-6 (Fig. 5, D and F). In the absence of calcin A, siRNA for PP-1α had little effect on expression of PP-1α (Fig. 6D). When the siRNA for PP-1β was used for knockdown of PP-1β expression, 15% of the 46-kDa Pax-6 and also ~80% of the 32-kDa Pax-6 were hyperphosphorylated (Fig. 6, E and F). Thus, both PP-1α and PP-1β are involved in dephosphorylation of Pax-6. When specific siRNA for PP-2Aα was used to knock down the expression of the endogenous PP-2Aα, no hyperphosphorylation of Pax-6 was detected (Fig. 7). This result further suggests that PP-2Aα is not responsible for the dephosphorylation of Pax-6.

**Mutation Imitating Constant Phosphorylation or Constant Dephosphorylation of Pax-6 Modulates Its Function**—To explore whether dephosphorylation of Pax-6 may have any effects on its function, we have overexpressed the wild type, TS360/361DD, and TS360/361AA mutant Pax-6 in human lens treatment, the 46-kDa Pax-6 appeared in a single dephosphorylation band, and the 32-kDa Pax-6 appeared in two bands, both hypo- and hyperphosphorylation. Under treatment with 2 nM calcin A, the single dephosphorylation band of Pax-6 (p46) was reduced, and about 5% hyperphosphorylated Pax-6 (p46) appeared (Fig. 5, D and E); at the same time, about 70% hyperphosphorylated Pax-6 (p32) became visible (Fig. 5, D and F). However, a treatment with 5 nM calcin A led to further increase in hyperphosphorylation of both forms of Pax-6 (Fig. 5D). About 60% of p46 Pax-6 (Fig. 5E) and 90% of p32 Pax-6 became hyperphosphorylated (Fig. 5F). These results further confirm that PP-1α is the protein phosphatase responsible for dephosphorylation of Pax-6 in human lens epithelial cells.

**Silence of PP-1α and PP-1β but Not PP-2Aα by Specific siRNAs Substantially Enhances the Hyperphosphorylation of Pax-6**—To further confirm that PP-1α is responsible for dephosphorylation of Pax-6, we examined the phosphorylation status of Pax-6 with endogenous PP-1 and PP-2A knocked down by specific siRNAs against PP-1α or PP-1β or PP-2Aα. As shown in Fig. 6A, siRNA for PP-1α down-regulated up to 70% of PP-1α expression. When the endogenous PP-1α was down-regulated, about 20% of the 46-kDa Pax-6 became hyperphosphorylated (Fig. 6B), and 80% of the 32-kDa Pax-6 was found hyperphosphorylated (Fig. 6C). The
epithelial cells. As shown in Fig. 8A, the three types of Pax-6 are all expressed in HLE cells with similar mRNA levels (panel 1 of Fig. 8A) and protein levels (panel 3 of Fig. 8A). With the same mRNA extracted from the human lens epithelial cells expressing either mutant or wild type Pax-6, we analyzed expression of one of the downstream target genes, αB-crystallin. Previous
FIGURE 6. Silence of PP-1α or PP-1β by specific siRNA enhances hyperphosphorylation of Pax-6. A, specific siRNA to PP-1α reduced 70% of PP-1α expression (lane 2) compared with the control siRNA (mutated PP-1α siRNA). In contrast, the siRNA to PP-1β had little effect on PP-1α expression (lane 3) but did cause hyperphosphorylation of Pax-6, because it knocked down PP-1β (described in D). Silence of PP-1α leads to appearance of a prominent hyperphosphorylation band of the 46-kDa Pax-6 (p46) and enhanced hyperphosphorylation of the 32-kDa Pax-6 (p32). Treatment of the cells with calyculin A resulted in a stronger level of Pax-6 hyperphosphorylation in both p46 and p32. B, quantitation of phosphorylation of the 46-kDa Pax-6 (p46) in A and unpresented Western blots. Density scanning analysis revealed that about 20% of the 46-kDa Pax-6 became hyperphosphorylated due to PP-1 knockdown by siRNA. About 35% of the 46-kDa Pax-6 became hyperphosphorylated due to PP-1 inhibition by calyculin A. C, quantitation of phosphorylation of the 32-kDa Pax-6 (p32) in A and unpresented Western blots. Density scanning analysis revealed that about 80% of the 32-kDa Pax-6 became hyperphosphorylated due to PP-1 knockdown by siRNA. About 90% of the 32-kDa Pax-6 became hyperphosphorylated due to PP-1 inhibition by calyculin A. D, specific siRNA to PP-1β reduced 85% of PP-1β expression in comparison with the control siRNA (mutated PP-1β siRNA). On the other hand, the siRNA to PP-1α had little effect on PP-1β expression (lane 1) but did cause hyperphosphorylation of Pax-6, because it knocked down PP-1α (described in A). Silence of PP-1β led to the hyperphosphorylation of Pax-6 (both 46-kDa Pax-6 and 32-kDa Pax-6). E, quantitation of phosphorylation of the 46-kDa Pax-6 (p46) in D and unpresented Western blots. Density scanning analysis revealed that about 15% of the 46-kDa Pax-6 became hyperphosphorylated due to PP-1 inhibition by siRNA. About 40% of the 46-kDa Pax-6 became hyperphosphorylated due to PP-1 inhibition by calyculin A. F, quantitation of phosphorylation of the 32-kDa Pax-6 (p32) in D and unpresented Western blots. Density scanning analysis revealed that about 80% of the 32-kDa Pax-6 became hyperphosphorylated due to PP-1 knockdown by siRNA. A similar percentage of the 32-kDa Pax-6 became hyperphosphorylated due to PP-1 inhibition by calyculin A.
PP-1 Dephosphorylates Pax-6

Co-transfection of PP-1 and Pax-6 Can Down-regulate the Function of Pax-6, whereas Knockdown of PP-1 Enhances Pax-6 Transcriptional Activity—Together, these results demonstrate that PP-1 is the major phosphatase dephosphorylating Pax-6 and thus actively modulating Pax-6 functions.
PP-1 Dephosphorylates Pax-6

both forms of Pax-6; 6) overexpression of human Pax-6 mutant imitating constant dephosphorylation at residue 360/361 substantially attenuates its ability to regulate its downstream target gene, αB-crystallin transcription; 7) co-transfection of PP-1α but not PP-2Aα with Pax-6 could abolish the translational function of a Pax-6 downstream gene promoter, the αB-crystallin promoter, whereas knockdown of the transcription of PP-1 could enhance the transcription of the Pax-6 downstream gene, αB-crystallin. These results confirm that PP-1 but not PP-2A directly dephosphorylates Pax-6 in human lens epithelial cells and that dephosphorylation by PP-1 modulates Pax-6 function.

PP-1 Is a Major Phosphatase That Dephosphorylates Pax-6 in Human Lens Epithelial Cells—Pax-6 is an important transcription factor that regulates eye development in a variety of organisms from Drosophila to humans (14–31). Mutations of the Pax-6 gene result in the absence of eyes in humans (30), and targeted expression of Pax-6 induces ectopic eye formation in Drosophila (31). Pax-6 appears in multiple forms with different molecular weights: p48, p46, p43, and p32 (32, 33). Although the N terminus is heterogeneous in different forms, all of the forms have a conserved homeobox domain in the central region and an activating PST in the C terminus (Fig. 1). Sequence alignment of the PST domains revealed very high amino acid sequence homology among different vertebrate species and the relatively rich proline, serine, and threonine residues in this region. These features suggest that Pax-6, as a transcription factor, could be modulated by post-translational modifications, such as phosphorylation and dephosphorylation. Indeed, it has been shown that the function of Pax-6 can be modulated by several kinases, including p38, ERK (34), and homeodomain-interacting protein kinase 2 (35). In the present study, we have demonstrated that although both PP-1 and PP-2A are able to dephosphorylate Pax-6 in the in vitro dephosphorylation assays, in the immunoprecipitation-linked Western blot analysis, PP-2A and Pax-6 failed to form interactive complex, and thus, it is unlikely that PP-2A dephosphorylates Pax-6 in vivo. The fact that in the reciprocal immunoprecipitation-linked Western blot analysis, we observed that more than 90% of 46-kDa Pax-6 and 100% of 32-kDa Pax-6 proteins were

DISCUSSION

In the present study, we have demonstrated the following: 1) PP-1 and PP-2A dephosphorylate Pax-6 in the in vitro dephosphorylation assays; 2) the catalytic subunits for PP-1α and PP-1β (but not the catalytic subunit nor the scaffold subunit, A, and the regulatory subunit, B, of PP-2A) are able to form interactive complex with Pax-6, and more than 90% of Pax-6 are bound to PP-1 within normal human lens epithelial cells; 3) PP-1 directly dephosphorylates Pax-6 in the in vitro dephosphorylation assays; 4) two forms of Pax-6 (p46 and p32) are present in human lens epithelial cells (whereas the p46 exists in hypophosphorylated status, p32 exists in both hyper- and hypophosphorylated status); 5) although overexpression of PP-1α promotes dephosphorylation of Pax-6 (p32), inhibition of PP-1 activity with calyculin A or knockdown of PP-1 expression with specific siRNAs enhances hyperphosphorylation of

FIGURE 9. Demonstration that dephosphorylation of Pax-6 by PP-1 changes its transcriptional activity in vivo. A, overexpression of PP-1α but not PP-2Aα abolishes Pax-6-induced increase in the reporter gene activity. The luciferase reporter gene construct, pαB-Luc, driven by the promoter of the αB-crystallin, a downstream gene of Pax-6, was co-transfected with either a vector, pCI-Neo, or a Pax-6 expression construct without any other construct or together with a PP-1α expression construct (pCI-PP-1α) or together with a PP-2Aα expression construct (pCI-PP-2Aα) in the presence of a control plasmid pSV40-Luc into mouse lens epithelial cells, αTN4–1, or rabbit lens epithelial cells, N/N1003A. Overexpression of Pax-6 enhanced the luciferase activity from the basic level in both types of cells. Such enhancement was abolished by co-overexpression of PP-1α but not by co-overexpression of PP-2Aα. Luciferase activity was assayed as described under “Experimental Procedures.” B, knockdown of PP-1α and PP-1β but not PP-2Aα enhances expression of the Pax-6 downstream gene encoding αB-crystallin in human lens epithelial cells. RNA interference and RT-PCR were conducted as described under “Experimental Procedures.” Note that knockdown of PP-1α and PP-1β barely changed the Pax-6 mRNA level but significantly enhanced expression of the αB-crystallin mRNA. C, quantitation of the αB-crystallin mRNA levels from three different experiments. The relative mRNA level for αB-crystallin was obtained through dividing the total αB-crystallin mRNA pixel by the Pax-6 mRNA pixel after normalization against β-actin. Note that knockdown of PP-1α and PP-1β led to more than 50% increase in the αB-crystallin mRNA expression. In contrast, knockdown of PP-2Aα had little effect on the αB-crystallin mRNA expression. Thus, dephosphorylation of Pax-6 by PP-1 significantly changes its transcriptional activity. The corresponding number in B and C represents the same conditions.
bound to PP-1 suggests that protein phosphatase-1 seems to be the major phosphatase that dephosphorylates Pax-6 (Fig. 3B).
This conclusion is further supported by the fact that knockdown of either PP-1α or PP-1β by specific siRNAs leads to hyperphosphorylation of a significant portion of the 46-kDa Pax-6 and a majority of the 32-kDa Pax-6 (Fig. 6). The fact that siRNA only decreased up to 85% PP-1 expression and, moreover, only a small portion of cellular PP-1 was bound to Pax-6 (Fig. 3B) explains why a large portion of 46-kDa Pax-6 is still in hypophosphorylation status in the PP-1 knockdown cells (Fig. 6).

In the present studies, we also observed that two forms of Pax-6 are present in human lens epithelial cells. Although the p46 Pax-6 only exists as the hypophosphorylated form, the p32 Pax-6 appears in both hyperphosphorylated and hypophosphorylated forms. Since the 32-kDa Pax-6 has slight high affinity to PP-1 (Fig. 3B), its presence of hyperphosphorylation status suggests the possibility that the two forms of Pax-6 proteins are differentially phosphorylated by the cognate kinases. Differential phosphorylation status of the two forms of Pax-6 may indicate a functional difference. First, they might have different target genes. Consistent with this possibility is the fact the 32-kDa Pax-6 contains only the homeodomain for DNA binding; in contrast, the 46-kDa Pax-6 contains two DNA binding regions: the paired domain and the homeodomain. Second, it is also possible that the two forms of Pax-6 may have similar target genes but different activation potential. These possibilities are currently under investigation.

Dephosphorylation Acts as a Molecular Switch—It has been well established that reversible phosphorylation and dephosphorylation at the serine and threonine residues on proteins play important roles in regulating gene expression, cell cycle progression (43), and apoptosis (44). Although protein phosphorylation through activation of various kinases has been the central subject of signal transduction studies and thus the field is well advanced, the study of protein dephosphorylation by protein phosphatases is now becoming established. Results from recent studies in numerous laboratories have shown that protein dephosphorylation has an important impact in regulating different cellular functions.

Dephosphorylation generally inactivates functions of a target molecule. In the present study, we demonstrate that PP-1 but not PP-2A directly dephosphorylates Pax-6. Dephosphorylation of Pax-6 has important functional consequence, as demonstrated from three aspects. First, a mutant imitating constant dephosphorylation at Thr-360/Ser-361 negatively regulates its downstream target gene coding for αB-crystallin (Fig. 7). In contrast, a mutant imitating constant phosphorylation at the same residues enhances expression of the same target gene. Second, reporter gene luciferase activity assays through the αB-crystallin promoter revealed that co-transfection of pCI-Pax-6 significantly increases the reporter gene activity. This increase derived from Pax-6 overexpression was abolished by PP-1α overexpression but not by PP-2Aα overexpression (Fig. 9A). Finally, knockdown of PP-1α or PP-1β but not PP-2Aα by specific siRNAs enhances hyperphosphorylation of Pax-6 (Fig. 6) and also up-regulates the transcription of the Pax-6 target gene, αB-crystallin (Fig. 9, B and C).

Consistent with the fact that dephosphorylation of Pax-6 attenuates its transcriptional activity in human lens epithelial cells, dephosphorylation of p53, as we and others have recently shown, attenuates its transcriptional activity and also its pro-apoptotic activity in both lens and nonlens cells (11, 45). More recently, Lin et al. (46) also demonstrated that dephosphorylation of Smad2/3 by another serine/threonine phosphatase, PPM1A/PP-2Ca, terminates TGFβ signaling pathway. In addition, PP-2A dephosphorylation of ERK negatively modulates the ERK signaling pathway (47).

On the other hand, dephosphorylation may also activate functions of the target molecules. It has been shown that dephosphorylation of the Na+/,K+−ATPase by calcineurin enhances its enzyme activity (48). Similarly, dephosphorylation of Rb, a tumor suppressor at Thr-821, by protein serine/threonine phosphatase-1 promotes its ability to bind to members of the E2F family (49). Together, these studies suggest that dephosphorylation can act as a molecular switch, which regulates multiple cellular functions.

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