Analysis of Expression Patterns of Protein Phosphatase-1 and Phosphatase-2A in Rat and Bovine Lenses

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Purpose. The reversible phosphorylation and dephosphorylation at the serine and threonine residues on proteins play distinct roles in regulating multiple cellular activities. Whereas the protein serine-threonine kinases have been well studied in the lens system, very little is known about the expression and function of the serine-threonine phosphatases. The present article reports the expression patterns of protein phosphatase (PP)-1 and -2A in adult rat and bovine lenses.

Methods. Total RNAs and proteins were extracted from the epithelial and fiber cells of rat and bovine lenses. RT-PCR and Northern blot analysis were used to detect the mRNA expression levels in the epithelial cells and different fractions of fiber cells of these two types of lenses. Western blot was used to examine the protein expression levels in these different samples. An enzymatic assay was used to detect the activity distribution of PP-1 and -2A in these samples.

Results. The mRNAs for the PP-1 catalytic subunit (PP-1cs) and PP-2A catalytic subunit (PP-2Acs) were expressed in both epithelial and fiber cells of rat and bovine lenses. A detailed examination of the expression patterns of the two mRNAs in different fractions of fiber cells revealed that the cortical fiber cells (F1) contain the highest level of PP-1cs and -2Acs mRNAs (similar to those in the epithelial cells) among different fractions of fiber cells. The levels of the two mRNAs were sequentially decreased in the next layers of fiber cells (F2 and F3) and became barely detectable in the inner layers of fiber cells (F4 and N). In contrast to the mRNA expression patterns, the PP-1cs protein was mainly found in the epithelial cells. Among different layers of fiber cells, only cortical (F1) fiber cells contained detectable level of PP-1cs protein (bovine lenses contained a relatively higher level of PP-1cs than rat lenses in this region). In the remaining fiber cells, the PP-1cs protein was hardly detectable in rat lenses and slightly detectable in bovine lenses. The PP-2Acs protein was detectable only in the lens epithelial cells. Enzymatic assays revealed that the distribution patterns of PP-1 and -2A activities were similar to those of PP-1cs and -2Acs proteins. Furthermore, PP-1 activity was approximately four to five times higher than PP-2A activity in the lens epithelial cells.

Conclusions. This study demonstrates that active PP-1 and -2A are mainly distributed in the lens epithelial cells, with PP-1 as a major phosphatase. The mRNAs and proteins for PP-1cs and -2Acs are differentially expressed in the epithelial and fiber cells of rat and bovine lenses. (*Invest Ophthalmol Vis Sci.* 2001;42:2603–2609)

The reversible phosphorylation and dephosphorylation at the serine and threonine residues on proteins play important roles in regulating gene expression,[1] cell cycle progression,[2] apoptosis,[3–6] and other cellular activities. In the eukaryotes, dephosphorylation at the serine-threonine sites is largely executed by four major protein phosphatases—phosphatase (PP)-1, -2A, -2B, and -2C[7,8] although other recently identified protein phosphatases—PP-4, -5, -6, and -7 also contribute to this process.[9–13] The majority of intracellular protein phosphatase activity has been attributed to PP-1 and -2A.[7,8,14,15]

PP-1 and -2A are involved in regulation of many important cellular activities. Both phosphatases can regulate expression of other genes.[1] For example, by dephosphorylating Ser-133 of the cAMP response element-binding protein (CREB), PP-1 can downregulate expression of the somatostatin gene.[16] Through dephosphorylation of the elongation factor-2, PP-2A regulates gene expression at the translation level.[17] PP-1 and -2A exert control of the cell cycle by interacting with certain key regulators. The human PP-1α interacts with the hypophosphorylated form of the retinoblastoma (RB) protein from mitosis to the G1 stage.[18] In *Xenopus* egg extracts, PP-2A dephosphorylates another phosphatase, cdc25-C, to negatively regulate p34cdc2/cyclin B which is an important cell-cycle activator.[19] PP-1 and -2A are also actively engaged in other cellular processes such as cell differentiation and programmed cell death.[1,3]

In the lens system, although the expression and function of various serine-threonine kinases have been well documented,[20–38] much less is known regarding the serine-threonine phosphatases. Nevertheless, the few available studies suggest that the serine-threonine phosphatases may play important roles in normal lens function and during pathogenesis. For example, Chiesa and Spector[39] showed that in bovine lens, the α-crystallin A chain is dephosphorylated by PP-2B. More recently, using differential display, Kantorow et al.[40] demonstrated that the regulatory subunit of PP-2A was downregulated in human cataractous lens. We have recently shown that in rabbit and rat lens epithelial cells, inhibition of PP-1, but not PP-2A, by okadaic acid induces apoptosis of the treated lens epithelial cells.[41,42]

To further extend studies on the expression and function of protein serine-threonine phosphatases in the lens system, we examined the expression patterns of the two important phosphatases, PP-1 and -2A, in rat and bovine lenses. Our results demonstrate that the genes encoding PP-1cs and -2Acs are differentially expressed at the mRNA and protein levels in the epithelial and fiber cells of rat and bovine lenses. Moreover, both PP-1 and -2A are mainly localized in lens epithelial cells, with PP-1 as a major phosphatase.

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Supported by a Grant EY 11372 from the National Eye Institute (DW-CL).

Submitted for publication March 21, 2001; revised June 11, 2001; accepted June 22, 2001.

Commercial relationships policy: N.

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METHODS

Sources of Rat and Bovine Lenses

Rats used in this study were handled in compliance with the "Guide for the Care and Use of Laboratory Animals" and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Four-week-old Sprague-Dawley rats weighing approximately 100 g were ordered from Harlan Sprague-Dawley (Indianapolis, IN). Bovine eyes were obtained from abattoirs. The age of the cattle ranged from 18 to 24 months.

Preparation of Total RNAs

For rat lens epithelial and fiber cell collections, the rats were killed by CO_2 inhalation. The eyeballs were removed and the lenses were carefully dissected by a posterior approach.[43] The lens capsule and epithelial cells were removed immediately and transferred into a tube (Eppendorf, Fremont, CA) containing 500 μl RNA extraction buffer (Trizol; Gibco BRL, Grand Island, NY). The epithelia were homogenized on ice with a tube micropestle (Brinkman Instruments, Inc., Westbury, NY). The remaining fiber mass from a combination of 10 lenses was immediately transferred into a 20-ml beaker containing 2 ml RNA extraction buffer with constant stirring using a 5-mm magnetic stirring bar at 4°C. After 20 minutes, the dissolved fiber cell solution was collected and homogenized for 20 strokes with a glass homogenizer (Kimax; Fisher Scientific, Fairlawn, NJ). The remaining part of the lens was transferred to a new beaker containing 2 ml extraction buffer, and the next layers of fiber cells were dissolved by another 20-minute stirring. This process was repeated another two times. The first collection of the dissolved fiber cells was designated F1 (the cortical layers of secondary fibers) and the fourth collection, F4 (the inner layers of secondary fibers). After four collections, the final part of the lens (the lens nucleus mainly consisting of primary fibers) was dissolved by homogenization with the glass homogenizer, and the sample was labeled N. The homogenates of both epithelial and fiber cells were immediately processed for RNA extraction, as previously described.[41–44] The same procedure was used for RNA extraction from bovine lenses, except that a single lens with 4 ml extraction buffer was used, and the stirring time was 40 minutes for each fraction.

Reverse Transcription-Linked Polymerase Chain Reaction

Reverse transcription was conducted using a kit (catalog no. 18085-019; Gibco) as previously described.[42,44] Briefly, 3 μg total RNA was used in a total reaction volume of 25 μl. For PCR amplification, the following primers were used: β-actin, 5′-GTGGGGCGCCCCAG-GCACCA-3′ (forward) and 5′-CTCCTTAATGTCACGCACG-ATTTC-3′ (reverse); rat PP-1, 5′-TCCATGGAGCAGATTAGACG-3′ (forward) and 5′-GCTTTGGCAGAATTGCGG-3′ (reverse); and rat PP-2A, 5′-GTTCGT-TACCGAGA-GCGTATCA-3′ (forward) and 5′-GCTTGGTTACCACAAC-GATAGC-3′ (reverse). Two microliters of the reverse transcription reaction mixture was used for the PCR reaction. For PCR, both the β-actin and the PP-1 primers or the β-actin actin and PP-2A primers were added at the same time, for a total 30-cycle amplification. Each cycle was run with the following program: denaturing at 94°C, 30 seconds; annealing at 52°C, 30 seconds; chain extension at 72°C, 1 minute. At the end of each reaction, the PCR products were separated by agarose gel (1.5%) electrophoresis and photographed under UV illumination.

Preparation of Total Proteins

After dissection of rat lenses, the lens capsule and epithelial cells were removed immediately and transferred into a tube (Eppendorf) containing 300 μl extraction buffer (50 mM Tris-HCl [pH 7.0]; 0.1% β-mercaptoethanol; 0.1 mM EDTA, 0.1 mM EGTA, 2 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM benzamidine-HCl, 2 mM dithiothreitol [DTT], 0.5% Triton X-100). The epithelia were homogenized on ice with the tube micropestle (Eppendorf; Brinkman Instruments, Inc.). The remaining fiber mass was immediately transferred into a 10-ml beaker containing 1 ml protein extraction buffer with constant stirring, using a 5-mm magnetic stirring bar at 4°C. During the extraction, we found that the lens fiber cells were more easily dissolved in protein extraction buffer than in the RNA extraction buffer; thus, the stirring time used to collect the similar layers of fiber cells was shorter. After 10 minutes, the dissolved fiber cell solution was collected and homogenized for 20 strokes with the glass homogenizer (Kimax; Fisher Scientific). The remaining part of the lens was transferred to a new beaker containing 1 ml extraction buffer, and the next layers of fiber cells were dissolved by another 10-minute stirring. This process was repeated another two times. The first collection of the dissolved fiber cells was again designated F1 (the cortical layers of secondary fibers) and the fourth collection, F4 (the inner layers of secondary fibers). After four collections, the final part of the lens (the lens nucleus mainly consisting of primary fibers) was dissolved by homogenization and labeled N. The homogenates of both epithelial and fiber cells were centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatant of each sample was collected in aliquots and frozen with liquid nitrogen and then stored at −80°C. The same procedure was used for protein extraction from bovine lenses, except that 2 ml of extraction buffer was used and the stirring time was 20 minutes for each fraction.

Assays of PP-1 and -2A Activities

The protein phosphatase activities were assayed using a kit (10638-017; Gibco) as previously described.[41,42] Specific inhibitor 1 for PP-2A (50% inhibitory concentration [IC_{50}] = 30 nM,[45]) or specific inhibitor 2 for PP-1 (IC_{50} = 2 nM[46]) was used to distinguish PP-1 from PP-2A. The procedure used ^{32}P-labeled glycogen phosphorylase b as a substrate. Briefly, 5 μl extract of either epithelial cell sample or various fractions of fiber cell samples was mixed with 5 μl assay buffer and also 5 μl ^{32}P-labeled glycogen phosphorylase b, incubated for 10 minutes at 30°C. To distinguish PP-1 activity from PP-2A, the following reactions were also conducted. PP2A-I1 (90 nM) or PP1-I2 (6 nM) or a mixture of both was incubated with 5 μl extract from either epithelial cell sample or various fractions of fiber cell samples for an period of 30 minutes on ice. At the end of the incubation, 10 μl assay buffer and 5 μl ^{32}P-labeled glycogen phosphorylase b were added to each mixture and incubated for 10 minutes at 30°C. After the dephosphorylation reaction, 125 μl 20% trichloroacetic acid (TCA) was added into the mixture and incubated for 10 minutes on ice. The mixture was then centrifuged at 14,000 rpm for 5 minutes at 4°C, and 80 μl or 90 μl (in the presence of inhibitors) of the supernatant was withdrawn, to count the free ^{32}P. At the same time, the protein concentration in each sample was determined according to Peterson.[47] The value shown in Figure 3 in the Results section represents counts per minute per microgram protein. All the assays were repeated at least four times with four and six pairs of bovine and rat lenses, respectively.

Western Blot Analysis

Fifty micrograms of total proteins from epithelial and fiber cells (fractions F1 to N) of rat and bovine lenses were separated by 10% SDS-PAGE and transferred into supported nitrocellulose membranes (Gibco). The protein blots were blocked with 5% milk in Tris-buffered saline (TBS; 10 mM Tris [pH 8.0], 150 mM NaCl) overnight at 4°C. Each blot was then incubated with an anti-PP-1cs or PP-2Acs antibody (primary antibody, 1:1000 dilution in 5% milk prepared in TBS; Transduction Laboratories, Lexington, KY) for 60 minutes at 4°C with mild shaking. After three washes with TBS-T (TBS with 0.05% Tween-20), 10 minutes each, each blot was incubated with a secondary antibody (anti-mouse IgG; Amersham, Arlington Heights, IL) at a dilution of 1:1000 for 45 minutes. After two washes with TBS-T followed by another two washes with TBS (5 minutes each), the PP-1cs and -2Acs proteins were detected with an enhanced chemiluminescence detection kit, according to the manufacturer's instructions.

TABLE 1. Total RNA in Each Fraction of the Ocular Lens

Species	Epithelium*	F1	F2	F3	F4	N
Rat†	2.6 ± 0.2	14.4 ± 2.1	4.1 ± 0.4	1.8 ± 0.2	0.9 ± 0.1	1.2 ± 0.1
Bovine	6.8 ± 0.4	87.5 ± 12.5	25.8 ± 2.4	5.1 ± 0.48	2.6 ± 0.2	3.6 ± 0.3

Data are expressed as micrograms per lens ± SD.

* $OD_{260/280}$ was 2.2 ± 0.1 in the epithelium and F1 fiber cells, 1.8 ± 0.1 in the F2 and F3 fiber cells, and 1.5 ± 0.1 in the F4 and nuclear (N) fiber cells.

† The fiber mass from 10 rat lenses was combined, to extract total RNAs from different layers of the fiber cells, and the results were divided by 10 to give the amount of total RNAs per lens.

RESULTS

Expression of the mRNAs for PP-1cs and -2Acs

Although the lens epithelial cells are the center of metabolism and the fiber cells have either degenerating or no nuclei,[48] our previous studies have demonstrated that the mRNAs for certain genes are present in both epithelial cells and fiber cells.[49] To determine the mRNA levels for PP-1cs and -2Acs in rat and bovine lenses, we dissected 4-week-old rat lenses and 18- to 24-month-old bovine lenses into epithelial and five fractions of fiber cells. The total RNAs isolated from each fraction are presented in Table 1. Compared with the fiber mass, the epithelial cells from each lens contained only a small amount of total RNAs (Table 1). Among the different fractions, the cortical (F1) fiber cells (in the bow region, most of them have degenerating nuclei) contained the major portion (60%–70%) of total RNAs in rat and bovine lenses. The amount of total RNAs substantially decreased in F2 (the subcortical layers of secondary fiber cells) and F3 (in the middle region of the fiber mass) fractions, and became only detectable in F4 (the inner layers of secondary fiber cells toward the lens nucleus) and N (the nuclear primary fiber cells).

The quality of the total RNAs from different fractions was further reflected by the ratio of their optical absorbance at 260 nm and 280 nm wavelength ($OD_{260/280}$; Table 1). The total RNAs from epithelial (E) cells and cortical (F1) fiber cells had an $OD_{260/280}$ of 2.2 ± 0.1, those from F2 and F3 fiber cells had an $OD_{260/280}$ of 1.8 ± 0.1, and those from F4 and N fiber cells had an $OD_{260/280}$ of 1.5 ± 0.1. The decreasing $OD_{260/280}$ in the total RNAs from F1 to N fiber cells reflect an increase of impurity in these RNA samples.

RT-PCR analysis was conducted with an equal amount (3 μg) of total RNAs from the epithelial cells and different fractions of fiber cells in rat lenses. After RT-PCR reaction, an equal amount of the amplified product was analyzed by agarose gel electrophoresis. As shown in Figure 1, an amplified DNA band of 440 bp from the PP-1 specific primers was observed at similar levels in both epithelial and cortical (F1) fiber cell samples. As an internal control, RT-PCR was conducted with β-actin primers in the same reaction for PP-1 amplification. The predicted DNA band of 540 bp for β-actin was also equally amplified in both the epithelial and the cortical (F1) fiber cell samples (Fig. 1). In the remaining fiber cells, the PP-1cs mRNA was slightly decreased in subcortical (F2) fiber cells, and substantially decreased in the next layers (F3) of fiber cells (Fig. 1). The PP-1cs mRNA was barely detectable in the inner layers (F4) of fiber cells and nuclear (N) fiber cells. Associated with the sequential decrease of the PP-1cs mRNA in the different fractions of fiber cells, the mock control mRNA for β-actin was also sequentially decreased. These results are not difficult to understand, considering that the amount of total RNAs from subcortical (F2) fiber cells to nuclear (N) fiber cells is substantially reduced.

Compared with the expression pattern of PP-1cs mRNA, the mRNA level for PP-2Acs in the epithelial cells appeared slightly higher than that in the cortical (F1) fiber cell samples (as reflected by the density of the 505-bp band). However, the level of the control RNA for β-actin was also higher in the epithelial cell sample than that in the F1 fiber cells. Thus, the observed results may reflect a difference in the amplification efficiency, because the quality of the total RNAs from these two fractions are similar, as reflected by the $OD_{260/280}$. On the other hand, the possibility could not be ruled out that the F1 fiber cells indeed contained less mRNAs for PP-2Acs and β-actin, observed in other layers of fiber cells (fractions F4 to N). The PP-2Acs mRNA concentration was gradually decreased from the F2 to F3 fractions of fiber cells and became barely detectable in the F4 and N fractions. A similar change was observed with β-actin mRNA for the reason discussed above.

Similar expression patterns of the mRNAs for PP-1cs and PP-2Asc were observed in bovine lenses by Northern blot analysis (data not shown).

Expression of PP-1cs and -2Acs Proteins

After determination of the mRNA expression patterns for PP-1cs and -2Acs in rat and bovine lenses, we next examined their protein expression patterns. Again, both rat and bovine lenses were separated into lens epithelial cells and five different fractions of fiber cells as described in the Methods section. Western blot analysis using antibodies against the PP-1cs and -2Acs proteins demonstrated distinct protein expression patterns (Fig. 2). The PP-1cs protein was predominantly expressed in the epithelial cells of both rat and bovine lenses (Figs. 2A, 2C). Among the five fractions of fiber cells, only the cortical cells had a detectable level of PP-1cs protein in rat lenses (Fig. 2A; lane F1). In bovine lenses, the PP-1cs protein in F1 fiber

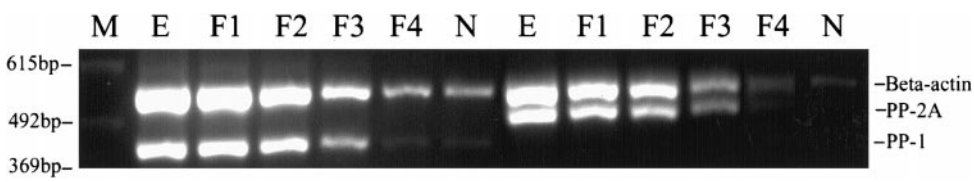

FIGURE 1. RT-PCR to detect the mRNA levels for the catalytic subunits of PP-1 and -2A in rat lenses. The PP-1 or -2A primers and the primers for β-actin were added to the reactions at the same time. The 440-bp DNA band for the PP-1cs mRNA (*bottom band*; *lanes 2–7*) and the 505-bp DNA band for PP-2Acs mRNA (*bottom band*; *lanes 8–13*) were mainly expressed in the epithelial cells (*lane E*) and the F1 to F3 fractions of the fiber cells (*lanes F1–F3*), but were barely detectable in the inner layers of secondary (*lane F4*) and nuclear (*lane N*) fiber cells of rat lenses. As an internal control, a β-actin DNA band of 540-bp (*top band*) was also amplified. Distribution of β-actin mRNA was also decreased from the cortical fiber (F1) to nuclear (N) fiber cells, because of the substantial decrease in the amount of total RNAs in these different fractions (Table 1). *Lane M*: 123-bp marker.

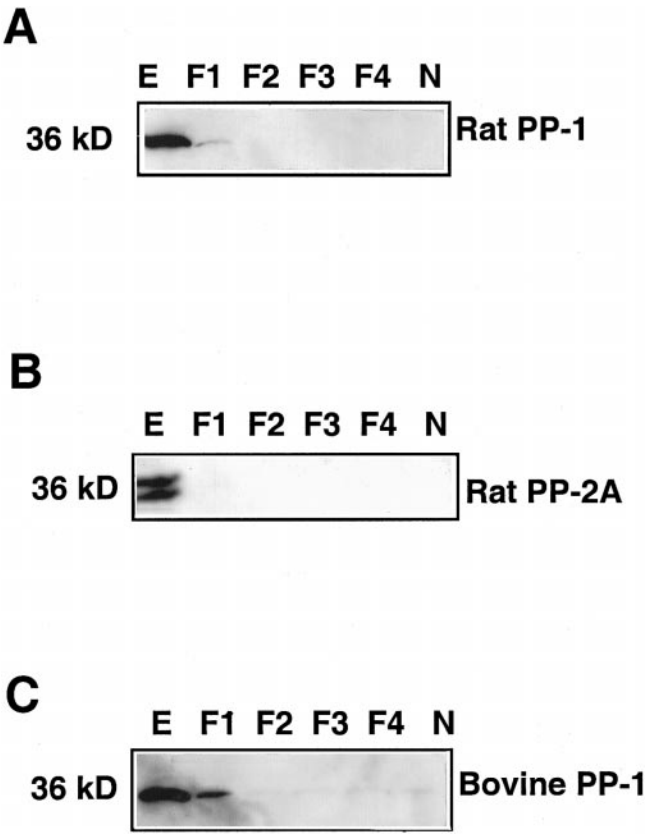

FIGURE 2. Western blot analysis of the protein for PP-1cs and -2Acs in rat (**A**, **B**) and bovine (**C**) lenses. Fifty micrograms of total proteins extracted from either epithelial cells (*lane E*) or different layers of fiber cells (*lanes F1–N*) were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, probed with (**A**) anti-PP-1cs– or (**B**) anti-PP-2Acs–specific antibody (1:1000 dilution) for 60 minutes. After three washes with TBS-T, the blots were incubated with anti-mouse IgG (1:1000 dilution) linked to peroxidase for 45 minutes. At the end of incubation, the blots were washed twice with TBST followed by another two washes with TBS and finally visualized with ECL. PP-2Acs always appeared in two bands, because of different phosphorylation status. Molecular weight was determined according to a commercial protein standard (Bio-Rad, Richmond, CA).

cells was relatively higher than that in rat lenses (lane F1; Fig. 2C). Moreover, in the remaining fiber cells (Fig. 2C; lanes F2 to N), PP-1cs protein was not detectable in rat lenses and was only slightly detectable in bovine lenses. The PP-2Acs protein was detectable only in the lens epithelial cells of both rat and bovine lenses (Fig. 2B and data not shown). We repeated the same Western blot analysis four times with the protein samples extracted from four different groups of rat and bovine lenses, and the obtained results were essentially unchanged. The presence of two bands for PP-2Acs was derived from phosphorylation, as previously described.[50] Therefore, the PP-1cs and -2Acs proteins were mainly localized in the lens epithelium.

Relative PP-1 and -2A Activities

Because PP-1cs and -2Acs were differentially expressed in the epithelial and fiber cells at the mRNA and protein levels, we next determined the distribution of enzyme activity in rat and bovine lenses. Again, both rat and bovine lenses were separated into lens epithelial cells and five different fractions of fiber cells as has been described. The enzymatic assay detected the total activity of both PP-1 and -2A in the absence of specific inhibitors, because the ^{32}P-labeled glycogen phosphorylase b could be used as substrates for both phosphatases. The majority (80%) of PP-1 and -2A activity was found in the epithelial cells of rat lenses (Fig. 3). Among the five different layers of fibers, the activity for PP-1 and -2A gradually decreased from the F1 to F4 fractions (Fig. 3). No activity was detectable in the nuclear fibers (Fig. 3). The PP-1 and -2A activities from F1 to F4 added together were equal to approximately 20% of the total activity assayed in the whole lens. To distinguish the relative activity levels of PP-1 and -2A in the epithelial and fiber cells of rat lenses, enzymatic assays in the presence of either specific PP-1 or specific PP-2A inhibitor or both were also performed. As shown in Figure 3, 90 nM of the specific PP-2A inhibitor blocked 18% of the total phosphatase activity. In contrast, 6 nM of the PP-1 inhibitor blocked 82% of the total phosphatase activity. A combination of both inhibitors almost completely inhibited the phosphatase activity. Thus, the rat lens epithelial cells contained approximately 82% of PP-1 activity and 18% of PP-2A activity. Using the same assay and calculation, we found that the cortical fiber cells contained approximately 95% PP-1 activity and 5% PP-2A activity.

In bovine lenses, the same enzymatic assay demonstrated a slightly different PP-1 and -2A activity pattern from that in rat lenses. Although the epithelial cells still contained the majority of PP-1 and -2A activity (66%), the phosphatase activity in the F1 fiber cells was about half the activity observed in the epithelial cells (32%). Thus, the detectable phosphatase activity ratio between F1 fiber cells and the epithelial cells in bovine lenses was much higher than that in rat lenses. As in rat lenses, the next layers of secondary fibers (F2, F3, and F4) had extremely low levels of phosphatase activity. In the nuclear fiber cells (N), no detectable phosphatase activity was observed (data not shown). Further examination of the phosphatase activity in the epithelial cells revealed that 80% was contributed by PP-1 activity and 20% by PP-2A activity, as demonstrated with specific PP-1 and -2A inhibitors. In the fiber cells, again, 95% phosphatase activity came from PP-1 activity and 5% from PP-2A activity (data not shown).

Comparison of PP-1 and -2A

Because active PP-1 and -2A were mainly distributed in the lens epithelium, we further compared their expression pattern in rat and bovine lenses. A similar amount of PP-1cs protein was

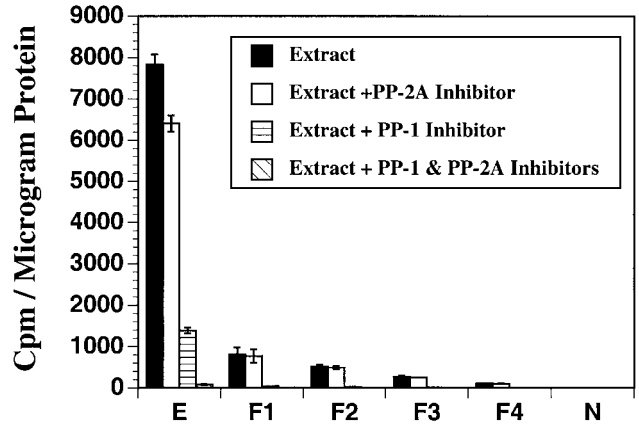

FIGURE 3. Distribution of PP-1 and -2A activity in rat lenses. Both epithelial and different layers of fiber cell samples were collected. PP-1 and -2A activity was assayed without or with various inhibitors (PP-2A inhibitor, PP2A-I1, IC_{50} = 30 nM; PP-1 inhibitor, PP1-I2, IC_{50} = 2 nM) as described in the Methods. All assays were repeated at least four times with four pairs of rat lenses. The bars represent total activity of PP-1 and -2A (in the absence of PP-1 or -2A inhibitors), or PP-1 activity (in the presence of PP-2A inhibitor), or PP-2A activity (in the presence of PP-1 inhibitor). E, capsule epithelium; F1 to F4, the first to the fourth layers of fiber cells (secondary fibers); N, the nucleus of the lens containing mainly primary fiber cells.

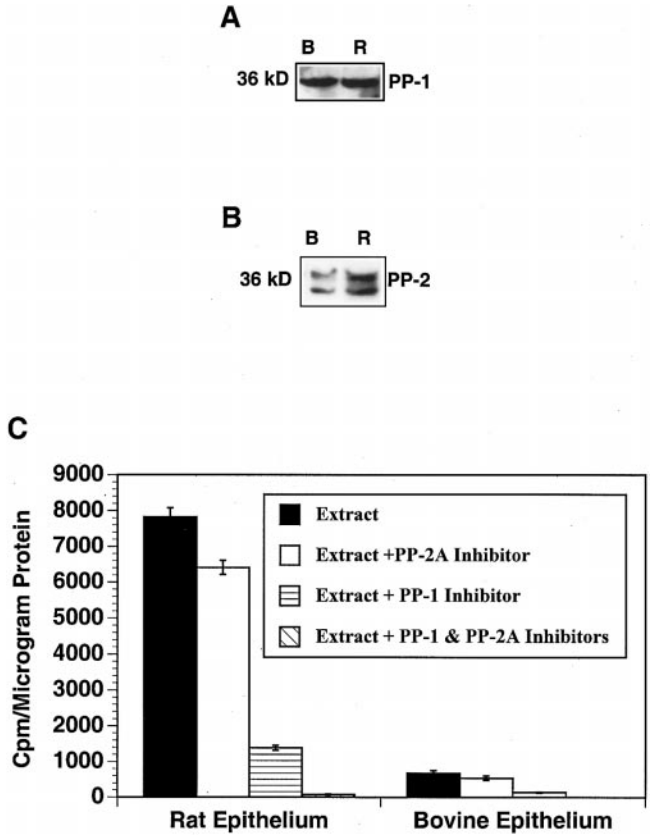

FIGURE 4. Comparison of expression of PP-1 and -2A in the epithelial cells of rat and bovine lenses. (**A**) Western blot analysis of the PP-1cs protein in 50 μg total proteins from bovine (*left lane*) or rat (*right lane*) lens epithelial cells. A similar level of PP-1cs was found in the epithelial cells of bovine and rat lenses. (**B**) Western blot analysis of the PP-2Acs protein in 50 μg total proteins from bovine (*lane B*) or rat (*lane R*) lens epithelial cells. PP-2Acs in rat lens was approximately two times higher than that in bovine lenses. (**C**) PP-1 and -2A activity in rat and bovine lens epithelium. The protein phosphatase activity was determined as described in Figure 3. The bars represent total activity of PP-1 and -2A (in the absence of PP-1 or -2A inhibitors), or PP-1 activity (in the presence of PP-2A inhibitor, PP2A-I1), or PP-2A activity (in the presence of PP-1 inhibitor, PP1-I2).

present in the same amount of total proteins extracted from either rat or bovine lens epithelial cells (Fig. 4A). However, rat lens epithelium contained more than two times PP-2Acs protein than bovine lens epithelium when the same amount of total proteins were analyzed by Western blot analysis followed by densitometric scanning (Fig. 4B). When PP-1 and -2A activities in equal amount of total proteins were compared between rat and bovine lenses, to our surprise, we found that PP-1cs and -2Acs in rat lens epithelial cells displayed approximately 10 times higher activity than that in bovine lens epithelial cells (Fig. 4C).

DISCUSSION

In the present study, we investigated the expression patterns of the two genes encoding PP-1cs and -2Acs and obtained the following results: First, the levels of the mRNAs for PP-1cs and -2Acs were similarly distributed in the epithelial and the cortical (F1) fiber cells of rat and bovine lenses. In the remaining fiber cells, the mRNAs for PP-1cs and -2Acs were slightly decreased in F2 fiber cells and substantially decreased in F3 fiber cells and became barely detectable in F4 and N fiber cells (Fig. 1, and data not shown). In contrast, PP-2Acs protein was expressed only in the epithelial cells of rat and bovine lenses (Fig. 2B, and data not shown). The PP-1cs protein was mainly expressed in the epithelial cells (Figs. 2A, 2C). The F1 fiber cells also contained a detectable level of PP-1cs protein in rat lenses and a relatively higher level of PP-1cs protein in bovine lenses. From the F2 to N fiber cell fractions, PP-1cs protein was hardly detectable in rat lenses and slightly detectable in bovine lenses (Fig. 2A, 2C). Finally, active PP-1 and -2A were mainly found in the lens epithelial cells (Fig. 3, and data not shown). Moreover, in the epithelial cells of rat and bovine lenses, the PP-1 activity was approximately four to five times higher than the PP-2A activity. In the fiber cells, PP-1 activity dominated, and PP-2A activity was just barely detectable.

It is well established that the protein phosphorylation and dephosphorylation at the serine-threonine residues are important biochemical events regulating key cellular processes.[1–6] The protein serine-threonine kinases mediating phosphorylation have been well studied in the lens system.[20–38] Several families of serine-threonine kinases have been examined. These include the cyclin-dependent kinase family,[20–24] the protein kinase C family,[25–32] the casein kinase family,[33,34] the TGF-β receptor family,[35–36] the kinase mediating myotonic dystrophy,[37,38] and others. These different families of serine-threonine kinases play important role during lens development, differentiation, and pathogenesis.

Every phosphorylation reaction catalyzed by protein serine-threonine kinases is reversed by protein serine-threonine phosphatases. Thus, it is conceivable that protein phosphatases also have important functions in lens cells. The few previous studies show that this is indeed the case. For example, α-crystallins are important lens proteins that act as molecular chaperones,[51–54] anti-apoptotic regulators,[42,55–57] and autokinases.[58] Previous studies from a number of laboratories have shown that α-crystallins are phosphorylated by different serine-threonine kinases[59–61] and dephosphorylated by PP-2B[39] and other unknown phosphatases. Change of the phosphorylation status is closely linked to its functional ability. For example, Hoover et al.[62] demonstrated that phosphorylation of the serine residue at position 59 in αB crystallin is critical for its cellular protection. In another study, Kantorow et al.[40] observed that the mRNA for the regulatory subunit of PP-2A is downregulated in the epithelial cells of the human cataractous lenses, suggesting possible roles of PP-2A activity in maintaining normal lens transparency. Our recent studies[41,42] demonstrated that PP-1 activity is very important in maintaining the viability of rabbit and rat lens epithelial cells. Inhibition of the PP-1 activity by okadaic acid leads to upregulated expression of the proapoptotic genes, which leads to eventual apoptosis of the treated lens epithelial cells.[41,42]

To further understand the protein serine-threonine phosphatases in the lens system, we examined the expression patterns of the two genes encoding the catalytic subunits of PP-1 and -2A, the two major intracellular phosphatases. The mRNA levels for PP-1cs and -2Acs were present at similar levels in the epithelial and the cortical (F1) fiber cells of rat and bovine lenses (Fig. 1, and data not shown). In the remaining fiber cells, the two mRNAs were slightly decreased in the F2 fiber cells, substantially reduced in the F3 fiber cells, and became barely detectable in F4 and N fiber cells. A similar change was also observed for the β-actin mRNA in these different layers of fiber cells. Analysis of the total RNAs in different fractions of fiber cells reveals an explanation. As shown in table 1, the cortical (F1) fiber cells contained 60% to 70% of the total RNAs found in the whole fiber mass, with $OD_{260/280}$ of 2.2 $\pm$ 0.1, similar to those of the epithelial cell RNAs. The amount of total RNAs decreased to 20% in the subcortical (F2) fiber cells, and to 4% to 8% in the middle layers (F3) of fiber cells, with $OD_{260/280}$ of 1.8 $\pm$ 0.1. On the other hand, the total RNAs in F4 and N fiber cells were reduced to 2%

to 4%, with $OD_{260/280}$ of 1.5 ± 0.1. Such a low $OD_{260/280}$ clearly suggests impurity in these RNA samples. Thus, the decrease in the mRNA levels for PP-1cs, PP-2Acs, and β-actin paralleled the reduction in the amount of total RNAs and their $OD_{260/280}$ from subcortical (F1) to nuclear (N) fiber cells.

In contrast to its RNA expression pattern, the PP-2Acs protein was only detectable in the epithelial cells in our repeated Western blot analysis. The presence of two bands for PP-2Acs was derived from phosphorylation, as previously described.[50] Again, different from its RNA expression pattern, the PP-1cs protein was predominantly expressed in the epithelial cells. In the fiber cells, only the cortical (F1) fiber cells contained a detectable level of PP-1cs protein in rat lenses and a relatively higher level of PP-1cs protein in bovine lenses. The PP-1cs protein was hardly detectable in other fiber cells of rat lenses and was only slightly detectable in the same fiber cells of bovine lenses (Fig. 2). Thus, the genes encoding PP-1cs and -2Acs were differentially expressed at the mRNA and protein levels in the epithelial and fiber cells of rat and bovine lenses.

Analysis of enzyme activity of PP-1 and -2A in the epithelial cells and different layers of fiber cells confirmed that active PP-1 and -2A were mainly distributed in the lens epithelium. Although PP-2Acs protein was undetectable in the lens fiber cells with Western blot analysis, enzymatic assay suggests there was still some PP-2A activity there. This discrepancy probably reflects the sensitivity of the experimental conditions. Apparently, an enzymatic assay is more sensitive than Western blot analysis. Nevertheless, compared with PP-1 activity, PP-2A was almost negligible in the fiber cells of rat and bovine lenses.

The much higher level of PP-1 activity than that of PP-2A found in the lens epithelial cells suggests that PP-1 plays an essential role in this tissue. This also explains why inhibition of PP-1 but not PP-2A by okadaic acid in the lens epithelial cells leads to apoptosis.[41,42] The presence of PP-1 but not PP-2A in the cortical fibers suggests that PP-1 may play a role during fiber cell differentiation.

Although the cortical (F1) fiber cells of rat and bovine lenses contain substantial mRNAs for PP-1cs and -2Acs, translation of these mRNAs is restricted, as indicated by the much lower level of PP-1cs protein (in comparison with that in the lens epithelial cells) and undetectable PP-2Acs protein (at least under our repeated Western blot analysis). Similar patterns are observed for the genes encoding the members of the metallothionein family.[63] The absence of PP-1cs and -2Acs proteins in the inner layers of fiber cells (fractions F2 to N) is consistent with the disappearance of cellular organelles and also protein synthesis machinery in these cells.[64,65] However, in the cortical differentiating fibers, the translation machinery is still functional.[66] The likely differential expression of PP-1cs and -2Acs proteins in these fiber cells suggests the possibility of translational control. Of course, it is also possible that PP-1cs and -2Acs proteins may have different half-lives.

Comparative analysis reveals that PP-1cs and -2Acs proteins in rat lenses have approximately 10 times higher activity than those in bovine lenses (Fig. 4). What accounts for this distinct difference? First, the rat lenses we used were dissected from 4-week-old animals, and bovine lenses used were obtained from 18- to 24-month-old animals. The age difference of these animals may have contributed partially to the different activity levels. Second, it is possible that bovine lenses contain more endogenous PP-1 and -2A inhibitors. The physiological significance of this difference in PP-1 and -2A activities between rat and bovine lenses remains to be investigated.

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