Expression and Activity of the Signaling Molecules for Mitogen-Activated Protein Kinase Pathways in Human, Bovine, and Rat Lenses

David Wan-Cheng Li,[1,2] *Jin-Ping Liu,*[1] *Juan Wang,*[2,3] *Ying-Wei Mao,*[2,4] *and Li-Hui Hou*[2,5]

Purpose. The mitogen-activated protein kinase (MAPK) pathways play distinct roles in the lens. However, the expression patterns and activity levels of various components for these pathways have not been well-documented in vertebrate lenses, especially human lens. In the present study, the expressions and activities of extracellular signal-regulated kinase (ERK)-1/2/3, c-Jun NH2-terminal kinase (JNK)-1/2, p38 kinase, mitogen-activated protein kinase kinase (MEK)-1/2, and RAF1 were recorded in human, bovine, and rat lenses.

Methods. Human, bovine, and rat lenses were isolated from intact eyes. The epithelia and different layers of fiber cells were isolated from these lenses. Total proteins extracted from these samples were subject to analysis of the expression patterns and activity levels of the MAPKs and the activating kinases of ERK1/2.

Results. ERK1 and ERK2 were the most abundant MAPKs in terms of both protein and activity levels in all lenses. JNK1 and JNK2 were highly expressed in bovine lens, which differed from the pattern shared by human and rat lenses. p38 kinase was similarly expressed in bovine and rat lenses, but different from that in human lens. However, p38 kinase activity was exclusively detected in the epithelia. All lenses had MEK1/2 activity in their epithelia but the expression patterns of MEK1 and MEK2 differed in these lenses. RAF1 was expressed in the epithelia of all lenses, but its activity was detected only in rat lens.

Conclusions. ERK1 and ERK2 are the most abundant MAPKs in the ocular lens, providing the basis for their multiple functions in lens development and pathogenesis. The dominant epithelial distribution of JNK1/2 and p38 kinase suggests that the lens epithelium is a major site for stress response. ERK1, p38 kinase, and PKCα can be used as molecular markers for aging. (*Invest Ophthalmol Vis Sci.* 2003;44:5277–5286) DOI: 10.1167/iovs.03-0348

From [1]The Hormel Institute, University of Minnesota, Austin, Minnesota; and the [2]Department of Molecular Biology, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey.

Present affiliations: [3]Division of Endocrinology, Diabetes, and Metabolism, Departments of Medicine and Genetics and The Penn Diabetes Center, University of Pennsylvania, Philadelphia, Pennsylvania; the [4]Department of Pharmacology, University of Michigan, Ann Arbor, Michigan; and the [5]Department of Molecular and Cellular Biology and Biochemistry, Brown University, Providence, Rhode Island.

Supported by the Hormel Foundation, University of Minnesota Graduate School, and Grant EY11372 from the National Eye Institute (DW-CL).

Submitted for publication April 5, 2003; revised July 3 and August 25, 2003; accepted August 28, 2003.

Disclosure: **D.W.-C. Li**, None; **J.-P. Liu**, None; **J. Wang**, None; **Y.-W. Mao**; None, **L.-H. Hou**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: David Wan-Cheng Li, The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912; dwcli@hi.umn.edu.

The mitogen-activated protein kinase (MAPK) pathways play important roles in regulating embryogenesis, cell proliferation and differentiation, and programmed cell death (apoptosis).[1] The MAPK pathways are organized in a conserved three-kinase architecture, including an MAPK, an activator of the MAPK (MAPK kinase, MKK or MEK), and an activator of the MAPK activator (MAPK kinase kinase, MKKK or MEKK).[2] So far, three distinct types of mammalian MAPKs have been characterized: the extracellular signal-regulated kinases (ERKs),[3] c-Jun NH_2-terminal/stress-activated protein kinases (JNK/SAPKs),[4–6] and p38.[7] MAPKs are activated by dual phosphorylation of threonine and tyrosine residues. The ERK MAPKs are phosphorylated on the Thr-Glu-Tyr amino acid motif by MEK1 and MEK2. The JNK/SAPK phosphorylation on the Thr-Pro-Tyr motif is mediated by MEK4 and MEK7. MEK3 and MEK6 phosphorylate p38 MAPKs on the Thr-Gly-Tyr motif. The activated MAPKs are then translocated from the cytoplasm to the nucleus, where they activate nuclear transcription factors leading to changes of expressions of many different genes[1,2] and thus regulating cell proliferation and differentiation,[1–3] inflammation and stress responses,[4–7] and programmed cell death.[8–10]

In the lens system, MAPKs mediate signaling pathways for lens diseases as well as development. Several studies have suggested that MAPK pathways may be linked to cataractogenesis. First, Gong et al.[11] have shown that overexpression of MEK1 in transgenic mouse induces postnatal macrophthalmia with cataract. Zatechka and Lou[12,13] have demonstrated that galactose at concentrations causing sugar cataract, activates all three types of MAPK. We have previously shown that calcimycin-induced apoptosis contributes to cataractogenesis.[14] More recently, we observed that calcimycin-induced apoptosis requires activation of ERK1/2 (Li et al., manuscript submitted).

The ERKs also actively participate in the signaling pathways for cell proliferation and differentiation in the lens system. Several laboratories have reported that fibroblast growth factor (FGF)-induced cell proliferation and differentiation in mouse, rat, or chicken lenses require activation of ERKs.[15–18] In addition, epidermal growth factor (EGF) activates ERK1/2 in chicken and porcine lens epithelial cells.[12,13,19,20] Mechanistically, ERKs have been shown to modulate activities of several transcription factors, including Maf A and L-maf in the lens system.[21,22]

Because MAPK pathways play crucial roles in lens development and pathogenesis, we determined the protein expression patterns and enzyme activity levels of the three types of MAPKs in different fractions of the adult human, bovine, and rat lenses. Our results revealed distinct features of their expression patterns and activity levels, which provide valuable references for future studies.

Methods

Sources of Human, Bovine, and Rat Lenses

Four pairs of intact human eyeballs were provided by the National Disease Research Interchange (NDRI, Philadelphia, PA) and the Oregon Lion Eye Bank (OLEB). The ages of the donors were between 60

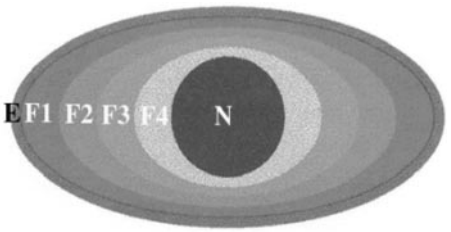


FIGURE 1. The lens capsular epithelium and the different fractions of fiber cells, which were isolated stepwise, are illustrated with different shading. Note that the actual thickness of each fraction of fiber cells varies from fraction to fraction and from species to species. E, capsular epithelium; F1, the cortical layer; F2, the subcortical layer; F3, the intermediate layer; F4, the inner layer; and N, the nuclear layer of fiber cells.

and 88 years. Six pairs of bovine eyes were obtained from the slaughterhouses in Peterson and Berlin (NJ). The ages of the cattle were 18 to 24 months. Rats used in this study were handled in compliance with the Guide for the Care and Use of Laboratory Animals and also with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ten 4-week-old and six 2-year-old Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN).

Isolation of Lens Epithelia and Different Fractions of Fiber Cells

After dissection of human, bovine, and rat lenses from the intact eyeballs, the lenses were measured in size and weight. The capsular epithelium from each lens was removed immediately after measurement (Fig. 1) and transferred into a prechilled Eppendorf tube containing protein extraction buffer (300 μL for human and rat lenses, 500 μL for bovine lens, 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], and 0.5% Triton X-100). The de-epithelialized lens was measured. After measurement, the fiber mass was immediately transferred into a 20-mL beaker (for human and bovine lenses) or a 10-mL beaker (for rat lens) containing protein extraction buffer (5 mL for human lens, 10 mL for bovine lens, and 1 mL for rat lens) with constant stirring with a magnetic stirring bar at 4°C. After 15 (for human lens), 20 (for bovine lens), or 10 (for rat lens) minutes, the dissolved fiber cells were collected and recorded as F1 (Fig. 1). The remaining part of the lens was measured as described earlier and transferred to a new beaker containing extraction buffer for another round of stirring (the same condition for each lens). The dissolved fiber cells were then collected and recorded as F2 (Fig. 1). This process was repeated twice to collect fiber fractions F3 and F4 (Fig. 1). After four collections, the remainder of the lens was difficult to dissolve in the same condition and thus was homogenized as described later and recorded as the nuclear fraction (N; Fig. 1). The weight and size of the intact human, bovine, and rat lenses, de-epithelialized lens, and remaining fiber mass after each collection are recorded in Table 1.

Extraction of Total Proteins from the Epithelial and Fiber Cells

The epithelia were homogenized on ice with an Eppendorf tube micropestle (Brinkmann Instruments, Inc., Westbury, NY). Various fractions of fiber cells were homogenized for 20 strokes with a glass homogenizer (Kimax, Alphuretta, GA). An additional 10 strokes were necessary for the lens nucleus from human and bovine lenses. The homogenates of both epithelial and fiber cells were then centrifuged at 10,000*g* 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 for further analysis.

Analysis of Total Proteins for Various Kinases, GAPDH, and α-Crystallin

For each sample, the protein concentration was determined according to Peterson.[23] Western blot analysis of total proteins was conducted as described before.[24,25] Briefly, 50 μg (for α-crystallin) or 100 μg (for kinases and GAPDH) total proteins in each sample were resolved on a 10% polyacrylamide gel. The protein blots were blocked with 5% milk in TBS (10 mM Tris-HCl [pH 8.0] and 150 mM NaCl) overnight at 4°C and incubated with anti-ERKs, JNK1/2, p38 kinase, and MEK1/2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-PKCα and anti-RAF1 antibodies (Transduction Laboratories, Louisville, KY), anti-αA and αB-crystallin antibodies (StressGen, Biotechnologies, Victoria, British Columbia, Canada), and anti-GAPDH antibody (Advanced Immunochemicals, Long Beach, CA) at a dilution of 1:250 to 1:2000 (in micrograms per milliliter). The secondary antibody is anti-mouse IgG (for analysis of JNK2, MEK1, RAF1, and GAPDH), or anti-rabbit IgG (for analysis of ERK1/2/3, JNK1, MEK2, and αA- and αB-crystallins), or anti-goat IgG (for analysis of p38) at a dilution of 1:500 to 1:1000. Immunoreactivity was detected with an enhanced chemiluminescence kit according to the manufacturer's instructions (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Detection of Activities for MAPKs, MEKs, and RAF1

Detection of the activities of various MAPKs, MEK1/2, and RAF1 also was conducted with Western blot analyses. After the immunoblots were hybridized with the antibodies against the total proteins for different kinases, they were stripped and then blocked with 5% bovine serum albumin (BSA) in TBST (10 mM Tris-HCl [pH8.0], 150 mM NaCl, and 0.05% Tween 20) for 1 hour at 4°C, and incubated with anti-p-ERKs, p-JNK1/2, p-MEK1/2, and p-RAF1 (Santa Cruz Biotechnology), or p-p38 (Cell Signaling Technology, Beverly, MA) at a dilution of 1:100 to 1:1000 (in micrograms per milliliter). The secondary antibody is anti-mouse IgG (for analysis of pERK1/2 and p-JNK1/2), or anti-rabbit IgG (for analysis of p-p38), or anti-goat IgG (for analysis of p-MEK1/2

TABLE 1. Weight and Size of the Intact lens, Various Fiber Masses after Removal of the Epithelium, and F1 to F4 Fractions of Fiber Cells

	Human Lens		Bovine Lens		Rat Lens	
	Weight (mg)	Diameter (mm)	Weight (mg)	Diameter (mm)	Weight (mg)	Diameter (mm)
Intact Lens	110.00 ± 5.00	9.00 ± 0.28	2060.00 ± 48.00	16.00 ± 1.80	20.00 ± 1.00	4.00 ± 0.15
With removal of:						
Epithelium	90.00 ± 3.60	7.50 ± 0.24	2000.00 ± 102.00	14.00 ± 1.40	19.00 ± 0.50	3.00 ± 0.12
F1 fiber layer	61.00 ± 3.00	5.30 ± 0.23	1282.00 ± 76.00	9.50 ± 1.20	12.40 ± 0.40	2.00 ± 0.10
F2 fiber layer	42.00 ± 2.40	3.90 ± 0.21	843.00 ± 54.00	6.80 ± 0.91	8.40 ± 0.30	1.40 ± 0.08
F3 fiber layer	31.00 ± 2.20	3.10 ± 0.18	620.00 ± 48.00	5.40 ± 0.62	6.30 ± 0.20	1.10 ± 0.06
F4 fiber layer	23.00 ± 2.00	2.60 ± 0.15	490.00 ± 38.00	4.60 ± 0.48	4.90 ± 0.20	0.95 ± 0.05

The data are expressed as mean ± SD, $n = 8$ for human lens, 12 for bovine and rat lenses.

and p-RAF1) at a dilution of 1:250 to 1:1000. Immunoreactivity was detected as described earlier.

Quantitation of the Total Protein and Activity Levels for Different Kinases

After exposure, the x-ray films were analyzed with an automated digitizing system (Silk Scientific Corp., Orem, UT). Total pixel data were averaged from three or more different groups of samples of each species after normalization against the background.

Results

Isolation of Total Proteins from the Epithelial Cells and Different Layers of Fiber Cells

To characterize the distribution of the protein and activity levels of various MAPKs and the upstream activating kinases for ERKs in human, bovine, and rat lenses, we developed a method to isolate various fractions of fiber cells from the cortical to the nuclear layer after removal of the lens capsular epithelium. Figure 1 depicts the capsular epithelium and different fractions of fiber cells that were isolated as described in the Methods section. After removal of the lens epithelium or each fraction of fiber cells, the remaining fiber mass was measured in both weight and size. The results are shown in Table 1. To further characterize different fractions, we examined the levels of αA- and αB-crystallin, and then compared them with the distribution of PKCα and GAPDH in the isolated lens epithelium and the different layers of fiber cells (Fig. 2). Although the weight of each fraction of fiber cells varied in human, bovine, and rat lenses (Table 1), the expression patterns of αA- and αB-crystallins in these lenses were common: both crystallins became gradually decreased from the cortical (F1) layer of fiber cells toward the N fiber cells (Fig. 2A, 2–3, 6–7, 10–11; in citations of Figures 2–8, the numbers after the figure number correspond to the numbers to the right of the Western blots in panel A). Quantitative analysis of the two crystallins in each fraction of the fiber cells with an automated digitizing system revealed that the F1 fiber cells of the lenses from three different sources all contained approximately 40% to 50% of the total fiber cell αA- or αB-crystallin. In contrast, less than 2% of the total fiber cell αA- or αB-crystallin was found in the N fiber cells of these lenses (Fig. 2B). The expression pattern of GAPDH was also similar in the lenses of three different sources. Higher level of GAPDH was found in their lens epithelia and relatively lower level of GAPDH was found in their F1 fiber cells (Fig. 2). GAPDH was hardly detectable in F3 to N fiber cells (Fig. 2). Compared with αA- and αB-crystallin, and GAPDH, PKCα was differentially expressed in the fiber cells of human, bovine, and rat lenses. In human lens, PKCα was absent in the lens fiber cells (Figs. 2A, 1; 2B, top). In the fiber cells of bovine lens, PKCα was detected only in the F1 fiber cells (Figs. 2A, 5; 2B, middle). In contrast, PKCα was expressed in all fractions of the fiber cells except for the nucleus of 4-week-old rat lens (Fig. 2A, 9). The PKCα level was gradually decreased from the epithelial cells to F3 fiber cells and became barely detectable in F4 fiber cells (Fig. 2B, bottom). Although it was difficult to find a unique marker for each fraction of the isolated fiber cells, our determination of the weight, size, and relative expression of αA- and αB-crystallins in these fractions provided necessary characterization for each fraction of fiber cells.

Expression and Activity of the ERKs in Human, Bovine, and Rat Lenses

After characterizing the different fractions of the lenses, we next compared the expression patterns and activity levels of the ERKs in human, bovine, and rat lenses. As shown in Figure 3A, the ERK1 (44 kDa) in human lens was predominantly expressed in the lens epithelium, and the expression dropped approximately 26-fold in the F1 fiber cells (Fig. 3B, top). It was absent in other layers of fiber cells (Fig. 3A, 1). In contrast, ERK2 (42 kDa) was expressed at very high levels in both the epithelium and the F1 fiber cells (Fig. 3A, 1). The expression of ERK2 significantly decreased in the F2 fiber cells, became barely detectable in the F3 and F4 fiber cells, and disappeared in the N fiber cells (Figs. 3A, 1; 3B, top). The activity levels of ERK1 and ERK2 in human lens were closely parallel to the protein expression levels in the E, F1, and F2 cells (Figs. 3A, 2; 3B, top). ERK1 activity was absent in the F2 fiber cells. ERK2 activity was barely detectable in the F3 fiber cells and became undetectable in the F4 and N fiber cells (Fig. 3A, 2).

Compared with the ERKs in human lens, bovine lens displayed similar expression patterns and activity levels of ERKs in the lens epithelium (Figs. 3A, 3; 3B, middle). In the fiber cells, however, the pattern was somewhat different. ERK1 in the F1 fiber cells was expressed in a level similar to that in the epithelium, and the level was substantially decreased in the F2 fiber cells and became hardly detectable in the F3, F4, and N fiber cells (Figs. 3A, 3; 3B, middle). The level of ERK2 was gradually decreased from F1 to F3, and then was substantially reduced in the F4 and N fiber cells (Fig. 3B, middle). The activities of ERK1 and ERK2 were very strong in both the epithelium and F1 fiber cells. Their activities were significantly decreased in F2 fiber cells and barely detectable in the F3, F4, and N fiber cells (Figs. 3A, 4; 3B, middle).

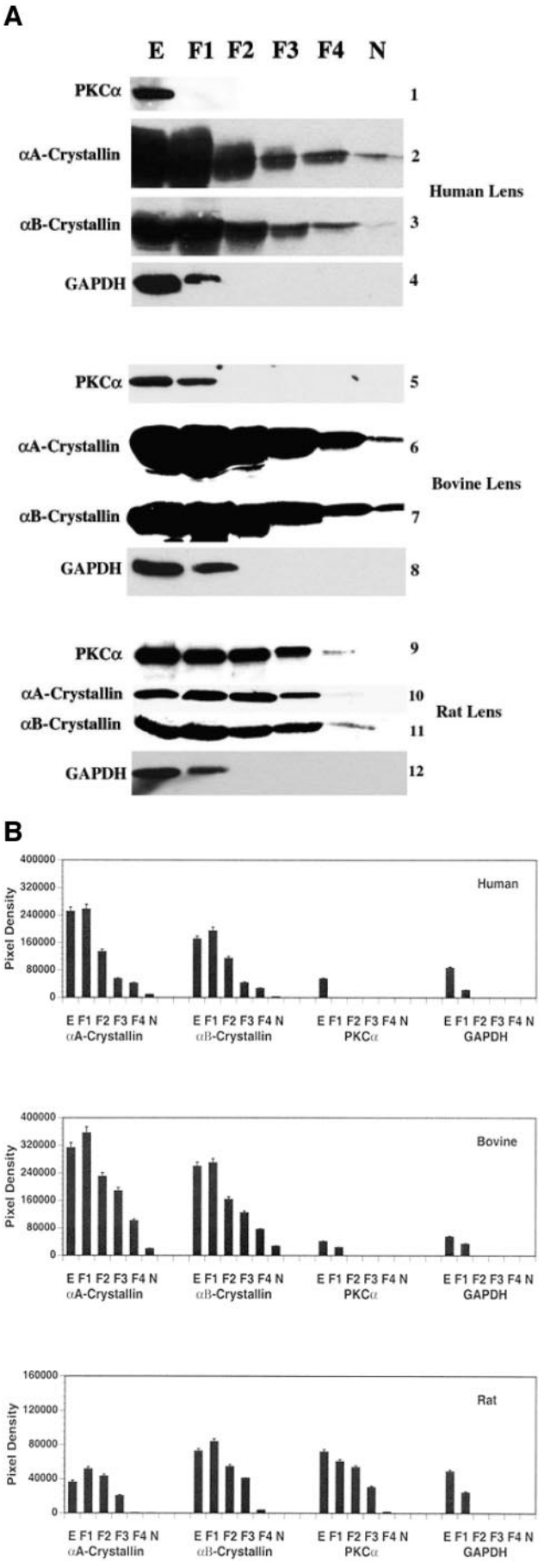
The ERKs in rat lens displayed some striking features. First, both ERK1 and ERK2 were present in the epithelium and all fractions of fiber cells (from F1 to N). Second, the ERK2 protein was present at a very high level in all the fractions of the lens fiber cells. Third, another ERK, ERK3 protein and its activity, was observed in the lens epithelium and F1 fiber cells of 4-week-old rats (Fig. 3A, 5–8; 3B, bottom; see also Fig. 8). Finally, the activities of ERK1 and ERK2 were higher in the F1 layer of fiber cells than in the epithelium (Fig. 3A, 6; 3B, bottom).

Further comparison of ERK1/2 expression and activity in the lenses from three different species revealed that the expression level of ERK2 was generally higher than that of ERK1 in each fraction of all lenses (Fig. 3B). When the epithelial samples from human, bovine, and rat lenses were compared in the same gel, it was found that the ERK1/2 expression and activity in human lens were approximately 10% higher than that in bovine and rat lenses (data not shown).

Expression and Activity of JNK1 and JNK2 Proteins in Human, Bovine, and Rat Lenses

Next, we examined the expression patterns and activity levels of both JNK1 and JNK2, the MAPK for stress response. As shown in Figure 4A, both JNK1 (46 kDa) and JNK2 (54 kDa) were expressed in the epithelium and the F1 fiber cells of human lens (Fig. 4A, 1). In the epithelium, the JNK1 level was approximately five times lower than that of JNK2. In contrast, in the F1 fiber cells, the level of JNK1 was two times higher than that of JNK2 (Fig. 4B, top). When the activities for JNK1 and JNK2 were analyzed, only JNK1 activity was detected, and its activity was found only in the epithelium of human lenses (Fig. 4A, 2).

The expression patterns of JNK1 and JNK2 in bovine lens differed from that in human lens in several aspects. First, both JNK1 and JNK2 were expressed at the highest levels in the epithelium (Figs. 4A, 3; 4B, middle). Second, the F1 fiber cells displayed substantial amounts of JNK1 and JNK2. Third, the F2, F3, and F4 fiber cells also contained detectable levels of JNK1 and JNK2 (Fig. 4A, 3; Fig. 4B, middle). Finally, JNK1 and JNK2 activities were found in both the epithelium and the F1 fiber



cells, and the JNK1 activity was also detectable in the F2 fiber cells (Figs. 4A, 4; 4B, middle).

As in human lens, expression of JNK1/2 in rat lenses was found only in the epithelium and F1 fiber cells (Figs. 4A, 5; 4B, bottom). Moreover, the level of JNK1 was approximately five times lower in the epithelium and three times lower in the F1 fiber cells than that of JNK2 (Fig. 4B, bottom). Both JNK1 and JNK2 activities were detectable in the lens epithelium and F1 fiber cells (Fig. 4A, 6).

In the epithelia of the lenses from three different species examined, the highest levels of JNK1/2 expression and activity were found in bovine lens (Fig. 4, and data not shown).

Expression and Activity of p38 Kinase in Human, Bovine, and Rat Lenses

p38 kinase was the third category of MAPK, responsible for inflammatory, heat, and osmotic shock responses. In human lens, p38 kinase is expressed in lens epithelium (Fig. 5A, 1), and sometimes is also detectable in the F1 fiber cells (Fig. 5B, top). However, p38 kinase activity was detected only in the lens epithelium (Fig. 5A, 2).

The expression pattern of p38 kinase in bovine lens was different from that in human lens. p38 kinase was expressed in the F1 and F2 fiber cells, and traces of p38 kinase may be sometimes found in F4 fiber cells (Fig. 5A, 3). Compared with human lens, bovine lens has a relatively high level of p38 kinase activity, which was detected only in the epithelium (Figs. 5A, 4; 5B, middle).

The p38 kinase in the 4-week-old rat lenses was expressed similarly to that in bovine lens. The epithelium and F1 and F2 fiber cells displayed a relatively high level of p38 kinase expression (Figs. 5A, 5; 5B, bottom). The p38 kinase was substantially decreased in the F3 fiber cells, became barely detectable in the F4 fiber cells, and disappeared in the N fiber cells (Figs. 5A, 5; 5B, bottom). As in human and bovine lenses, the p38 activity was detected only in the lens epithelium (Fig. 5A, 6; 5B, bottom).

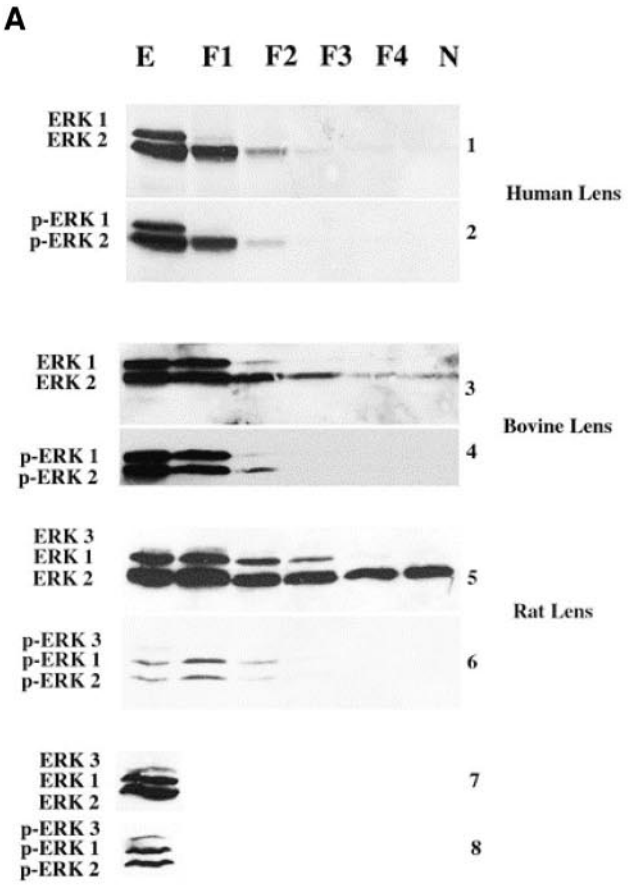
When the epithelia from all the lenses were compared for p38 kinase expression and activity, both bovine and rat lenses showed a similar level of p38 kinase expression that was relatively higher than that in human lens (Fig. 5B and data not shown).

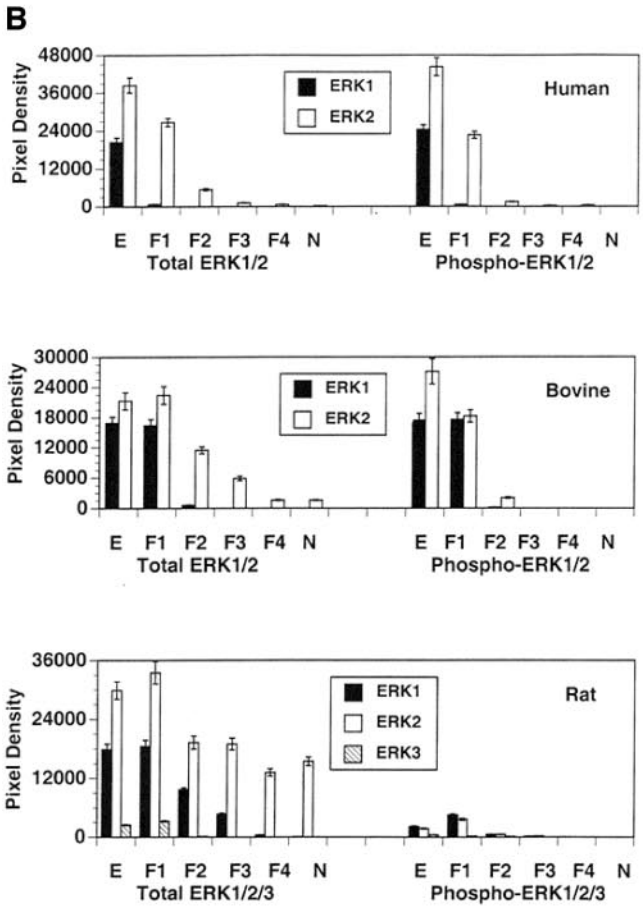
Expression and Activity of the MEK1 and MEK2 in Human, Bovine, and Rat Lenses

Because the ERKs are the most abundant MAPKs and mediate multiple functions in the ocular lens, we next examined the expression patterns and activity levels of their activating kinases, MEK1 and MEK2, in human, bovine, and rat lenses.

As shown in Figure 6A, 1 and 2, both MEK1 and MEK2 were exclusively expressed in the human lens epithelium, with the

FIGURE 2. Characterization of the different fractions of fiber cells through detection of the relative levels of two structural proteins, αA- and αB-crystallins, and nonstructural proteins, PKCα and GAPDH. **(A)** Typical results of three Western blot analyses. Total proteins were extracted from different fractions of the lenses and subjected to Western blot analysis. Note that the two α-crystallins were gradually decreased from F1 to the F4 fiber cells, and greatly decreased in the N fiber cells of human and bovine lenses. In rat lens, they were distinctly decreased in the F4 fiber cells and barely detectable in the N fiber cells. **(B)** Quantitative results of expression of αA- and αB-crystallin, PKCα, and GAPDH in human, bovine, and rat lenses. After exposure, the bands on each x-ray film were processed with an automated digitizing system. Total pixels for each band were recorded and averaged from three or more experiments after subtracting the corresponding background. Cell layer designations are as in Figure 1.





amount of MEK2 protein five times higher than that of MEK1 (Fig. 6B, top). A low level of MEK1/2 activity was detected in the lens epithelium (Fig. 6A, 3).

In bovine lens, MEK1 and MEK2 were expressed in both lens epithelium and F1 fiber cells (Fig. 6A, 4, 5). In contrast to the relative levels of MEK1 and MEK2 in human lens epithelium, MEK1 was approximately six times higher than MEK2 in bovine lens epithelium. In the F1 fiber cells, however, the protein level of MEK2 was five times higher than that of MEK1 (Fig. 6B, middle). As in human lens, the MEK1/2 activity was detected only in the lens epithelium (Fig. 6A, 6).

The MEK1/2 expression patterns in rat lens differed from those in human and bovine lenses. First, a substantial amount of MEK1 protein was detected in the F1 fiber cells (Fig. 6A, 7). Second, MEK2 was expressed in lens epithelium, F1 and F2 fibers in similar levels (Figs. 6A, 8; 6B, bottom). Finally, much higher levels of MEK1 and MEK2 activities were detected in the epithelium of rat lens (Figs. 6A, 9; 6B, bottom; data not shown).

Expression and Activity of the RAF1 Kinase in Human, Bovine, and Rat Lenses

Because both MEK1 and MEK2 are directly activated by RAF kinases, we next determined the expression patterns and activity levels of these kinases. As shown in Figure 7, Western blot analysis with anti-RAF1 revealed that the RAF1 (74 kDa) protein was present only in the epithelia of lenses from the three species. Western blot analyses with anti-RAF-A and -B antibodies revealed that these RAF proteins were not detectable in all lenses from the three species examined (data not shown). Analysis of the RAF1 activity in the lenses demonstrated that only rat lens epithelium possessed RAF1 activity (Figs. 7A, 6; 7B, bottom).

Discussion

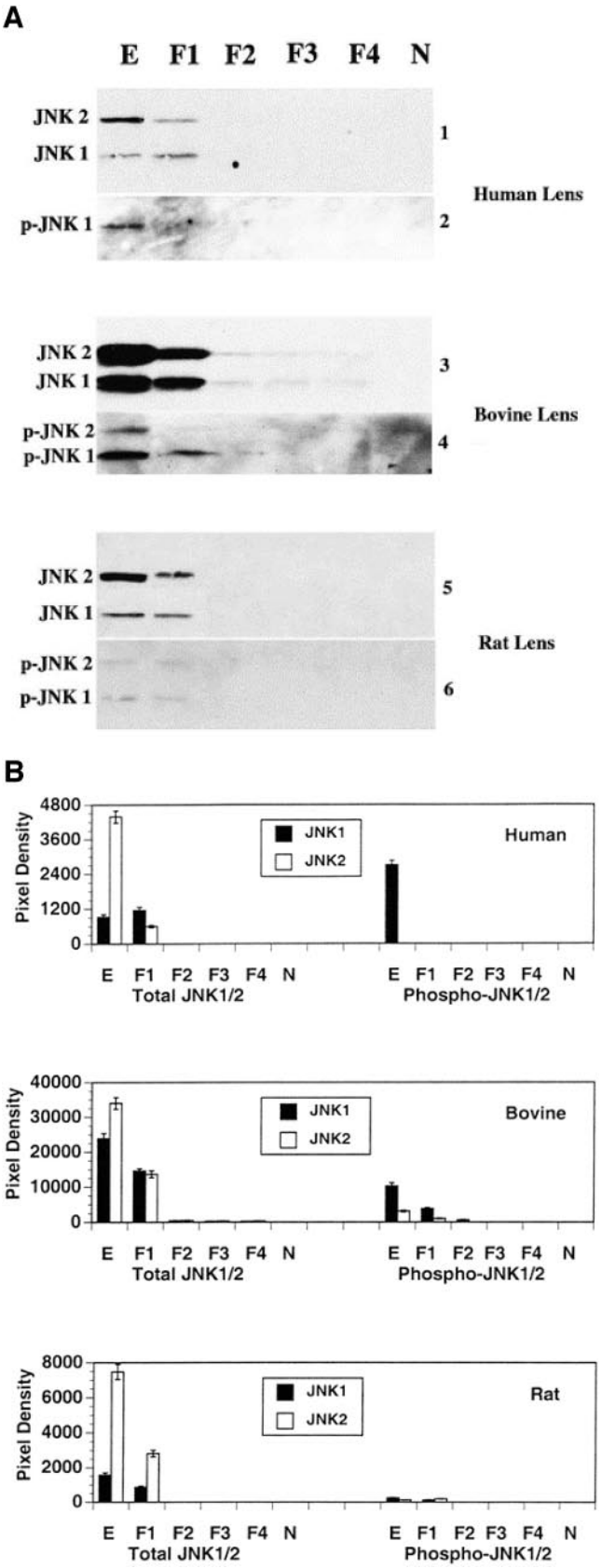
ERKs as the Major MAPKs in Ocular Lenses Required for their Multiple Functions

It is well established that MAPKs constitute important intracellular signaling components and mediating signaling pathways initiated by a large number of factors.[1–2] The three types of MAPKs—ERKs, JNKs, and p38s—have different yet overlapping roles. The ERK MAPKs expressed in many tissues, especially differentiated cells,[26] respond to growth factors (e.g. FGF, EGF), cytokines, transforming agents, and ligands for certain receptors to regulate cell proliferation, differentiation, survival, and apoptosis.[1–3]

In the present communication, among the three types of MAPKs, ERKs were the most abundant in human, bovine, and rat lenses in both protein levels and activity status. ERK1 and ERK2 were expressed at very high levels in both lens epithelium and F1 fiber cells of all lenses (except for ERK1 in human

Figure 3. Expression and activity levels of ERKs in human, bovine, and rat lenses. **(A)** Typical results of four Western blot analyses. Total proteins extracted from different fractions of the lenses were subjected to Western blot analysis. Note that the expression and activity levels of ERK1 and ERK2 were very high in both epithelium and the F1 fiber cells in all lenses except for ERK1 in the F1 fiber cells of human lens. ERK2 was present in other fractions of fiber cells at a very high level in rat lens, a modest level in bovine lens, and a detectable level in human lens. ERK3 was present only in the epithelium and F1 fiber cells of the 4-week-old rat lens. Panel 7 and 8 show the epithelial levels of ERK1/2/3 and p-ERK1/2/3 from a different rat. **(B)** Quantitative results of ERK1/2/3 in lenses from three species as indicated in each panel, analyzed as described in Figure 2B. Cell layer designations are described in Figure 1.

lens). Such high-level expression is important to accommodate their multiple functions in regulating cell proliferation, differentiation, and apoptosis of lens cells.



First, ERKs participate in FGF-induced cell proliferation and differentiation in the lens system.[15–18] In transgenic mice overexpressing a truncated FGF receptor R3, downregulation of ERK1/2 activity is associated with delayed lens fiber cell differentiation.[15] ERK activity is necessary and sufficient to mediate FGF-induced upregulation of intercellular communication through gap junctions in embryonic chicken lens epithelial cells[17] or to induce cell proliferation and differentiation in rat lens epithelial explants.[16] Our demonstration that the activity of ERK1/2 in the F1 fiber cells of rat lens is higher than that in the epithelial cells suggests that the activity is induced during fiber cell differentiation. It is likely that the epithelial activities of ERK1 and ERK2 are not homogenously distributed, and the epithelial cells within the germinal zone may have ERK1 and ERK2 activities similar to those observed in F1 fiber cells. ERK3 was initially identified in rat.[27] Later, a similar sequence was isolated from human HeLa cells.[28] Although the function of ERK3 remains unknown, its exclusive expression and activation in the epithelium and F1 fiber cells of 4-week-old rat lens (Figs. 3A, 8A) suggest its implication in cell proliferation and differentiation.

Second, factors such as pleiotrophin and TGFα also require activation of ERK MAPKs to mediate their functions.[29,30] Treatment of the primary cultures of bovine lens epithelial cells with pleiotrophin, a member of the heparin-binding factor family, stimulates DNA synthesis. Part of this stimulation is mediated by ERK1/2 MAPKs.[29] Under treatment with TGFα, chicken lens epithelial cells express the differentiation marker filensin. This induction is blocked by the ERK inhibitor.[30] ERK activation is also observed during EGF-initiated signal transduction.[12,13,19,20]

Finally, the ERKs are implicated in control of apoptosis. Activation of ERK1 and ERK2 is necessary and sufficient to mediate calcimycin-induced, p53-dependent apoptosis in lens epithelial cells (Li et al., manuscript submitted), which contributes to cataractogenesis.[14]

Our demonstration that the ERK2 is present in F3, F4, and N fiber cells suggests that it has additional functions. It is likely that through phosphorylation of different targets, ERK2 modulates the functions of other proteins such as ion channels, transporters, or metabolic enzymes and thus regulates the homeostasis of the F3, F4, and N fiber cells.

Lens Epithelium as a Major Site for Stress Response

In the present study, we have shown that JNK1 and JNK2 activities are mainly detected in the epithelia of bovine and rat lenses and are exclusively detectable in this compartment of the human lens. For p38 kinase, this is even more striking. p38 kinase activity was present only in the epithelia of all lenses examined. In contrast to the ERKs, the JNK1/2 and p38 kinase are activated by cytokines and many stress factors, such as the DNA-damaging agents oxidative stress and heat and osmotic shock.[4–7] The dominant epithelial localization of JNK activity and exclusive epithelial distribution of p38 kinase activity suggests that the lens epithelium is capable of acting as a major

FIGURE 4. Expression and activity levels of JNK1/2 in human, bovine, and rat lenses. (**A**) Typical results of four Western blot analyses. Total proteins extracted from different fractions of the lenses were subjected to Western blot analysis. Note that in the lens epithelium, the expression level of JNK2 was generally higher than that of JNK1. In contrast, the activity of JNK1 was higher than that of JNK2. JNK1 and JNK2 were also detectable in the F2, F3, and F4 fiber cells of bovine lens. (**B**) The quantitative results of JNK1/2 in lenses of three species as indicated in each panel, analyzed as described in Figure 2B. Cell layer designations are described in Figure 1.

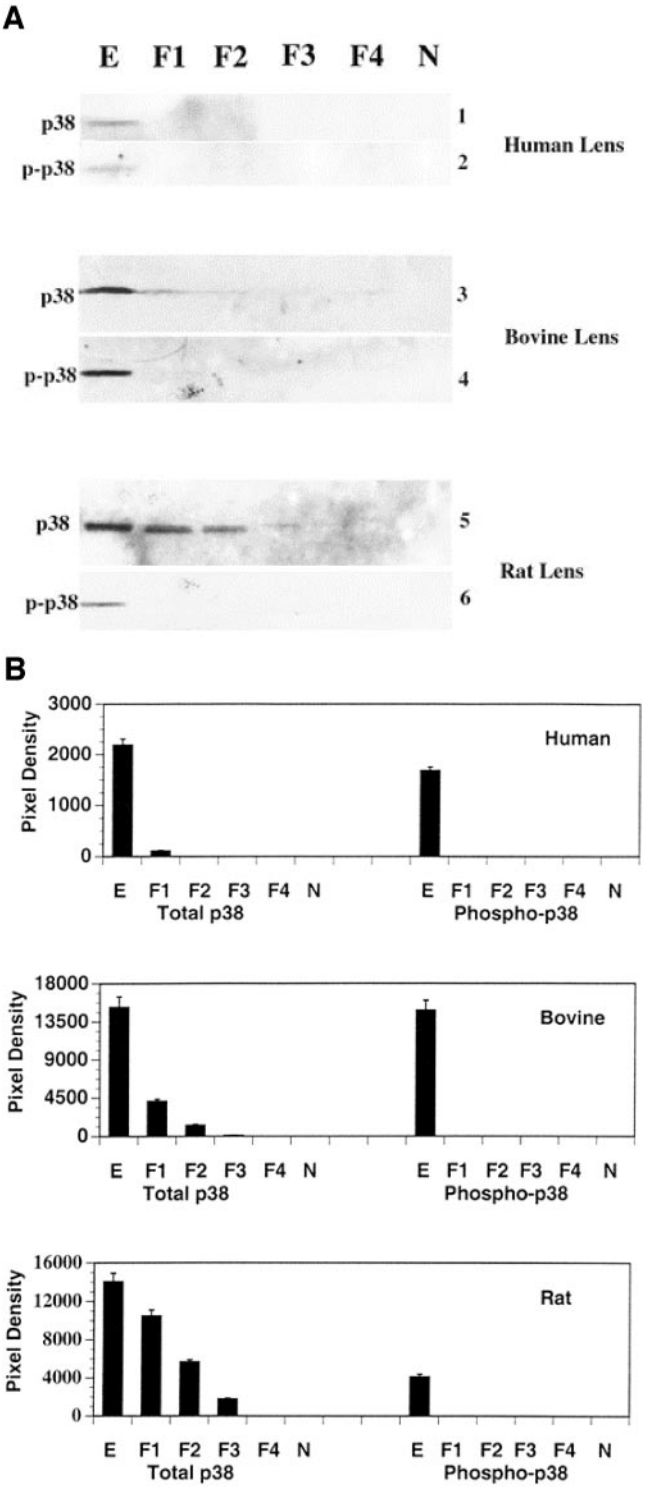


FIGURE 5. Expression and activity levels of p38 kinase in human, bovine, and rat lenses. **(A)** Typical results of four Western blot analyses. Total proteins extracted from different fractions of the lenses were subjected to Western blot analysis. Note that human lens expressed p38 kinase only in the epithelium. In contrast, bovine and rat lens expressed p38 kinase in the epithelium as well as in the F1, F2, and F3 fiber cells. Rat lens may express p38 in F4 fiber cells. However, p38 kinase activity was detected only in the lens epithelia of all lenses. **(B)** The quantitative results of p38 kinase in lenses of three species as indicated in each panel, analyzed as described in Figure 2B. Cell layer designations are described in Figure 1.

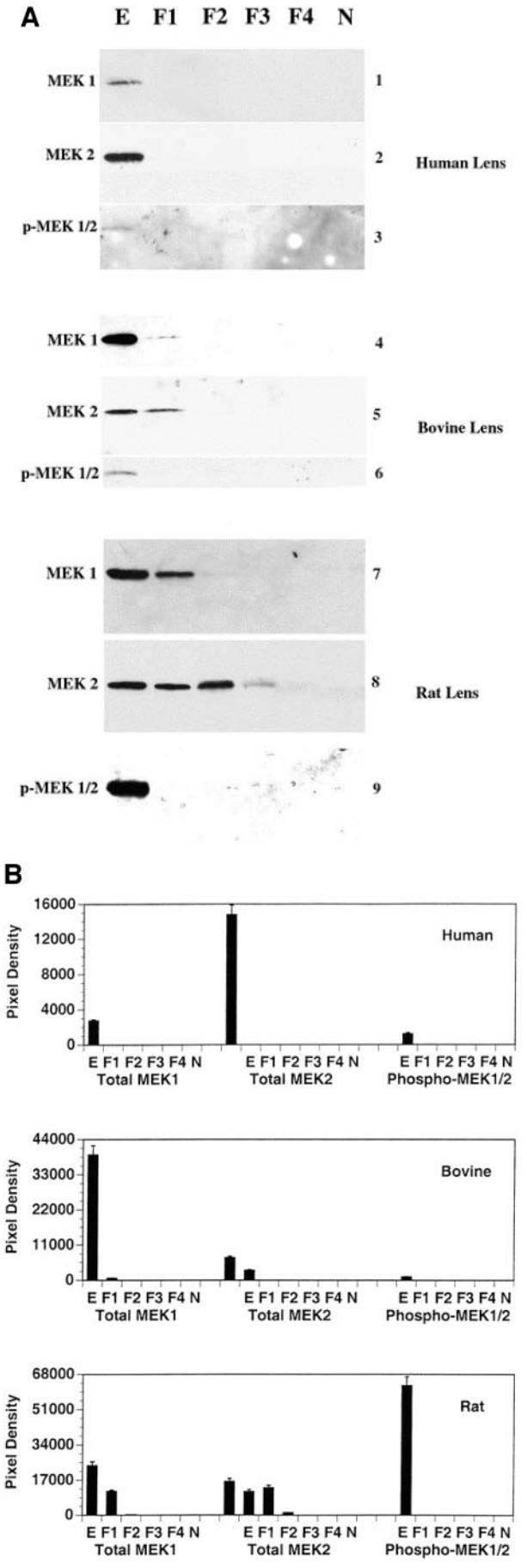
mediator of stress responses. In this regard, a recent study has demonstrated that in response to osmotic stress, p38 kinase is substantially activated and JNK1 and JNK2 are also induced in porcine lens epithelial cells under organ culture.[12,13] In addition, we have demonstrated that in response to hydrogen peroxide, activator protein (AP)-1 transactivity is substantially induced in rabbit lens epithelial cells, and this induction requires involvement of protein kinases such as JNK1/2.[31,32] Through AP-1, JNK1/2 may be also involved in control of cell proliferation and differentiation and regulation of gene expression. In the primary cultures of chicken embryonic lens, overexpression of c-Jun (substrate of JNK1/2) promotes cell proliferation, greatly delays the appearance of lentoid bodies and also downregulates expression of βA3- and A1-crystallin mRNA.[33] In human lens epithelial cells, oxidative stress–regulated expression of the human thiotransferase gene is also mediated through JNK-induced activation of AP-1.[34] AP-1 may be implicated in the regulation of the αA, βA3/A1-, βB1-, γB-, γF-, and S-crystallin genes of mouse, chicken, human, rat, and squid and thus is a common thread that unifies the diverse population of crystallin genes in several different species.[35]

Our observations that JNK2 is active only in bovine and rat lenses and that the p38 kinase activity in bovine and rat lenses is higher than that in human lens suggest induction of these kinases by the stress conditions experienced by bovine and rat lenses during sample collection (bovine eyes were stored in ice for 30 to 60 minutes before dissection, thus experiencing cold shock, rats were killed through CO_2 inhalation, therefore undergoing oxygen depletion).

Activation of ERKs by the Upstream Activating Kinases in Ocular Lenses

ERK1 and ERK2 are normally activated by the upstream kinases MEK1 and MEK2.[1,2] Although ERKs, especially ERK2, are expressed in the epithelium and all fractions of fiber cells, expression of MEK1/2 is more restricted. Moreover, the activities of ERK1 and ERK2 are detected in the lens epithelium and the F1 and F2 layers of fiber cells. In contrast, MEK1/2 activity is found only in the lens epithelium. RAF1 kinase is expressed only in the lens epithelium. These results suggest several possibilities regarding activation of ERK. First, in the epithelia of all the lenses examined, RAF1 and MEK1/2 are all expressed. Thus, ERK1/2 can be activated by MEK1/2, which can be further activated by RAF1. Although RAF1 activity was not detected in human and bovine lenses, it is possible that RAF1 activity is normally present in these lenses but disappeared during collection of the lens samples. In the fiber cells, both RAF1 and MEK1/2 were undetectable. Therefore, other upstream kinases other than MEK1/2 may be involved in activating ERKs. In this regard, MEK6 has been found to phosphorylate chimeras of p38 kinase and ERK2 in an in vitro assay.[36] In addition, Grammer and Blenis[37] suggest that ERKs are activated by an MEK-independent pathway through PKC and PI3K. Of course, it is also possible that the fiber ERKs, once activated, may stay in their activated status for a long time. This is especially true in the human lens. Human ERK2 expression and activity are very high in the F1 fiber cells, yet neither MEK1 nor MEK2 is detectable in these fiber cells. Prolonged ERK activation has been observed in other cell types under certain conditions, unlike the finding of the current prolonged ERK activation, which occur in a normal, unstimulated condition.[38,39] Finally, the possibility that MEK1/2 and RAF1 proteins were partially or completely degraded and thus their activities were lost during collection of lens samples cannot be ruled out.

In the epithelium, the relative expression levels of MEK1 versus MEK2 vary in human and rat lenses. Such variations are probably derived from the differential expression of the corre-



sponding genes. Because both MEKs can activate either ERK1 or ERK2,[1,2] the ERK1/2 activation patterns do not necessarily parallel the MEK1/2 expression patterns, at least in bovine and rat lenses. Activation of the MEK1 and MEK2 kinases is normally mediated by RAF kinases.[40,41] The RAF kinase family consists of three members: RAF1, RAFA, and RAFB. RAF1 is ubiquitously expressed, RAFA is primarily expressed in urogenital tissues, and RAFB in neuronal tissue and testis.[41] Our results showed that RAF1, but not RAFA and RAFB, was detectable in lens epithelial cells; thus, RAF1 is the only one that activates MEK1/2 in the lenses from these different species. RAF1 has been detected in cultured bovine and porcine lens epithelial cells, and its activity can be regulated by galactose.[12–13,42] Another kinase that probably activates MEK1/2 is MOS (the oncogene isolated from Moloney Sarcoma Virus).[1] Whether MOS is present in lens system remains to be explored.

ERK1, p38 Kinase, and PKCα as Molecular Markers for Aging

In the present study, our results show that expressions of ERK1, p38 kinase, and PKCα all fell into a similar pattern in different lenses. These kinases were either barely detectable or completely absent in the fiber cells of human lens or were expressed in much lower levels in F1 and F2 fiber cells of the bovine lens. In rat lens, however, they were not only expressed in F1, F2 and F3 fiber cells in relatively high levels but may also be detectable in the F4 and N fiber cells. When this pattern is linked to the age difference of these lenses (human lenses from donors aged 60 to 88 years, bovine lenses from animals 18 to 24 months old, and rat lenses from animals 4 weeks old), it appears that during the aging process, these kinases are gradually degraded—first in the N fiber cells, then in the F4 fiber cells, followed by their loss in F3, F2, and F1 fiber cells. This hypothesis was further supported when the protein levels of these kinases were compared in the lenses from 4-week- and 2-year-old rats (Fig. 8). PKCα is substantially decreased in F2 fiber cells and completely disappeared in the F3 and F4 fiber cells in 2-year-old rats compared with those in the 4-week-old rats. Similar differential expressions of ERK1 and p38 kinase are found in rat lenses of different ages. Thus, age seems to have an impact on the stability of these kinases. The ocular lens is a unique organ, in that it contains cells of different ages and thus can be used as an excellent system to study aging. Our finding that the expression patterns of ERK1, p38 kinase, and PKCα change with age suggests that these kinases may be used as molecular markers for aging. Whether MAPK pathways are involved in control of lens aging remains to be explored.

In summary, the present studies demonstrate that ERKs are the major MAPKs in the lens system, providing the ocular lens with the ability to mediate multiple signaling pathways essential in cell proliferation, differentiation, apoptosis, and pathogenesis. The presence of ERK1 and ERK2, especially ERK2 in F2 to N fiber cells suggests these kinases may regulate ho-

FIGURE 6. Expression and activity levels of MEK1/2 in human, bovine, and rat lenses. **(A)** Typical results of three Western blot analyses. Total proteins extracted from different fractions of the lenses were subjected to Western blot analysis. Note that human lens epithelium expressed a higher level of MEK2 than of MEK1 than did the bovine lens. Much less difference was observed in rat lens between MEK1 and MEK2. The MEK1 and MEK2 were expressed in the F1 fiber cells in bovine lens and in the F1 and F2 fiber cells in rat lens. Traces of MEK2 were also found in F3 fiber cells of rat lens. However, MEK1/2 activity was only detected in the lens epithelium of all lenses. **(B)** The quantitative results of MEK1/2 in lenses of three species as indicated in each panel, analyzed as described in Figure 2B. Cell layer designations are described in Figure 1.

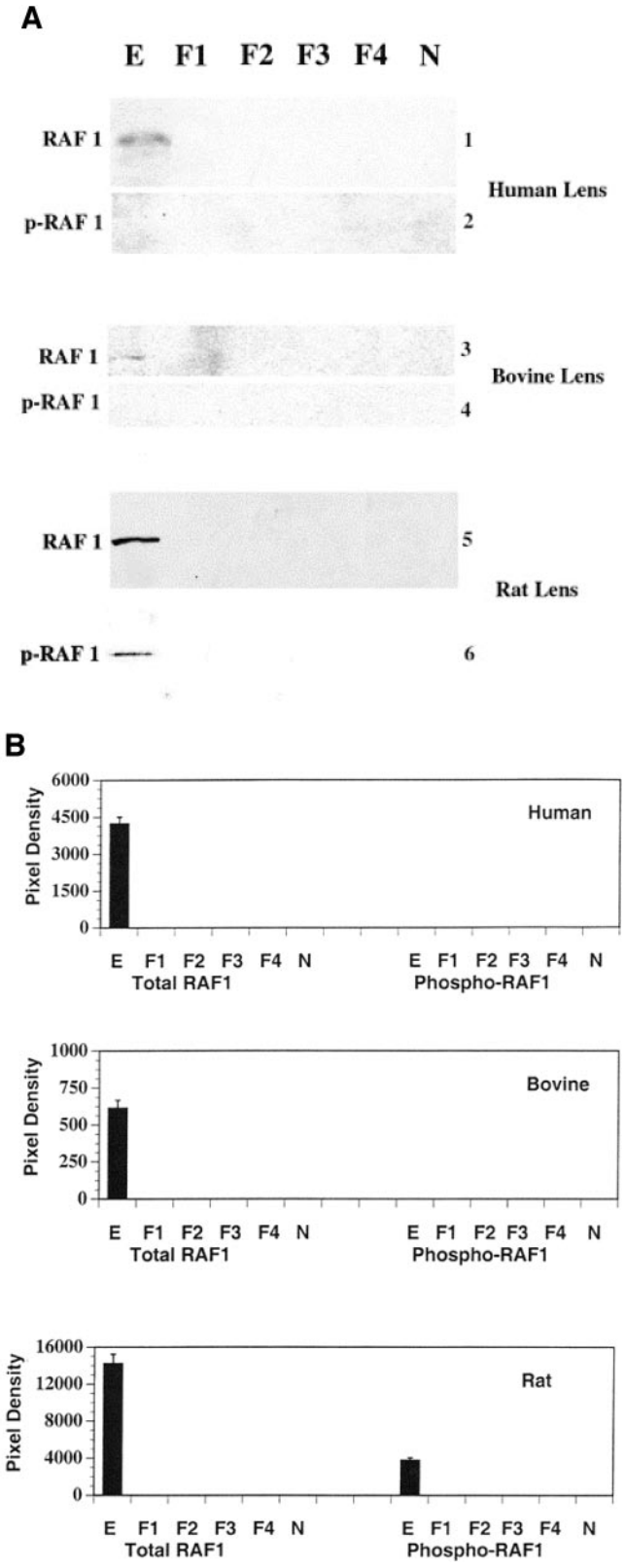


FIGURE 7. Expression and activity levels of RAF1 in human, bovine, and rat lenses. **(A)** Typical results of three Western blot analyses. Total proteins extracted from different fractions of the lenses were subjected to Western blot analysis. Note that the RAF1 protein was expressed in the epithelia of all lenses but its activity was detected only in the epithelium of rat lens. **(B)** The quantitative results of RAF1 in lenses from three species, as indicated in each panel, analyzed as described in Figure 2B. Cell layer designations are described in Figure 1.

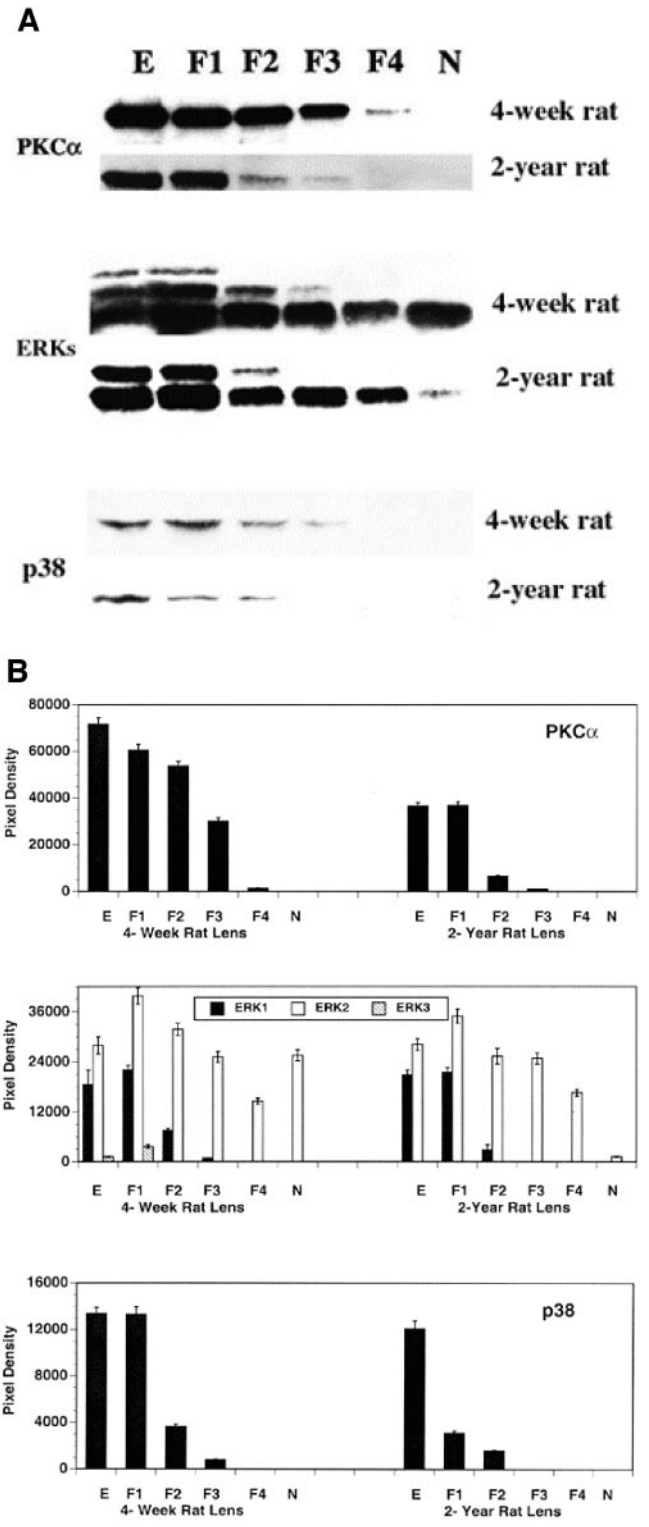


FIGURE 8. Comparative analysis of expression of PKCα; ERK1/2/3 and p38 kinase in 4- and 2-year-old rat lenses. **(A)** Typical results of three Western blot analyses. Note that PKCα, when compared with its expression in 4-week-old rat lens, was substantially decreased in F2 fiber cells and disappeared in the F3 and F4 fiber cells of the 2-year-old rats. Similar patterns were found for ERK1 and P38 kinase. **(B)** The quantitative results of PKCα; ERK1/2/3 and p38 kinase in 4-week and 2-year old rat lenses, analyzed as described in Figure 2B. Cell layer designations are described in Figure 1.

meostasis of the inner and nuclear fiber cells in addition to the functions in the epithelia and F1 fiber cells described herein. The dominant distribution of JNK1/2 activities and the exclusive localization of p38 kinase activity in the epithelia of human, bovine, and rat lenses indicate that the lens epithelium is a major site for stress response. Finally, the demonstration that the expression patterns of PKCα, ERK1, and p38 kinase change with age suggests that these kinases can be used as molecular markers for aging.

References

1. Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev.* 2001;22:153–183.
2. Rincon M. MAP kinase signaling pathways in T cells. *Curr Opin Immunol.* 2001;13:339–345.
3. Schaeffer HJ, Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol.* 1999; 19:2435–2444.
4. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell.* 2000;103:239–252.
5. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev.* 2001;81:807–869.
6. Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Genet Dev.* 2002;12:14–21.
7. Han J, Ulevitch RJ. Emerging targets for anti-inflammatory therapy. *Nat Cell Biol.* 1999;1:E39–E40.
8. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science.* 1995;270:1326–1331.
9. Franklin RA, McCubrey JA. Kinases: positive and negative regulators of apoptosis. *Leukemia.* 2000;14:2019–2034.
10. Ballif BA, Blenis J. Molecular mechanisms mediating mammalian mitogen- activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Diff.* 2001;12:397–408.
11. Gong X, Wang X, Han J, Niesman I, Huang Q, Horwitz J. Development of cataractous macrophthalmia in mice expressing an active MEK1 in the lens. *Invest Ophthalmol Vis Sci.* 2001;42:539–548.
12. Zatechka SD Jr, Lou MF. Studies of the mitogen-activated protein kinases and phosphatidylinositol-3 kinase in the lens. 1. The mitogenic and stress responses. *Exp Eye Res.* 2002;74:703–717.
13. Zatechka SD Jr, Lou MF. Studies of the mitogen-activated protein kinases and phosphatidylinositol-3 kinase in the lens. 2. The intercommunications. *Exp Eye Res.* 2002;75:177–192.
14. Li WC, Kuszak JR, Wang GM, Wu ZQ, Spector A. Calcimycin-induced lens epithelial cell apoptosis contributes to cataract formation. *Exp Eye Res.* 1995;61:91–98.
15. Govindarajan V, Overbeek PA. Secreted FGFR3, but not FGFR1, inhibits lens fiber differentiation. *Development.* 2001;128:1617–1627.
16. Lovicu FJ, McAvoy JW. FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signaling. *Development.* 2001;128:5075–5084.
17. Le AC, Musil LS. A novel role for FGF and extracellular signal-regulated kinase in gap junction-mediated intercellular communication in the lens. *J Cell Biol.* 2001;154:197–216.
18. Le AC, Musil LS. FGF signaling in chick lens development. *Dev Biol.* 2001;233:394–411.
19. Seth RK, Haque MS, Zelenka PS. Regulation of c-fos induction in lens epithelial cells by 12(S)HETE-dependent activation of PKC. *Invest Ophthalmol Vis Sci.* 2001;42:3239–3246.
20. Zhou J, Fariss RN, Zelenka PS. Synergy of epidermal growth factor and 12(s)-hydroxyeicosatetraenoate on protein kinase C activation in lens epithelial cells. *J Biol Chem.* 2003;278:5388–5398.
21. Benkhelifa S, Provot S, Nabais E, Eychene A, Calothy G, Felder-Schmittbuhl MP. Phosphorylation of MafA is essential for its transcriptional and biological properties. *Mol Cell Biol.* 2001;21:4441–4452.
22. Ochi H, Ogino H, Kageyama Y, Yasuda K. The stability of the lens-specific Maf protein is regulated by fibroblast growth factor (FGF)/ERK signaling in lens fiber differentiation. *J Biol Chem.* 2003;278:537–544.
23. Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem.* 1977;83: 346–356.
24. Li DW, Xiang H, Fass U, Zhang XY. Analysis of expression patterns of protein phosphatase-1 and phosphatase-2A in rat and bovine lenses. *Invest Ophthalmol Vis Sci.* 2001;42:2603–2609.
25. Mao YW, Xiang H, Wang J, Korsmeyer S, Reddan J, Li DW. Human bcl-2 gene attenuates the ability of rabbit lens epithelial cells against H_2O_2-induced apoptosis through down-regulation of the alpha B-crystallin gene. *J Biol Chem.* 2001;276:43435–43445.
26. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res.* 1998;74:49–139.
27. Boulton TG, Nye SH, Robbins DJ, et al. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell.* 1991;65:663–675.
28. Gonzalez FA, Raden DL, Rigby MR, Davis RJ. Heterogeneous expression of four MAP kinase isoforms in human tissues. *FEBS Lett.* 1992;304:170–178.
29. Souttou B, Ahmad S, Riegel AT, Wellstein A. Signal transduction pathways involved in the mitogenic activity of pleiotrophin: implication of mitogen-activated protein kinase and phosphoinositide 3-kinase pathways. *J Biol Chem.* 1997;272:19588–19593.
30. Chen F, Mrock LK, Ireland ME. A role for endogenous TGFalpha and associated signaling pathways in the differentiation of lens fiber cells. *J Cell Physiol.* 2001;186:288–297.
31. Li WC, Wang GM, Wang RR, Spector A. The redox active components H_2O_2 and N-acetyl-L-cysteine regulate expression of c-jun and c-fos in lens systems. *Exp Eye Res.* 1994;59:179–190.
32. Li DW, Spector A. Hydrogen peroxide-induced expression of the proto-oncogenes, c-jun, c-fos and c-myc in rabbit lens epithelial cells. *Mol Cell Biochem.* 1997;173:59–69.
33. Rinaudo JA, Vacchiano E, Zelenka PS. Effects of c-Jun and a negative dominant mutation of c-Jun on differentiation and gene expression in lens epithelial cells. *J Cell Biochem.* 1995;58:237–247.
34. Krysan K, Lou MF. Regulation of human thioltransferase (hTTase) gene by AP-1 transcription factor under oxidative stress. *Invest Ophthalmol Vis Sci.* 2002;43:1876–1883.
35. Lagan JG, Cvekl A, Kantorow M, Piatigorsky J, Sax CM. Regulation of alphaA-crystallin gene expression: lens specificity achieved through the differential placement of similar transcriptional control elements in mouse and chicken. *J Biol Chem.* 1999;274: 19973–19978.
36. Wilsbacher JL, Goldsmith EJ, Cobb MH. Phosphorylation of MAP kinases by MAP/ERK involves multiple regions of MAP kinases. *J Biol Chem.* 1999;274:16988–16994.
37. Grammer TC, Blenis J. Evidence for MEK-independent pathways regulating the prolonged activation of the ERK-MAP kinases. *Oncogene.* 1997;14:1635–1642.
38. Qui MS, Green SH. PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. *Neuron.* 1992;9:705–717.
39. Giambartolomei S, Covone F, Levrero M, Balsano C. Sustained activation of the Raf/MEK/Erk pathway in response to EGF in stable cell lines expressing the hepatitis C virus (HCV) core protein. *Oncogene.* 2001;20:2606–2610.
40. Kyriakis JM, App H, Zhang XF, et al. RAF1 activates MAP kinase-kinase. *Nature.* 1992;358:417–421.
41. Hagemann C, Rapp UR. Isotype-specific functions of Raf kinases. *Exp Cell Res.* 1999;253:34–46.
42. Takemoto DJ. Decreases in RAF1 levels in galactosaemic lens epithelial cells are partially reversed by myo-inositol. *Acta Diabetol.* 1998;35:145–149.