Lens Major Intrinsic Protein (MIP)/Aquaporin 0 Expression in Rat Lens Epithelia Explants Requires Fibroblast Growth Factor-induced ERK and JNK Signaling*

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Lens major intrinsic protein (MIP), exclusive to the vertebrate lens, otherwise known as MIP26 and Aquaporin 0, is abundantly expressed as a lens fiber membrane protein. Although relatively less efficient compared with other aquaporins, MIP is suggested to function as a water channel, as an adhesion molecule, and is required for lens transparency. Because MIP is specifically expressed in lens fiber cells, we investigated in this study the activation of Mip expression after triggering differentiation of rat lens epithelia explants by fibroblast growth factor (FGF)-2. Here, we show that Mip expression in the lens cells is regulated by FGF-2. Using Real time PCR we demonstrate that endogenous Mip levels in the explants were up-regulated upon FGF-2 stimulation, in a concentration-dependent manner. Up-regulation of Mip at the transcriptional level was simultaneous with the activation of the FGF downstream signaling components, ERK1/2 and JNK. Specific inhibitors, U0126 for ERK1/2 and SP600125 for JNK, abrogated Mip expression in response to FGF-2 in the explants. This inhibition pattern was recapitulated in reporter assays for transfection of the rat lens epithelia explants, driven by the Mip promoter (~1648/-44). Our studies show that ERK1/2 and JNK signaling pathways are required for Mip expression in lens epithelia explants induced to differentiate by FGF-2.

The vertebrate lens is composed by an anterior layer of cuboidal epithelial cells and differentiated fiber cells that comprise the bulk of the tissue (1). During embryonic development and throughout the life of the organism, fiber cells are added via differentiation of the lens epithelial cells. Spatial and temporal regulation of gene expression is required to maintain the transparency and correct refractive index of the ocular lens.

Major intrinsic protein (MIP) is a polypeptide found exclusively in the membranes of lens fiber cells. It was first isolated as the most abundant protein of the fiber cell membrane (2).

Subsequent to the identification of AQP1 as a water channel in red blood cells, MIP (also known as AQP0) was shown to be permeable to water (3–5), although this permeability is much lower than for most other AQPs. MIP may also have structural roles in the lens fibers, functioning as a cell to cell adhesion molecule (6, 7), playing a role in gap junction formation (8, 9) and in organizing γ-crystallins (10). In this way, MIP may play an important role in maintaining lens transparency by reducing the inter-fiber space and is required for the normal focusing properties of the lens (11). During cataractogenesis and aging, MIP undergoes selective proteolysis that may modulate gating of the MIP channels (12) and its adhesive properties (13). Moreover, mutations in the Mip gene have been linked to the mouse genetic cataracts Fraser (CatFr), lens opacity (lop), Hfi, Tohm (14–16), and two human cataracts (17, 18). Although expression of the Mip gene in the lens is tightly regulated temporally and spatially during embryogenesis, signaling cascades involved in this process remain unidentified.

Growth factors such as FGFs, insulin-like growth factor-1, platelet-derived growth factor, epidermal growth factor, and insulin are shown to promote proliferation of lens epithelial cells and differentiation into fiber cells in rats and chicken (19–24). Recently, BMP was shown to participate in fiber cell differentiation (25, 26). The binding of growth factors to their cell surface receptors leads to the activation of the receptor tyrosine kinase eventually transducing the signals into cellular targets via distinct downstream signaling molecules (27). FGFs regulate a multitude of biological phenomena including adhesion, migration, proliferation, and differentiation (19, 20, 28).

Overexpression of secreted FGF-1, -3, -4, -7, -8, and -9 in the lens of transgenic mice causes the premature differentiation of anterior epithelial cells into fiber cells (29–31). However, overexpression of secreted FGF-2 suggests a role in modulating fiber cell differentiation and survival (32). Experiments using dominant-negative FGF receptors also provide insights into the role of FGFs in lens cell survival (29, 33, 34).

It has been demonstrated that FGF-1 or FGF-2 can stimulate either proliferation or differentiation of explanted rat lens epithelia in a dose-dependent manner (19, 20). In this study, we have used explanted rat lens epithelia induced to differentiate by FGF-2 to address the molecular mechanisms that determine how FGF signaling regulates Mip expression. Here we show that FGF-2 induces Mip expression in explanted lens epithelia. We provide evidence that both FGF-induced ERK1/2 and JNK activation result in the up-regulation of Mip at the transcriptional level. FGF-2 ability to up-regulate Mip gene expression is obliterated in the presence of a specific JNK inhibitor. We further demonstrate that an Mip active promoter (~1648/+44 bp) responds to ERK and JNK inhibitors in a similar way as the
endogenous Mip gene. Our results suggest that both ERK and JNK signaling pathways downstream of FGF activation are required for Mip expression and JNK activation is pivotal in addition to ERK activation.

EXPERIMENTAL PROCEDURES

Reagents—The MIP-specific polyclonal antiserum was obtained from Alpha Diagnostic (San Antonio, TX). Antibodies to total ERK (rabbit polyclonal), total JNK (rabbit polyclonal), phospho-ERK (mouse monoclonal), and phospho-JNK (mouse monoclonal) were purchased from Cell Signaling Technology Inc. (Beverly, MA). U0126, an inhibitor of ERK activation, was purchased from Promega (Madison, WI), and SP600125, an inhibitor of JNK activation, was obtained from Calbiochem (San Diego, CA).

Plasmids—pMIP1648-luc, pMIP598-luc, and pMIP461-luc reporter plasmids were constructed by cloning the DNA fragment containing the 1648-, 598-, or 461-bp 5′-flanking regions, respectively, plus 44-bp exon 1 sequence of a mouse Mip genomic clone into the pGL3-Basic vector (Promega) upstream of the luciferase gene. For internal control and positive controls, phRL-CMV and pGL3-Control (SV40 promoter) vectors were used (Promega).

DNA Sequencing—DNA sequencing of plasmid constructions was performed with a commercially available system (CEQ DTC5 Quick Start Kit; Beckman-Coulter, Hialeah, FL) and an automated DNA analysis system (CEQ 2000XL; Beckman-Coulter), according to the manufacturer’s instructions.

Lens Epithelia Explants Culture—Eyes were dissected from 3-day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). The lens capsule with adhering epithelial cells was microdissected from the lens fibers and pinned down around its periphery with forceps under the microscope and lysed. The dual luciferase activity was measured according to the protocol described by the manufacturer. The reporter luciferase activity was normalized by calculating its ratio to the Renilla luciferase activity.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction—RNA was isolated by using the Absolutely RNA Nanoprep Kit (Stratagene, Cedar Creek, TX) according to the manufacturer’s protocol. cDNA was prepared with the SuperScript First-strand synthesis system for RT-PCR (Invitrogen).

Relative Quantification of Gene Expression Using Real Time PCR—Relative amounts of Mip mRNA and that of the control gene were quantified by real time PCR using the ABI Prism 7900 Sequence Detection system. Primers and probe design for the real time PCR was made with Primer Express version 2 from Applied Biosystems. The Mip-specific primer pair was 5′-CACCAGCTGTCCGGAGAAA-3′ (forward) and 5′-GGCTCAAGAGATCTCCAGAT-3′ (reverse) and the probe (FAM-MGB) specific for Mip was FAM-5′-TCAACACGTCATGC-3′-MGB. Primers for mouse 18 S ribosomal RNA (as control) were 5′-AGTCCCTGTCCCTTTGTACACA-3′ (forward) and 5′-CCGAGGCC- TCACTAGG-3′ (reverse), and the VIC-Tamra probe sequence for 18 S ribosomal RNA was 5′-CATTTATACACAGAGCGGACACT-3′. The Mip forward primer spans the exon 1–exon 2 boundary to avoid amplification of possible contaminating MIP genomic sequences. All runs were performed in triplicate according to the default PCR protocol (50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min) or the default one-step RT-PCR protocol (42 °C for 30 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min). Relative standard curves were generated for each primer set so that the input amount from unknown samples could be calculated. Expression of Mip was normalized with 18 S ribosomal RNA expression. Expression was compared and expressed in graph format (Excel; Microsoft, Redmond, WA).
**SDS-PAGE and Immunoblot Analysis**—Total cell lysates (containing 10 μg of total proteins) prepared from the harvested lens explants were separated by SDS-PAGE using 4–12% polyacrylamide gradient gels (Novex, San Diego, CA) and transferred to nitrocellulose membranes (Novex). Blots were blocked with a 5% solution of nonfat dry milk in TBST (Tris-buffered saline containing 0.1% Tween 20) and transferred to nitrocellulose membranes (Novex, San Diego, CA) and transferred to nitrocellulose membranes (Novex). Blots were blocked with a 5% solution of nonfat dry milk in TBST (Tris-buffered saline containing 0.1% Tween 20) for 1 h at room temperature. Next, the blots were incubated in buffer containing appropriate antisera (1:1000 for total ERK; 1:1000 for total JNK, 1:1000 for phospho-ERK and phospho-JNK) for 2 h at room temperature. After washing, the blots were incubated in buffer containing the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) at a dilution of 1:1000 at 1 h at room temperature. The blots were then developed using an ECL-Plus chemiluminescence kit (Amersham Biosciences) and exposed to x-ray films. The x-ray films were subsequently scanned (ScanJet 6100C; Hewlett-Packard, Palo Alto, CA).

**Confocal Immunofluorescence**—The lens explants were fixed in 4% formaldehyde in isotonic phosphate-buffered saline (pH 7.4) buffer for 2 h at room temperature. Cellular localization of MIP, phospho-ERK, and phospho-JNK in the explants was examined by indirect immunofluorescence labeling. Briefly, the explants were incubated in ICC buffer (phosphate-buffered saline containing 0.20% Tween 20, 0.05% sodium azide, and 0.5% bovine serum albumin, pH 7.3) for 20 min at room temperature. The explants were then incubated in ICC buffer with rabbit anti-MIP (1:50), rabbit anti-phospho-ERK (1:50), or rabbit anti-phospho-JNK (1:50) for 1 h at 37°C. The explants were washed in ICC buffer, followed by incubation for 1 h in ICC buffer containing fluorescent dye-conjugated secondary antibodies (anti-rabbit biotinylated and Alexa 488-streptavidin or Alexa 555-streptavidin, Molecular Probes, Eugene, OR). After washing, the explants were mounted with Tris-buffered saline/glycerol (1:1) containing p-phenylenediamine (Sigma) and covered by a coverslip. The explants were viewed with a confocal microscope (model SP2; Leica Microsystems, Exton, PA). Images were assembled for publication using computer software Corel Draw 9.0 (Corel Corp.).

**Scanning Electron Microscopy**—All lens epithelia explants in plastic culture dishes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperature for 48–72 h. They were postfixed in 1% aqueous OsO4 for 1 h at room temperature, then each explant was carefully cut out of the dish with a jewelers saw. Dehydration of the epithelia explants was done in a graded ethanol series followed by critical point drying. The samples were then mounted on specimen stubs, and subsequently sputter-coated with gold palladium alloy. Micrographs of epithelial cells were taken from the apical surface in peripheral regions of the explants using a JEOL 620 scanning electron microscope at 10 kV.

**RESULTS**

**FGF-2 Induces Mip Expression in Rat Lens Explants**—Previous studies have shown that FGF-1 and FGF-2 promote lens epithelial cell proliferation and differentiation in lens explant cultures (19, 20). We used the rat lens explant cultures to determine the effect of FGF-2 on MIP protein expression (Fig. 1). A lens epithelia explant in culture is shown in Fig. 1A. Because the expression of fiber cell markers in the explants occurs in 3 days of culture with 100 ng/ml FGF-2, we monitored MIP protein expression in the explants by immunocytochemical analysis after 3 days in culture. A polyclonal antibody specific to the MIP protein revealed the expression of MIP in the explants (Fig. 1B). No MIP expression is observed at time 0. A higher magnification of a demarcated region of the explant demonstrated that many MIP expressing cells present elongated morphology as opposed to the cuboidal morphology of the epithelial cells (Fig. 1C).

Next we examined the Mip mRNA levels in the FGF2-treated and untreated explants. RT-PCR analyses demonstrated the up-regulation of Mip mRNA as evidenced by the detection of a 693-bp amplicon of the Mip gene in the explants treated with 100 ng/ml FGF-2. Mip gene transcripts were absent in the control explants cultured without FGF-2 (Fig. 1D). In the ab-
sence of FGF-2, the lens epithelia explants were still viable as observed by the expression of AP2, a transcription factor expressed in lens epithelia and not in lens fibers (35) (Fig. 1E).

FGF-2 Activates Mip Promoter Activity in Lens Epithelia Explants—On the basis of the observation that the Mip mRNA level was up-regulated in the explants treated with FGF-2, a follow-up study was designed to determine whether the Mip gene promoter harbored the response elements required to drive transcription triggered by FGF-2. Mip 5′-flanking promoter constructs spanning 461, 598, and 1,648 bp upstream of the Mip transcription start site and 44 bp of exon 1 fused to the luciferase reporter gene were used for transfection assays. In the presence of 100 ng/ml FGF-2, the lens explants 3-day post-transfection with the reporter plasmids displayed activation of the Mip promoter (Fig. 2A). The Mip 5′-flanking promoter sequence −1,648 bp showed a −20-fold increase in reporter activity relative to the control empty vector. However, the Mip promoter activity drastically decreases when only 598 bp of Mip 5′-flanking sequence are used.

To test the specificity of the Mip promoter response to FGF-2 in lens epithelia explants, we also monitored the Mip promoter activity in the presence of FGF-2 in two cell lines that do not express Mip (RK13 and AMLE cell lines). Fig. 2B shows that in the presence of FGF-2, the −1648 Mip 5′-flanking sequence lacks an active promoter, whereas a control SV40 promoter is active in rat kidney (RK13) cells. We obtained similar results using AMLE1, a mouse lens epithelial cell line (36) that does not express endogenous MIP. Transfection of either the −461- or −1648-bp reporter constructs resulted in a null response to transcriptional activation by FGF-2 (100 ng/ml), whereas SV40 driven reporter activity was noticeable (Fig. 2C). These results confirm that not only the 5′-flanking sequences, at least up to −1648 bp, of the Mip gene harbor positive response element(s) to FGF stimulation but also that this response is specific to differentiating lens epithelia cells.

FGF-2 Concentration Is Pivotal for Lens Fiber Cell Differentiation—McAvoy and Chamberlain (20) have demonstrated that FGF induces proliferation and differentiation of cultured rat lens epithelia explants. Upon adopting this differentiation study model by FGF-2 stimulation in lens explants, our goal was to first demonstrate the optimal dose of FGF-2 to induce differentiation in our culture system. We confirmed this observation by using scanning electron microscopy on cultured explants in the presence of 0.05, 5, and 100 ng/ml FGF, respectively. After 14 days in culture, morphological analyses by scanning electron microscopy revealed an elongating fiber cell phenotype in the explants treated with high concentration (100 ng/ml) of FGF-2, whereas the explants treated with low doses of FGF-2 (0.05 and 5 ng/ml) retained their epithelial characteristics (Fig. 3). In Fig. 3, A and B, cuboidal morphology typical of lens epithelial cells can be contrasted with the appearance of apical protrusions in the differentiating fiber cells in Fig. 3C, and these cells eventually assume a typical elongated fiber cell phenotype after 14 days of culture (Fig. 3D). These results confirm that in our culture conditions, lens explants are capable of undergoing differentiation to fiber cells under the influence of 100 ng/ml FGF-2.

![Fig. 3. Morphological changes in rat lens explants during induction of differentiation.](image-url)

**Fig. 3.** Morphological changes in rat lens explants during induction of differentiation. Rat lens explants were cultured in the presence of varying concentrations of FGF-2 for 4–14 days. Morphological changes were recorded by scanning electron microscopy. A, when cultured in the presence of 0.05 ng/ml FGF-2 for 4 days, lens epithelial cells of the explant are still maintained in an undifferentiated epithelial morphology as viewed from their apical surface. B, an epithelial morphology is still visible after culture in the presence of 5 ng/ml FGF-2 for 4 days. C, in contrast, in the presence of 100 ng/ml FGF-2 for 4 days, the apical surface of the epithelial cells undergoes significant transformations including the formation of numerous apical protrusions, indicating that fiber cell differentiation has started. D, the differentiation of fiber cells is further recognized in the presence of 100 ng/ml FGF-2 for 14 days, an epithelial phenotype is completely lost and elongating differentiated fiber cells can be readily observed. Scale bar is 10 μm in all figures.

![Fig. 4. Effect of FGF-2 concentration on endogenous Mip mRNA level in rat lens explants.](image-url)

**Fig. 4.** Effect of FGF-2 concentration on endogenous Mip mRNA level in rat lens explants. Total RNA was obtained from FGF-2-treated rat lens explants and real time PCR was performed to quantitate the endogenous levels of Mip mRNA after culturing the explants in the presence of 0.01–100 ng/ml FGF-2 for 1–4 days (A) and at various doses of FGF-2 for 4 days (B). Mip expression increases significantly after 3 days in culture with 100 ng/ml FGF-2 (A). At the highest dose (100 ng/ml), FGF is significantly more potent in inducing Mip transcription (B).
Endogenous Mip mRNA Level in Lens Explants Is FGF-2 Dose-dependent—Results demonstrating that 100 ng/ml FGF-2, but not low doses of FGF-2, induces lens fiber cell differentiation were followed up by experiments to determine the Mip expression pattern in the presence of various concentrations of FGF. Real time PCR was performed to determine the endogenous Mip mRNA level in the cultured explants. Based on our prior observation that 100 ng/ml FGF-2 efficiently
induced Mip expression, we used this dose to demonstrate a time course response. Mip mRNA levels after 1, 2, 3, and 4 days are shown in Fig. 4A. The Mip expression in explants after 3 or 4 days in culture showed ~70-fold increase over day 1. The significant increase in Mip transcripts appears between 2 and 3 days in culture. These results indicate that Mip transcripts appear as fiber cell differentiation begins to be underway.

We performed a dose-response study to determine whether the Mip transcript level is induced by different concentrations of FGF-2 (0.01, 5 or 100 ng/ml) (Fig. 4B). Real time PCR results demonstrated that at the highest dose (100 ng/ml), FGF-2 is significantly more potent in inducing Mip transcription after 3 to 4 days in culture. These results demonstrate that the concentration of FGF-2 is critical both for the morphological changes and Mip expression associated with lens fiber cell differentiation.

FGF-2 Induces ERK1/2 Activation in Lens Explants in a Concentration-dependent Manner—FGF-2 is known to stimulate the MAPK (ERK) signaling cascade in various types of cells. In our study, we attempted to determine whether ERK is phosphorylated and thereby activated by FGF-2. Using a gradient of doses from very low to high concentrations of FGF-2, ERK phosphorylation was evaluated by immunoblot assays. An antibody specific to activated phospho-ERK was used to probe the proteins extracted from the explants and resolved on SDS-PAGE. Fig. 5 shows the total and activated ERK in explants treated for 30 min with either 0.01 ng, 5 ng, and 100 ng/ml FGF-2. The two isoforms, p44 (ERK1) and p42 (ERK2), are indicated. Compared against a non-variable level of total ERK1/2 in all the explants (Fig. 5A), a sparse and robust increase in phospho-ERK1/2 was evident following 5 ng and 100 ng/ml FGF2 treatments, respectively (Fig. 5B). Densitometric analyses revealed 2- and 5-fold increases in phospho-ERK1, and 2- and 12-fold increases in phospho-ERK2 in the 5 ng and 100 ng/ml FGF2-treated explants over the 0.01 ng/ml FGF2-treated explant (Fig. 5, C and D). Consistent with this observation, immunocytochemical analyses of the explants treated for 30 min with 0.01 ng, 5 ng, and 100 ng/ml FGF-2 and
imaged by confocal microscopy reveal the presence of activated ERK. In a low dose (0.01 ng/ml), activated phospho-ERK was present in the cell cytoplasm and represents the basal level (Fig. 5E). We did not observe any difference in ERK phosphorylation in the absence of FGF compared with 0.01 ng/ml FGF (data not shown). However, in 5 ng/ml, FGF-2 treatment enhanced the level of activated ERK in the cells. In explants treated with 100 ng/ml FGF-2, the intensity of the signal was higher than in the 5 ng/ml FGF-2-treated explants (Fig. 5F, G), confirming the results obtained by immunoblot. The immunocytochemical analysis of the explants treated for 3 days with 0.01, 5, or 100 ng/ml FGF-2; G, 100 ng/ml FGF plus JNK inhibitor SP600125 (20 μM). Nuclei were stained with 4,6-diamidino-2-phenylindole.

ERK and JNK Regulate MIP/Aquaporin 0 Expression

Fig. 7. JNK activity in rat cell explants. A, the rat lens epithelial explants were incubated 30 min in the presence of various concentrations of FGF-2 and harvested, and the proteins were resolved on an SDS gel. 10 μg of protein of each sample was loaded to perform the Western blot. Immunoblots using anti-JNK and anti-phospho-JNK antibodies show total JNK levels and activated JNK levels in rat lens explants treated with various doses of FGF. Arrows indicate the JNK1 p46 and JNK2 p54 bands. The band intensities were quantitated by scanning densitometry. Values represent mean ± S.E. of three independent experiments. Densitometric analyses revealed 2- and 5-fold increases in phospho-JNK1 in the 5 ng and 100 ng/ml FGF-treated explants, respectively, over 0.01 ng/ml FGF-treated explants (Fig. 7B), whereas phospho-JNK2 remained unchanged under the same conditions (Fig. 7C). D–F, immunocytochemistry (green) shows phospho-JNK levels in the explants treated for 30 min with 0.01, 5, or 100 ng/ml FGF-2; G, 100 ng/ml FGF plus JNK inhibitor SP600125 (20 μM). Nuclei were stained with 4,6-diamidino-2-phenylindole.
In contrast, as expected, untreated control cells were undergoing differentiation as shown by the formation of numerous apical protrusions and disappearance of their typical epithelial morphology (Fig. 6B).

Real time PCR results revealed a 5-fold decrease in the Mip transcript level in the presence of UO126 in FGF-treated explants (Fig. 6C). Explants transfected with the Mip promoter construct (p1648MIP-luc or pBasic cultures with 100 ng/ml FGF-2 in the absence or presence of SP600125 (20 μM). The endogenous Mip mRNA level is reduced in rat lens explants treated with JNK inhibitor (SP600125). D, transfection of the rat lens epithelia explants with p1648MIP-luc or pBasic cultures with 100 ng/ml FGF-2 in the absence or presence of SP600125 for 72 h. Relative luciferase activity assay shows inhibition of the Mip promoter activity in lens explants in the presence of 10 μM SP600125.

**Fig. 8. Effect of JNK on Mip expression in differentiating rat lens epithelia explants.** Immunohistochemical analysis of the rat lens epithelia explants cultured with 100 ng/ml FGF-2 in the absence (A) or presence (B) of SP600125 for 72 h shows reduction in the MIP level in rat lens explants treated with JNK inhibitor. Nuclei were stained with 4,6-diamidino-2-phenylindole. C, real time PCR analysis of the rat lens epithelia explants cultured for 3 days with 100 ng/ml FGF-2 in the absence or presence of SP600125 (20 μM). The endogenous Mip mRNA level is reduced in rat lens explants treated with JNK inhibitor (SP600125). D, transfection of the rat lens epithelia explants with p1648MIP-luc or pBasic cultures with 100 ng/ml FGF-2 in the absence or presence of SP600125 for 72 h. Relative luciferase activity assay shows inhibition of the Mip promoter activity in lens explants in the presence of 10 μM SP600125.
cytoplasm (Fig. 7E). In contrast, in the explants treated with 100 ng/ml FGF-2, activated JNK was mainly localized in the nucleus (Fig. 7F). The immunocytochemical analysis of the explants treated for 3 days with 0.01 ng, 5 ng, and 100 ng/ml FGF-2 revealed no activated JNK (data not shown). These data suggest that the activated JNK is mainly required at the very early stages in the FGF signaling pathway.

**JNK Inhibitor Reduces Mip Expression in Lens Explants**—To determine whether JNK is involved in the expression of Mip, we used a specific inhibitor of JNK, SP600125. Explants treated for 30 min with 100 ng/ml FGF-2 in the presence of the JNK inhibitor, SP600125 (10 μM), showed abrogation of the phospho-JNK level (Fig. 7G). Immunocytochemical analyses show the abrogation of MIP protein expression in SP600125-treated explants in the presence of 100 ng/ml FGF for 3 days (Fig. 8D), whereas in the absence of the inhibitor, MIP protein expression remains unperturbed (Fig. 8A). We have confirmed the specificity of the MIP antibody by doing several controls. Omission of either primary or secondary antibody resulted in lack of the streptavidin Alexa-555 immunofluorescence signal (data not shown).

To demonstrate that SP600125 inhibits specifically the JNK pathway and does not affect ERK phosphorylation, we performed immunoblot and immunostaining analyses. The ERK expression was unaffected by the JNK inhibitor either in immunoblot or immunostaining analysis (data not shown).

That the role of JNK on Mip expression may possibly be played at the transcriptional level was confirmed by using real time PCR to quantify the endogenous Mip transcript level. Fig. 8C shows a 3-fold decrease in the Mip mRNA level in the explants cultured in the presence of FGF2 and SP600125 for 3 days. The control explants treated only with FGF2 and Me2SO (vehicle) expressed Mip transcripts to the expected level.

Explants transfected with the Mip promoter construct pMIP1648-luc revealed a significant 4-fold decrease in promoter activity in the presence of 10 μM SP600125 (Fig. 8D). These results suggest that JNK activity is required for the activation of Mip expression in lens epithelia explants induced to differentiate in culture by FGF-2.

**DISCUSSION**

The activation and cross-talk between different signaling cascades control the regulation of gene expression during the differentiation process of lens epithelia into lens fibers. MIP/ aquaporin 0 is specifically expressed in lens fibers and its expression is repressed in lens epithelia. FGF-1 and FGF-2 promote cell proliferation and differentiation in cultured rat lens epithelia explants (19–22). How the two biological processes, epithelial cell proliferation and fiber cell differentiation, are regulated by the FGFs remain to be clarified. In this report, our goal was to elucidate whether FGF-2 regulates Mip expression in differentiating lens cells and additionally to delineate the signaling pathways that are associated with this regulation. We used the standardized procedure of the lens explant culture system to examine how FGF-2 could use some downstream pathways like the MAPK pathway that includes both ERK and JNK signaling to regulate Mip expression.

It has been reported that a specific inhibitor of ERK signaling blocks morphological changes, such as cell elongation and filensin expression in FGF-treated lens cells. However FGF-induced expression of β-crystallin is sustained (39). These results, suggestive of the interplay of two distinct pathways, ERK-dependent and -independent, raises the possibility that ERK activation is not the sole trigger of lens fiber cell differentiation and the associated expression of important biomarkers. Here, in this report we show that FGF-2 induces Mip expression in lens epithelia explants in a dose-dependent manner. The induction of Mip gene expression by FGF2 is at the transcriptional level; in addition, the Mip promoter −1648/+44 is highly responsive to FGF-2. Mip promoter activation was lent cell-specific and the endogenous Mip expression pattern in the explants followed an identical spiking with a high concentration of FGF-2. Like MIP, several crystallins proteins and transcription factors such as 1-Maf are undetected in proliferative lens epithelial cells, but are expressed in differentiated fiber cells although an activated form of ERK is found in the lens epithelial cells (40, 41).

To address the issue of how FGF-2 induces Mip expression, we focused our studies on the FGF/ERK activity. FGF-2 concentration-dependent activation of ERK1/2 was concomitant with the expression of Mip in the lens explants. Inhibiting the ERK activation by U0126 resulted in the downregulation of endogenous Mip expression in the lens explants. The ability of the Mip promoter to activate luciferase expression in response to FGF-2 was partially blocked by U0126. These results emphasize the importance of the FGF/ERK activity for Mip expression, but do not rule out the possibility of the contribution of additional pathways.

In the *in vivo* and *in vitro* lens cell systems, operating signal molecules, such as the ERK/MAP kinase, JAK/STAT, Smad, and PKC isoenzymes have been shown to be involved in cell proliferation and differentiation (42–47). A complex mechanism coordinating these two tightly regulated biological processes has been suggested. Cross-talk between the signal-transducing mediators activated by the growth factors is possible. Therefore, it was imperative to elucidate whether another major MAPK-related pathway (SAPK/JNK) besides the ERK pathway is involved in Mip expression in the lens explant cultures.

The biochemical evidence tracking the activated ERKs using immunohistochemical and immunoblot analyses are confirmed by reporter activity assays showing the regulation of *Mip* expression at the transcriptional level. The relevance of the JNK pathway, a parallel pathway in the MAPK signaling alongside ERK, is demonstrated in this report. FGF-2 in higher doses (100 ng/ml) was capable of inducing JNK activation up to 5-fold in the lens explants. Using similar assays for studying the ERK pathway, we demonstrate that JNK activity is required for *Mip* expression in the lens explants in culture under FGF-2 stimulation. A specific inhibitor to JNK activity, SP600125, totally abolished JNK activation by FGF and simultaneously attenuated *Mip* expression. This effect of FGF-2-mediated JNK activity was also confirmed at the transcriptional level when *Mip* mRNA was monitored by real time PCR. Reporter activity assay using an *Mip* promoter in the presence of SP600125 confirmed the importance of an intact JNK pathway in *Mip* promoter activity in response to FGF-2.

In the final stages of lens differentiation, nuclei and organelles are lost in a process resembling programmed cell death or apoptosis (48, 49). Cells may be susceptible to apoptosis if survival signaling mechanisms are down-regulated when ERK and JNK inhibitors were used. In our explant culture system, by using nuclear staining we did not observe nuclear condensation, and cell morphology was retained in the presence of the inhibitor. Therefore, the inhibition of *Mip* expression by these two inhibitors is not the result of apoptosis.

Our results indicate that the *Mip* promoter has responsive elements downstream of ERK and JNK pathways. It is possible that these two pathways, besides their ability to activate some common transcription factors, distinctly regulate specific transcription factors to activate the *Mip* promoter in the lens explants. We also demonstrate that ERK and JNK pathways are positive regulators of *Mip* transcription. Rather than being a
redundant presence, JNK signaling synergizes with the ERK pathway upon FGF-2 stimulation to bring about an efficient expression of Mip. Although how Mip expression is regulated by FGF-2 in vivo is still unclear, these experiments provide new insights into the signaling pathways regulating Mip expression in the lens fibers. Further studies are required to precisely delineate the transcription factors mediating the FGF/ERK and FGF/JNK signals and to identify the response elements in the Mip promoter.

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