The Stability of the Lens-specific Maf Protein is Regulated by Fibroblast Growth Factor (FGF)/ERK Signaling in Lens Fiber Differentiation*

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Haruki Ochi, Hajime Ogino†, Yuji Kageyama, and Kunio Yasuda‡

From the Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma 630-0101, Japan

Fibroblast growth factor (FGF) signaling is necessary for both proliferation and differentiation of lens cells. However, the molecular mechanisms by which FGFs exert their effects on the lens remain poorly understood. In this study, we show that FGF-2 repressed the expression of lens-specific genes at the proliferative phase in primary cultured lens cells. Using transfected cells, we also found that the activity of L-Maf, a lens differentiation factor, is repressed by FGF/ERK signaling. L-Maf is shown to be phosphorylated by ERK, and introduction of mutations into the ERK target sites on L-Maf promotes its stabilization. The stable L-Maf mutant protein promotes the differentiation of lens cells from neural retina cells. Taken together, these results indicate that FGF/ERK signaling negatively regulates the function of L-Maf in proliferative lens cells and that stabilization of the L-Maf protein is important for lens fiber differentiation.

The vertebrate lens consists of only two cell populations, and because of this simple tissue organization, it provides an excellent model of cell differentiation. The anterior surface of the lens is covered by a simple epithelium, whereas the remainder and bulk of the lens is composed of elongated lens fibers (1). During embryonic development and throughout the life of the organism, fiber cells are added via differentiation of the lens epithelial cells. The proliferative epithelial cells in the anterior germinative zone move across the lens equator to the posterior transitional zone followed by withdrawal of cell cycling and differentiation into fiber cells. Fiber cell differentiation is characterized by cell elongation and the eventual degradation of all cellular organelles including the nucleus. These morphological changes are accompanied by specific activation of crystallin proteins as well as intermediate filament components such as filensin and CP49 (reviewed in Ref. 2). Molecular genetic approaches have also recently identified a number of signaling molecules and transcription factors that are critical for lens cell development.

Fibroblast growth factors (FGFs) regulate a diverse range of biological activities including adhesion, migration, proliferation, and differentiation (reviewed in Ref. 3). FGF signaling is one of the most important regulators of differentiation from lens epithelial cells into fiber cells. Using lens explant cultures, it has been demonstrated that FGF-1 and FGF-2 can stimulate both proliferation of lens epithelial cells and differentiation of lens fiber cells (4, 5). In addition, in vivo gain-of-function studies have shown that misexpression of FGF-1, -4, -8, or -9 under the control of the lens-specific a-crystallin promoter causes lens abnormalities and the so-called microphthalmalism (small lens) phenotype (6, 8). These FGFs effectively induce cell cycle withdrawal, loss of the characteristic cuboidal morphology, elongation of the cell shape, and accumulation of β-crystallin, resulting in premature differentiation of lens epithelial cells into fiber cells. In addition, a truncated form of FGF receptor (FGFR) lacking the cytoplasmic kinase domain inhibits fiber cell differentiation when expressed in transgenic mice under the control of a lens-specific promoter (7), and a secreted form of FGFR can cause a delay in fiber differentiation (9). Together, these studies implicate FGFs as bifunctional molecules that regulate both proliferation and differentiation of the lens cells. The transcription factors that mediate FGF signaling in lens development and the molecular mechanisms used by FGFs to regulate the transcriptional factors remain to be identified.

Several transcription factors, including Pax6, Sox2, Six3, c-Maf, and L-Maf, are known to be important in lens development and in regulating the expression of lens-specific genes such as crystallin genes (10–17). Of these factors, L-Maf is one of the most attractive candidates for mediating FGF signaling, because L-Maf has been identified as a lens-specific regulator of avian a-crystallin expression (14), and c-maf-deficient mice have defective lens fiber differentiation and loss of crystallin gene expression (16, 17). There results show that Mafs are critical for expression of crystallin genes and lens fiber differentiation. L-Maf is a basic region/leucine zipper transcription factor and regulates the expression of aα-crystallin by binding the lens-specific enhancer element αCE2 (14, 18). In chicken, the expression of L-maf is initiated in the lens placode and is subsequently restricted to the developing lens (14, 19). In addition, ectopic expression of L-Maf induces the expression of several crystallin and filensin genes (14) and promotes the formation of lens-like structures called lentoid bodies in retinal primary cultures. Furthermore, a dominant-negative form of L-Maf inhibits crystallin gene expression and proper lens cell differentiation (20). Thus, L-Maf is clearly important for crystallin gene expression and for lens development.

To address the molecular mechanisms that determine how FGF signaling regulates transcription of the lens-specific genes...
in lens development, we investigated the effects of FGF signaling on the regulation of L-Maf function. Here we show that FGF-2 promotes proliferation of lens cells in the early stages of primary culture but represses expression of lens-specific genes at the same time points. In addition, FGF/ERK signaling represses activity of an L-Maf-dependent reporter in the lens cells. We also found that Thrombin-57 and Ser-65 of the L-Maf protein are important as phosphorylation sites for extracellular-regulated kinase (ERK) in vitro. Mutations at these sites reduce protein stability, and increased expression of an L-Maf protein, resulting in enhanced expression of δ-crystallin. These results indicate that FGF/ERK signaling controls terminal differentiation of the lens fiber cells through regulation of L-Maf stability.

**EXPERIMENTAL PROCEDURES**

**Lens Cell Culture**—Lens cell cultures were prepared as previously described (18). Lens cells from 14-day-old chick embryos were maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% chicken serum. When the cells reached confluence, they were harvested with trypsin and centrifuged to remove nonproliferating lens fiber cells. The cells were then cultured at a density of 1 × 10^6 cells per dish to establish primary cultures. Transfection with plasmids or treatment with FGF-2 (human fibroblast growth factor-2, PeproTech) were carried out 1 day after establishment of the primary cultures.

**Plasmid Construction**—Plasmids used to express FLAG epitope-tagged L-Maf (pEFX3-fg-L-Maf) constructs in lens cells have been described previously (14). pEFX-β-gal containing the human EF-1α promoter and β-galactosidase open reading frame inserts and pCAGGS-GFP carrying the cytomegalovirus enhancer, β-actin basal promoter, and green fluorescent protein open reading frame sequences were used as controls for normalization (14). Single amino acid mutations of the L-Maf protein were carried out by subcloning an Eag I–Eco RI end of the L-Maf protein, resulting in enhanced expression of δ-crystallin. The stability of the L-Maf was analyzed by incubating the cells for 3 h in the presence of proteasome inhibitor. The inhibitors used were L-LN (2-Leu-Leu-Leu-aldehyde, 20 μg/ml), BostonBiochem), MG132 (BostonBiochem, 50 μM), and clasto-lactacystin-β-lactone (lactactatin, BostonBiochem, 10 μM). After the treatment, cells were washed to remove inhibitors followed by further incubation in fresh Dulbecco's-modified Eagle's medium, 10% fetal bovine serum, 1% chicken serum for various periods. Then the whole cell extracts were subjected to Western blot analysis.

**Northern Blot Analysis**—Northern blot analyses were carried out as described previously (23). In brief, total RNA was isolated from the cultured lens cells, and equal amounts of RNA were separated on formaldehyde-agarose gels. After electrophoresis, RNA was blotted onto Biodyne A nylon membranes (Pall) and hybridized with digoxigenin-labeled DNA probes. mRNAs were detected with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Molecular Biochemicals) and visualized by reaction with the chemiluminescent substrate, diastar lactose, 1,2-dioxetane 2.2' (5-chlorotriazole[3,1-b:3',1'-b']diazirine) (Applied Biosystems). The reaction mixture was then added to a 4% polyacrylamide gel. The gel was transferred to DEAE paper, dried, and subjected to autoradiography.

**RESULTS**

**FGF-2 Stimulates Lens Epithelial Cell Proliferation but Represses the Expression of Lens-Specific Genes**—Previous studies had shown that FGF-1 and FGF-2 promote proliferation and differentiation of lens epithelial cells in explant cultures (4, 24). However, the molecular mechanisms underlying FGF signaling in lens development are yet to be elucidated. To address these mechanisms, we used primary cultures enriched for proliferating lens epithelial cells. Initially, we aimed to confirm whether FGF-2 regulates both proliferation and differentiation of the lens cells. Cell counts were performed at different times during culture with or without FGF-2 treatment. The number of FGF-2-treated lens cells was greatly increased on days 1 and 2 compared with the untreated control cells (Fig. 1A). However, by days 3–6, neither the FGF-2-treated nor untreated lens cells showed significant proliferation. After 6 days of culture, we found abundant and large lentioid bodies in the FGF-2-treated lens cell cultures (Fig. 1B). Lentoid bodies are clusters of dif-
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FGF-2 treatment of primary cultured lens cells. A, primary cultured lens cells were plated at a density of 1 × 10⁵ cells/30-mm dish. One day after plating, FGF-2 was added (100 ng/ml) and retained in the medium during further culturing. At various stages of culture, cells were counted. B, after 6 days of culture, the cells were observed under phase contrast microscopy (b, c) and immunostained with anti-β-crystallin antibody (b’, c’). FGF-untreated (b, b’) and -treated (c, c’) lens cells differentiated into lens fiber cells and formed β-crystallin-positive lentoid bodies (arrowheads). C, primary cultured lens cells were cultured for the indicated periods of time in the presence or absence of FGF-2 (100 ng/ml). Equal amounts of total RNA were subjected to Northern blot analysis to examine the expression of lens-specific genes. The expression was repressed in FGF-2-treated lens cells at 1–2 days of culture.

The use of FGF-2 promotes both proliferation and differentiation of primary cultured lens cells, which is consistent with previous results using lens explants (4, 24).

Next we examined the effect of FGF-2 on the expression of marker genes for lens fiber differentiation, crystallin genes and filensin gene. Primary lens cells were incubated with or without FGF-2, and then total RNA was isolated and subjected to Northern blot analysis. After day 3, expression of both crystallin and filensin genes was enhanced by the addition of FGF-2 (Fig. 1C). Surprisingly, FGF-2 efficiently reduced the expression of Δ-, αA-, βB1-crystallin, and filensin genes at earlier culture points (Fig. 1C, days 1–2), which coincided with the period of lens cell proliferation (Fig. 1A). These results suggest that FGF-2 represses lens-specific genes at early stages of culture when the lens epithelial cells are proliferating, whereas the same growth factor promotes lens fiber differentiation to augment crystallin gene expression and lentoid body formation at later stages of cell culture.

FGF Represses L-Maf-mediated Reporter Activity—On the basis of FGF-2 treatment, we then proposed that transcription factors regulating the expression of crystallins should function under the control of FGF-2. One of the attractive candidates for this regulation is lens-specific Maf (L-Maf), which binds to chicken αA-crystallin enhancer (αCE2) and regulates expression of the αα-, Δ-, βB1-crystallin, and filensin genes (14, 18). In addition, a dominant-negative form of L-Maf that lacks its acidic domain (DN-L-Maf (20)) repressed expression of the αα-, Δ-, βB1-crystallin, and filensin genes in primary cultured lens cells (Fig. 2A) and chick embryos (20), showing that L-Maf is essential for the expression of the lens-specific genes. To assess the possibility that FGF signaling regulates the L-maf gene, we first examined the effect of FGF-2 on expression of L-maf mRNA. Lens cell primary cultures were incubated for 48 h with or without FGF-2, and then total RNA was isolated and subjected to Northern blot analysis. L-maf mRNA levels in the FGF-2-treated cells were comparable to those in the untreated control cells (Fig. 2B), indicating that FGF-2 does not significantly affect transcription of the L-maf gene.

We next examined the effect of FGF-2 on regulation of L-Maf at the protein level. Since we could not detect L-Maf protein by Western blot analysis (data not shown), we measured the transcriptional activity of L-Maf protein in primary cultured lens cells using a reporter gene construct containing six copies of a 25-bp L-Maf binding site in αCE2, β-actin basal promoter, and the luciferase coding sequence (cαALuc, Fig. 2C). This construct was transiently transfected into the lens cells, which were incubated with or without FGF-2 for 48 h. Luciferase activities provided a measure of transcriptional activity. FGF-2 clearly repressed the expression of the reporter gene (Fig. 2C), an effect that was dose-dependent (Fig. 2D, left panel). This repression was not due to a general effect on transcription because a luciferase reporter driven by either a thymidine kinase promoter or a β-actin basal promoter alone was not affected by the addition of FGF-2 (tkLuc and βLuc, Fig. 2C). We also examined the effect of a specific FGF receptor inhibitor, SU5402 (25). The reporter activity significantly increased in the presence of SU5402 (Fig. 2D, right panel), indicating that inhibition of FGF signaling promotes expression of the reporter gene. Taken together, these results indicate that FGF signaling inhibits L-Maf-dependent transcription in the primary cultured lens cell, resulting in repression of the lens-specific genes at the early culture stage.

MEKI Represses L-Maf-mediated Reporter Activity—ERK1 and ERK2, members of the mitogen-activated protein kinase (MAPK) family, play major roles in a variety of developmental processes and biological actions induced by FGF signaling (26, 27). Because activated ERK is detected in lens in vivo (28), FGF-2 may regulate L-Maf activity through ERK. To test this possibility, we first examined whether FGF signaling involves the ERK pathway in primary cultured lens cells. Because serum is a potent activator of the MAPK cascade, lens cells were serum-starved for 4 h followed by FGF-2 treatment for 1 h. In the whole cell extracts of unstimulated cultured lens cells,
A, PD98059, a specific inhibitor of MEK1, the ERK kinase (Fig. 2A), caused a decrease in expression of the reporter gene (Fig. 3A), lane 3). These results demonstrate that FGF-2 promotes phosphorylation of ERK through MEK1 in lens cells. We also confirmed that the constitutively active MEK1 inhibitor PD98059 promoted phosphorylation of MEK1/ERK in living cells, we introduced an expression plasmid encoding MEK1 into the COS-7 monkey kidney cell line. Ectopically expressed MEK1 protein was detected as a 42-kDa band in transfected lens cells (Fig. 2A). This phosphorylation of MEK1 was clearly inhibited by addition of PD98059, a specific inhibitor of MEK1, the ERK kinase (Fig. 2A, lane 2). These results demonstrate that FGF-2 promotes phosphorylation of ERK through MEK1 in lens cells.

We next examined the effect of constitutively active MEK1 on the activity of L-Maf in the primary cultured lens cells using the cαALuc reporter (Fig. 3B, left). Introduction of the constitutively active MEK1 caused a decrease in expression of the reporter gene. In contrast, MEK1 inhibitor PD98059 promoted expression of the reporter gene (Fig. 3B), indicating that L-Maf activity in lens cells is regulated through the MEK1/ERK pathway. We also confirmed that the constitutively active MEK1 phosphorylated ERK was detected only as a weak band by Western blot analysis with an anti-phospho-ERK antibody (Fig. 3A, lane 1). In contrast, phosphorylation of ERK was increased in the FGF-2-treated cells (Fig. 3A, lane 2). This phosphorylation of ERK was clearly inhibited by addition of PD98059, a specific inhibitor of MEK1, the ERK kinase (Fig. 3A, lane 3). These results demonstrate that FGF-2 promotes phosphorylation of ERK through MEK1 in lens cells.

Could activate Elk considerably, an ERK-regulated transcription factor, using the GAL4-Elk1 and UAS6 luciferase reporter gene assay (Fig. 3B, right), indicating that MEK1 is active in the cultured lens cells and the inhibitory effect of MEK1 is not ubiquitous to all transcription. These results indicate that MEK1/ERK mediates FGF-2 signaling to repress L-Maf-dependent transcription.

Thr-57 and Ser-65 Are Important Amino Acids of the L-Maf Protein for the Phosphorylation by ERK in Vitro—We identified three putative MAPK target sites in the L-Maf acidic region. To test whether L-Maf is directly phosphorylated by ERK, we first performed in vitro phosphorylation analysis. When incubated with purified ERK, recombinant L-Maf protein was clearly phosphorylated (Fig. 4A). To examine phosphorylation of L-Maf in living cells, we introduced an expression plasmid encoding wild-type L-Maf into the COS-7 monkey kidney cell line. Ectopically expressed L-Maf protein was detected as a 42-kDa band in transfected lens cells (Fig. 2A). A 34-kDa band, which is approximately the same molecular size as the in vitro translated L-Maf protein, appeared when the cell lysate was treated with calf intestine alkaline phosphatase, indicating that L-Maf is phosphorylated in living cells. To identify the ERK phosphorylation site, a series of L-Maf mutant proteins was made with substitutions to alanine residues at the three potential sites of Ser-14, Thr-57, or Ser-65 (designated as S14A, T57A, and S65A, respectively).
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Fig. 4. Residues Thr-57 and Ser-65 of the L-Maf protein are important for phosphorylation by ERK in vitro. A, GST-L-Maf proteins were incubated with purified ERK (New England Biolabs) and [γ-32P]ATP at 30 °C for 3 h followed by autoradiography or Western blot analysis with anti-GST antibody. B, mutation of amino acid residues in the S14A, T57A, and S65A proteins is shown schematically. Wild-type (WT) or mutant L-Maf protein fused to GST was incubated with purified ERK followed by autoradiography or Western blot analysis with anti-GST antibody. C, COS-7 cells were transfected with pEFX-fg-L-maf. After 48 h of transfection, nuclear proteins were isolated and incubated with calf intestinal phosphatase followed by Western blot analysis (WB) with anti-FLAG antibody.

respectively). The wild-type and mutant proteins were subjected to in vitro phosphorylation. Incorporation of [γ-32P]ATP was detected with the wild-type L-Maf protein and mutant S14A, suggesting that Ser-14 is not involved in phosphorylation by ERK (Fig. 4B). In contrast, T57A and S65A mutants showed a significantly reduced phosphorylation signal. These results implicate Thr-57 and Ser-65 as important in the phosphorylation of L-Maf by ERK.

L-Maf Activity Is Regulated by Phosphorylation at Thr-57 and Ser-65—We next evaluated the effects of mutating the phosphorylation sites on the transcriptional activation of L-Maf. The mutation on Ser-14 did not significantly affect the transcriptional activity of L-Maf (Fig. 5A), whereas the T57A and S65A L-Maf mutants showed ~5- and 7-fold higher levels of reporter activity than the wild-type protein, respectively. We also examined the importance of Thr-57 and Ser-65 for the ability of L-Maf to induce the expression of its in vivo target gene, δ-crystallin. When either T57A or S65A mutant L-Maf was introduced into primary cultured retinal cells, expression of endogenous δ-crystallin was significantly induced (Fig. 5C), and the number of δ-crystallin-positive cells increased 3-4-fold compared with wild-type L-Maf (Fig. 5C, Table I). To confirm that these mutational effects involve MEK1/ERK signaling, constitutively active MEK1 was cotransfected into cultured lens cells together with either wild-type or S65A mutant L-Maf. The transcriptional activity of endogenous L-Maf and overexpressed wild-type L-Maf were decreased by the co-expression of constitutively active MEK1 (Fig. 5B), whereas S65A mutant L-Maf was not sensitive to MEK1 signaling. Taken together, these results indicate that the ERK phosphorylation sites at Thr-57 and Ser-65 are important for the transcriptional activity of L-Maf and that phosphorylation at these sites negatively regulates L-Maf function in lens cells.

Unphosphorylated L-Maf Protein Is More Stable in Lens Cells Compared with Wild-type L-Maf—To elucidate how L-Maf function is regulated by phosphorylation, we measured the DNA binding activity of phosphorylated or unphosphorylated L-Maf protein by an electrophoretic mobility shift assay using the L-Maf response element (cMaf) that is in cα-crystallin enhancer as a probe (Fig. 6A). The wild-type L-Maf protein phosphorylated by ERK showed DNA binding activity similar to that of unphosphorylated L-Maf protein (Fig. 6A). We also confirmed that mutant L-Maf proteins could bind to the L-Maf response element (data not shown), indicating that the binding activity of L-Maf is not significantly affected by ERK phosphorylation.

We next examined the amounts of wild-type and mutant L-Maf proteins expressed in primary cultured lens cells. Although the mutant L-maf mRNA levels were equivalent to that of wild-type L-maf (Fig. 6B), both the T57A and S65A mutant L-Maf proteins accumulated in the cultured lens cells (Fig. 6B, lanes 4 and 5), but the wild-type L-Maf and S14A mutant protein did not (Fig. 6B, lanes 2 and 3). In addition, when proteasome-mediated degradation was blocked by MG132,
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Fig. 6. Stability of the L-Maf protein is regulated by phosphorylation. A, GST-L-Maf proteins were incubated in the presence or absence of purified ERK and subjected subsequently to electrophoresis mobility shift assay. B, primary cultured lens cells were transfected with either wild-type (WT) or mutant L-Maf expression plasmids. After 48 h of transfection, nuclear extracts were isolated, and equal amounts were subjected to Western blot analysis using anti-L-Maf antibodies. Northern blot analysis showed that expression levels of wild-type and mutant L-Maf mRNA were not affected. Green fluorescent protein (GFP) was used as a control of transfection. C, pEFX-L-Maf was transfected into lens cells, and the cells were incubated for 3 h in the presence of proteasome inhibitors, LLnL (20 μg/ml), MG132 (50 μM), or lactacystin (20 μM). Nuclear extracts were prepared and subjected to Western blot analysis with anti-L-Maf antibodies. DMSO, dimethyl sulfoxide. D, wild-type or mutant L-Maf expression plasmid was transfected into lens cells. The cells were treated for 3 h with the proteasome inhibitor LLnL (20 μg/ml), washed to remove the inhibitor, and incubated further for various periods. Nuclear extracts were prepared and subjected to Western blot analysis with anti-L-Maf antibodies. E, pEFX-L-Maf was transfected into lens cells, and the cells were incubated for 48 h in the presence of PD98059 (100 μM). L-Maf expression was analyzed by immunostaining.

LLnL, or lactacystin, ectopically expressed L-Maf protein accumulated in the cultured lens cells (Fig. 6C). We also compared the stability of the wild-type and S65A mutant L-Maf proteins in cultured lens cells. Because wild-type L-Maf protein is unstable in cultured lens cells, we used LLnL to prevent its degradation. Cells were incubated in the presence of LLnL for 3 h, washed to remove the inhibitor and further incubated for various periods. The wild-type L-Maf protein almost completely disappeared, even at 2 h (Fig. 6D), whereas the S65A mutant L-Maf protein was detected consistently for at least 3 h (Fig. 6D), indicating that the S65A mutant L-Maf protein is more stable than the wild type.

To test the importance of MAPK signaling in this stability in cultured lens cells, we examined the effect of the MEK1 inhibitor PD98059 on degradation of the L-Maf protein. L-Maf protein was not detected in untreated control lens cells but was detected in the PD98059-treated cells (Fig. 6E), indicating that MEK1 signaling regulates stability of the L-Maf protein. Taken together, these results indicate that MEK1/ERK signaling regulates stability of the L-Maf protein through Thr-57 or Ser-65 phosphorylation, and degradation of the L-Maf protein is dependent on the proteasome pathway.

L-Maf Protein Accumulates in Differentiated Fiber Cells—To further investigate the connection between L-Maf protein and ERK signaling, we examined expression patterns of the L-Maf protein and an active form of ERK in in vitro differentiating cultured lens cells. Previous studies have shown that primary cultured lens cells differentiate into lens fiber cells and form lens-like bodies (lentoid bodies) (29, 30), which appear to contain a few different cell types (30). Relatively large fiber cells in the central region of the lentoid bodies are surrounded by small cells, suggesting that the central cells are differentiated into fiber cells, whereas the edge cells remain undifferentiated. Double-immunostaining showed that L-Maf protein was expressed only in differentiated fiber cells that expressed δ-crystallin but not in epithelial cells (Fig. 7, A–C). In contrast, high resolution confocal microscopy revealed that phosphorylated ERK is found only at the edges of lentoid bodies but not in the central regions (Fig. 7, D and E). These results indicate that accumulation of L-Maf protein is involved in lens fiber differentiation and the stability of L-Maf protein is negatively regulated by an active form of ERK in the undifferentiated lens epithelial cells.

DISCUSSION

Previous studies showed that FGF-1 and FGF-2 promote lens cell proliferation and lens fiber differentiation in explant cultures (4, 31). Whether these two effects are achieved via identical or distinct mechanisms remains to be clarified. Morphological changes induced by FGF in fiber differentiation, such as cell elongation, are blocked by a specific inhibitor against ERK signaling in explant culture but FGF-induced β-crystallin expression is not (28). This suggests that FGFs exert their influences through at least two distinct (e.g. ERK-dependent and -independent) molecular mechanisms. This study shows that after 1–2 days of FGF treatment, the proliferation of lens cells is significantly enhanced, but the expression of lens-specific genes is repressed in primary cell cultures. Furthermore, during the later period of culture, expression of lens-specific genes is enhanced, and lentoid body formation and fiber cell elongation are promoted. Together, these results suggest that FGF-2 has a binary function that is essential for both the “early” events in which the lens cells proliferate and the “late” events in which differentiation of the cells proceed.

To address the issue of how FGF-2 represses lens differentiation in the early stages of culture, we focused on L-Maf, a protein that regulates expression of crystallin genes and differentiation of the lens fiber cells (14, 16, 17). By transcription assays using primary lens cell cultures, we showed that L-Maf-dependent reporter gene expression is repressed by FGF-2 treatment but is activated by the addition of an FGFR inhibitor, SU5402. In addition, ERK can phosphorylate L-Maf in vitro, and mutation at the ERK phosphorylation sites has a drastic effect on the activation of the L-Maf-dependent reporter gene in primary cultured lens cells, probably due to accumulation of the mutant L-Maf proteins. We also found that the mutant L-Maf proteins can induce lens fiber differentiation in primary cultured neural retina cells. These results demonstrate that L-Maf is negatively regulated by FGF-2 through phosphorylation by ERK at Thr-57 and Ser-65, resulting in...
degradation of the L-Maf protein. Consistent with this idea, L-Maf protein is undetected in proliferative lens epithelial cells but expressed in differentiated fiber cells in primary cultured lens cells, as an activated form of ERK is found in the lens epithelial cells (28).

Several transcription factors are regulated by phosphorylation not only in regard to degradation but also their transcriptional activity. Microphthalmia (Mi) is an essential transcription factor for melanocyte development, which is phosphorylated by MAPK/ERK. This phosphorylation triggers not only enhanced transcriptional activity of the microphthalmia protein but also its degradation (32). Because we normalized the luciferase activities against activities of the control tk reporter gene in the transcription assays but not against amounts of the wild-type or mutant L-Maf proteins, our results do not indicate whether L-Maf phosphorylation also affects its transcriptional activity. Several lines of evidence do, however, imply that phosphorylated L-Maf is transcriptionally more active. First, Benkhelifa and colleagues (33) recently reported that MafA, an L-Maf homolog in quail, is phosphorylated by ERK at Ser-14 and Ser-65 and that this phosphorylation is required for expression of QR1, a secreted glycoprotein in neural retina cells. Secondly, FGF-2 stimulates expression of lens-specific genes and characteristic morphological changes of lens cells during the later phases of primary culture; these FGF stimulative effects are also exerted by overexpression of L-Maf in primary neural retina cultures (this study and Ref. 14), implying that FGF-2 stimulates L-Maf activity. Therefore, FGF-2 regulates degradation of L-Maf protein during the early phase, and once L-Maf is stabilized at a later phase (discussed below), FGF-2 regulates transcriptional activity through phosphorylation by ERK at the same sites, Thr-57 and Ser-65.

On the basis of our present study using primary cultured lens cells, we propose that the stabilization of L-Maf protein is an important mechanism for the differentiation of lens epithelial cells into lens fiber cells. FGF-2 promotes proliferation of lens cells and represses the expression of crystallin genes when lens cells proliferate. During this period, L-Maf is negatively regulated by MEK1/ERK signaling under the control of FGF-2, and repression of the L-Maf could prevent the lens-specific genes from precocious activation. In fact, a bromodeoxyuridine incorporation assay using primary lens cell cultures shows that proliferative lens epithelial cells did not express the L-Maf protein (data not shown), indicating that L-Maf protein is unstable in proliferative cells. Once proliferating lens epithelial cells begin to differentiate, they cease cell cycling and initiate the late lens fiber differentiation events including enhanced expression of lens-specific genes, such as crystallins, and elongation of cell shape. Because L-Maf can activate lens-specific genes in chick embryos (14) and promote lentoid body formation and elongation of primary cultured neural retina cells (this study and Ref. 14), it is probable that L-Maf is critical for this late event. Using frozen sections, we examined L-Maf accumulation in the transitional zone in which cells elongate and undergo the earliest morphological changes associated with lens fiber differentiation. Reciprocally, phosphorylated ERK is expressed throughout the proliferative population of epithelial cells in neonatal rat lens, extending into the transitional zone (28). Although it is known that several transcriptional factors such as Pax6, Sox2, and Six3 also regulate the expression of crystallin gene (13, 34, 35), L-Maf is the first example of a differentiation factor that can be regulated directly by FGF signaling in lens development.

Because FGF-2 is expressed widely in both lens epithelial cells and fiber cells (1), there should be a certain mechanism that interrupts FGF-2 signaling and prevents the L-Maf protein from degradation. McAvoy and colleagues (1, 36) have reported that FGF receptors exhibit distinct but overlapping expression patterns throughout lens development. Therefore, the spatiotemporal pattern of the FGFRs may be important in stabilizing the L-Maf protein. Another possibility is that an antagonizing factor may function to block FGF-2 signaling and degradation of the L-Maf protein. Sprouty2 is a potential candidate; it is expressed in lens fiber rather than in lens epithelium during chick development (37). Sprouty2 is induced by ectopic FGF8 protein within the neural tube (38), suggesting that Sprouty2 can function in a negative feedback mechanism with FGF signaling. Therefore, Sprouty2 may respond to FGF-2 signaling and block protein degradation of L-Maf during differentiation of the lens fiber. Finally, we also signal the possibility of a decline in the proteasome-mediated pathway. Generally, target selection of proteasome-mediated protein degradation is ruled by E3 ubiquitin ligases, and each E3 enzyme has a specific target protein (39, 40). Recent studies have shed some light on the connection between ubiquitin-mediated protein degradation and development (reviewed in Ref. 41) (42). Because our results suggest that degradation of the L-Maf protein is mediated by the proteasome pathway, the ubiquitin E3 ligase specific to L-Maf may be repressed in an

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*a* H. Ochi and K. Yasuda, unpublished observations.
Fgf-dependent or -independent manner. Further studies are needed to elucidate the molecular mechanisms underlying the stabilization of the L-Maf protein.

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