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Differential Regulation of Components of the Ubiquitin-Proteasome Pathway during Lens Cell Differentiation

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Abstract

Purpose—To investigate the role for the ubiquitin-proteasome pathway in controlling lens cell proliferation and differentiation and the regulation of the ubiquitin conjugation machinery during the differentiation process.

Methods—bFGF-induced lens cell proliferation and differentiation was monitored in rat lens epithelial explants by bromodeoxyuridine (BrdU) incorporation and expression of crystallins and other differentiation markers. Levels of typical substrates for the ubiquitin-proteasome pathway, p21^{WAF} and p27^{Kip}, were monitored during the differentiation process, as were levels and activities of the enzymes involved in ubiquitin conjugation.

Results—Explants treated with bFGF initially underwent enhanced proliferation as indicated by BrdU incorporation. Then they withdrew from the cell cycle as indicated by diminished BrdU incorporation and accumulation of p21^{WAF} and p27^{Kip}. bFGF-induced cell proliferation was prohibited or delayed by proteasome inhibitors. Lens epithelial explants treated with bFGF for 7 days displayed characteristics of lens fibers, including expression of large quantities of crystallins. Whereas levels of E1 remained constant during the differentiation process, the levels of ubiquitin-conjugating enzyme (Ubc)-1 increased approximately twofold, and the thiol ester form of Ubc1 increased approximately threefold on 7 days of bFGF treatment. Levels of Ubc2 increased moderately on bFGF treatment, and most of the Ubc2 was found in the thiol ester form. Although levels of total Ubc3 and -7 remained unchanged, the proportions of Ubc3 and -7 in the thiol ester form were significantly higher in the bFGF-treated explants. Levels of Ubc4/5 and -9 also increased significantly on treatment with bFGF, and more than 90% of Ubc9 was found in the thiol ester form in the bFGF-treated explants. In contrast, levels of Cul1, the backbone of the SCF type of E3s, decreased 50% to 70% in bFGF-treated explants.

Conclusions—The data show that proteolysis through the ubiquitin-proteasome pathway is required for bFGF-induced lens cell proliferation and differentiation. Various components of the ubiquitin-proteasome pathway are differentially regulated during lens cell differentiation. The downregulation of Cul1 appears to contribute to the accumulation of p21^{WAF} and p27^{Kip}, which play an important role in establishing a differentiated phenotype.

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The lens is composed of epithelial cells and fiber cells. A single layer of epithelial cells covers the anterior surface of the lens and fiber cells occupy the rest of the volume of the lens. The fiber cells form on differentiation of the epithelial cells. During differentiation, lens epithelial cells exit the cell cycle¹ and undergo significant morphologic and biochemical changes that result in the formation of fully differentiated fiber cells, where virtually all organelles, including the nuclei, are removed. This unique pattern of differentiation occurs in the equatorial region of the lens, and fibers with increasingly advanced stages of differentiation accumulate concentrically at the interior of the lens.² Proper execution of the differentiation program and formation of mature fibers seem to be required for lens transparency, in that abnormalities that result in incomplete degradation of intracellular organelles are associated with various forms of cataract.^{3,4}

The ubiquitin-proteasome pathway (UPP) is a major cytosolic proteolytic pathway in most eukaryotic cells. There are two stages in the UPP: substrate-recognition by covalent ligation of ubiquitin to substrate proteins to form ubiquitin-protein conjugates and the subsequent degradation of the ubiquitin conjugates by the 26S proteasome.⁵ Ubiquitin-protein conjugates are formed by sequential actions of a series of enzymes. To initiate the UPP, ubiquitin is first activated by the formation of a high-energy thiol ester with ubiquitin-activating enzyme (E1). The ubiquitin is then transferred to one of many ubiquitin-conjugating enzymes (Ubc1 or E2s), also by formation of a thiol ester. Subsequently, ubiquitin is transferred directly to substrates or is transferred to substrates after reaction with one of several ubiquitin ligases (E3s). Multiple isoforms of E2s and E3s have been identified in each species. The multiplicity of E2 and E3 enzymes is responsible for the substrate specificity of the UPP. Usually, multiple molecules of ubiquitin attach to substrate proteins to form ubiquitin chains. Thus, most ubiquitin conjugates attain high masses. These ubiquitin conjugates are either recognized and degraded by the 26S proteasome or deconjugated by isopeptidases.

In prior studies, we demonstrated that lens epithelial cells have a fully functional UPP^{6–13} and the ubiquitin conjugating activity and proteolytic activity in lens epithelial cells are up-regulated during recovery from oxidative stress.^{10,13} We have also demonstrated that ubiquitin conjugation activity increased during early stages of lens fiber cell differentiation.¹¹ Because the UPP is involved in regulation of cell proliferation in other types of eukaryotic cells, we hypothesized that the UPP plays a role in controlling lens cell proliferation, establishing the differentiation phenotype, and executing the dramatic morphologic remodeling during lens cell differentiation. In this study, we tested the hypothesis by determining the effects of proteasome inhibition on the bFGF-induced lens cell proliferation and differentiation in rat lens explants. Levels and activities of enzymes involved in the ubiquitination process were also determined. We found that bFGF-induced proliferation could be prohibited or delayed by proteasome inhibitors and that levels of p21^{WAF} and p27^{Kip}, the typical substrates for the UPP, accumulated on differentiation. Protein levels of several E2s, including Ubc1 and -4/5, were upregulated during lens cell differentiation. Although the total levels of Ubc2, -3, and -7 either increased only moderately or decreased, the proportion of these E2s in the activated thiol ester form increased dramatically. In addition, Ubc9, the E2 involved in conjugation of small ubiquitin-like modifier (SUMO), also increased during lens cell differentiation. In contrast, levels of Cul1, the backbone of the Skp1–cullin–F-box (SCF) type of E3s, decreased on bFGF-induced differentiation. These data indicate that the UPP is required for lens cell proliferation and differentiation and that the expression of components of the ubiquitin conjugation machinery are differentially regulated during lens cell differentiation. This regulation is consistent with a role for ubiquitination in the temporal and spatial control of cell proliferation and cellular remodeling during lens differentiation.

Materials and Methods

Materials

The rabbit polyclonal anti-E1 and anti-Ubc1 antibodies were produced in this laboratory, as described previously.^{11,14} Rabbit polyclonal anti-Ubc2 antibodies were a kind gift from Simon Wing. Rabbit polyclonal antibodies to Ubc3, -4/5, -7, and -9, and *clasto*-lactacystin β -lactone were purchased from Boston Biochem (Cambridge, MA). Rabbit poly-clonal antibodies to Cul1 and p21^{WAF} and mouse monoclonal antibody to p27^{Kip} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal antibody to β -actin was purchased from Sigma-Aldrich (St. Louis, MO). If not otherwise specified, all chemicals were purchased from Sigma-Aldrich or Bio-Rad (Hercules, CA).

Preparation of Lens Epithelial Explants

All procedures involving the use of animals were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee (ACUC) of this Center. Four- or 5-day old Wistar rats were killed, and the lenses were dissected. The lens capsule together with the attached epithelial cells was peeled off the lens and pinned onto tissue culture dishes, as previously described.^{15–17} The lens epithelial explants were cultured with medium 199 (Gibco, Grand Island, NY), supplemented with 0.1% bovine serum albumin (BSA), 100 IU/mL penicillin, 100 μ g/mL streptomycin, 2.5 μ g/mL amphotericin B, and 25 mM HEPES at 37°C in 5% CO₂. To induce differentiation, bFGF (PeproTech, Rocky Hill, NJ) was added to the culture medium at a final concentration of 100 ng/mL. To investigate the effect of proteasome inhibitor on proliferation and differentiation of rat lens epithelial cells, *clasto*-lactacystin β -lactone was added to the culture medium at a final concentration of 10 μ M. Explants were incubated in this medium at 37°C for 3 to 21 days, and the medium was changed every 3 days.

To determine DNA synthesis, 100 μ M bromodeoxyuridine (BrdU) was added to the medium for 2 hours, and the explants were fixed in 10% neutral-buffered formalin for 1 hour and stored at 4°C in 70% ethanol. The explants were rinsed with PBS, pre-embedded in 3% agar, dehydrated through a series of ethanol gradients, and washed with xylene before being embedded in paraffin.¹⁸ The embedded explants were sectioned at 5 μ m, dewaxed, hydrated, and stained with hematoxylin and eosin. For detecting BrdU incorporation, the sections were incubated in 50% formamide and 2 $\times$ SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 2 hours. After the sections were washed with 2 $\times$ SSC and subsequent incubation in 2 M HCl for 30 minutes at 37°C, they were washed once with 0.1 M borate buffer (pH 8.5) for 10 minutes. The sections were blocked with 10% horse serum for 1 hour and incubated with monoclonal anti-BrdU antibody in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl [pH 7.4]) at 4°C overnight. After rinses in TBS, the sections were incubated with biotinylated horse anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) for 4 hours at room temperature. Avidin-biotin complex (ABC) reagent (Elite; Vector Laboratories, Burlingame, CA) was applied to the sections, and the sections were incubated for 1 hour. Diaminobenzidine was applied as a substrate for the peroxidase reaction for 5 minutes at a concentration of 0.25 mg/mL in TBS with 0.01% hydrogen peroxide and 0.04% nickel chloride. Sections were then thoroughly washed and mounted with coverslips for examination.

SDS-PAGE and Western Blot Analysis

Explants to be used for SDS-PAGE and Western blot analysis were rinsed once with cold PBS and lens proteins were extracted in SDS-gel loading buffer without β -mercaptoethanol to preserve thiol esters of ubiquitin-conjugating enzymes and ubiquitin. A fraction of the prepared samples were treated with dithiothreitol (DTT) to break the disulfide bonds and thiol ester bonds.

The expression of crystallins in explant extracts was measured by Coomassie brilliant blue staining after SDS-PAGE. Levels of E1, E2, E3 and some of the UPP substrates were determined by Western blot analysis using the respective antibodies stated earlier. In brief, the sample was electrophoresed through a 12% SDS-PAGE gel and then transferred to a nitrocellulose membrane. The membrane was blocked with TST (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.02% Treen-20) containing 2.5% milk proteins before overnight incubation at 4°C with the primary antibodies. The membrane was then washed four times with TST and incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature. Immunocomplexes were visualized by incubating the membrane with detection reagents (Super Signal; Pierce, Rockford, IL) and exposed to x-ray film. The autoradiograms were scanned with an imaging densitometer (Amersham Biosciences, Sunnyvale, CA) and evaluated by an image analyzer (Image-Quant software, ver. 3.3; Amersham Biosciences).

Results

Ubiquitin Proteasome–Dependent Proteolysis in bFGF-Induced Rat Lens Epithelial Cell Differentiation

Levels of many regulators of cell proliferation and differentiation are controlled by UPP-mediated degradation.^{19–21} However, there are no studies that document a role for the UPP in regulating lens cell proliferation and differentiation. To explore the relation between the function of the UPP and lens cell proliferation and differentiation, we used a lens explant model. The faithful recapitulation of differentiation in bFGF-treated rat lens explants is shown in Figure 1. bFGF promotes cell proliferation initially, as indicated by the enhanced BrdU staining (yellow-brown nuclei) and formation of multiple layers of cells on the explant (Fig. 1, compare 1B with 1A). Mitotic indexes of the explants were summarized in Table 1. Whereas only approximately 3.6% of nuclei were BrdU positive in explants cultured in the absence of bFGF for 3 days, treatment of the explants with bFGF for 3 days increased the proportion of BrdU-positive nuclei to approximately 21%. Elongation and enlargement of the cells were also observed after 3 days of bFGF treatment (Fig. 1B vs. 1A), and even more so at 7 days (Fig. 1E). But, by 7 days of bFGF-treatment, proliferation was no longer observed, as indicated by the diminished BrdU incorporation (approximately 1% of the nuclei were BrdU positive). These data show that bFGF exerts a proliferative effect initially and then results in withdrawal from the cell cycle. The withdrawal from cell cycle is thought to be required for the cells to transit into the differentiated phenotype.

Simultaneous treatment with *clasto*-lactacystin β -lactone, an inhibitor of ubiquitin dependent proteolysis,²² diminished the bFGF-induced proliferation and differentiation, as indicated by the nearly 73% reduction in BrdU incorporation at day 3 (Table 1, Fig. 1, compare 1C with 1B). Proteasome inhibitor had little effect on BrdU incorporation in explants not treated with bFGF (Table 1), which showed only basal levels of proliferation. Similarly, multilayering of cells, an early marker of differentiation, was also found to be limited in explants treated with proteasome inhibitor (Fig. 1, compare 1C and 1F with 1B and 1E, respectively). These data clearly indicate that UPP-dependent processes are necessary for the rapid proliferation and differentiation that is observed in bFGF-treated explants. Additional mechanisms may be involved in regulating basal levels of proliferation such as is observed in bFGF-untreated lens explants.

The bFGF-induced differentiation was corroborated by increased expression of crystallins, the major gene products of lens fiber cells. In explants not treated with bFGF for 3 days, there was a small amount of crystallins (Fig. 2, lane 1), and the ratio of crystallins (20–33 kDa, Crys) to cytoskeleton proteins (45–60 kDa, Cyto) was approximately 4. Western blot analysis confirmed that there was a small amount of α - and β -crystallins in explants not treated with bFGF (data not shown). This small amount of crystallins in the untreated explants appeared to

come from the equatorial epithelium, which is attached to the explant and where differentiation in vivo was in progress before the explants were obtained. In explants treated with bFGF for 3 days, the amount of crystallins increased slightly (Fig. 2, lane 2) and the ratio of crystallins to cytoskeleton proteins increased to approximately 6. In explants treated with bFGF for 7 days, the amount of crystallins increased dramatically (Fig. 2, lane 4) and the ratio of crystallins to cytoskeleton proteins increased to approximately 12. In the absence of bFGF, there was no increase in this ratio. In samples treated with lactacystin, the proportion of crystallin proteins was only half that found in the absence of lactacystin (Fig. 2, compare lane 6 with lane 5). Taken together, these data clearly show that this in vitro model of lens cell differentiation faithfully recapitulates the in vivo differentiation program and that UPP activity, including proteolysis, is required for the differentiation process.

Levels of p21^{WAF} and p27^{Kip} Increase during bFGF-Induced Differentiation

Initial proliferation and subsequent withdrawal from the cell cycle appear to be associated with lens fiber differentiation.^{23–25} Entry into and exit from the cell cycle are often associated with activation or inactivation of protein kinases known as the cyclin-dependent kinases (Cdk). Cdk activities are regulated by the concerted action of activators and inhibitors. Cdk inhibitors that prohibit progress beyond G1 phase include p21^{WAF}, p27^{Kip}, and p57^{Kip}.²¹ In most cell types these levels are regulated by degradation through the UPP. Levels of p21 and p27 in the explants cultured without bFGF were low (Fig. 3A, lane 1), and we hypothesized that the low levels of these Cdk inhibitors were due in part to degradation by UPP activity. This was confirmed by the fact that inhibition of the proteasome activity of the explants cultured without bFGF resulted in a 25-fold increase in p21^{WAF} and a 4-fold increase in p27^{Kip} (Figs. 3B, 3C). However, even with such large increases in levels of these Cdk inhibitors, there was little change in the proliferative index in the absence of bFGF. These data suggest that under these conditions p21^{WAF} and p27^{Kip} are not rate controlling with respect to basal proliferation and that other factors participate in the control of the limited cell proliferation in lens explants cultured in the absence of bFGF. Possible candidates for such regulatory molecules include p16 and p57^{Kip}.

To determine whether accumulation of p21^{WAF} and p27^{Kip} is associated with proliferation and/or the subsequent withdrawal from the cell cycle during bFGF-induced lens cell differentiation, we determined the protein levels of p21^{WAF} and p27^{Kip} in the bFGF-treated lens epithelial explants at 3 and 7 days. In contrast with the slow growth in the absence of bFGF, treatment of the explants with bFGF resulted in rapid proliferation until at least 3 days. Levels of p21^{WAF} and p27^{Kip} remained low during this time (Fig. 4A). The low level of these Cdk inhibitors during the time of rapid proliferation is consistent with similar observations in rapidly proliferating lens epithelial cells in culture.²⁶

By 7 days in culture, there were changes in the Cdk inhibitor profile in the explants. In comparison to similar levels of p27^{Kip} in explants treated without bFGF for 3 days (Figs. 4A, 4B, compare lane 3F with lane 3C), p27^{Kip} levels increased by approximately 3.2-fold in explants treated with bFGF for 7 days (Fig. 4B, compare lane 7F with lane 3F). In the explants cultured for 7 days, the levels of p27^{Kip} were 1.7 times higher in the presence of bFGF than in the absence of bFGF (Fig. 4B, compare lane 7F with lane 7C), and the latter had p27^{Kip} levels that were still approximately two times higher than p27^{Kip} levels in explants, which were cultured for only 3 days (Fig. 4B, compare lane 7C with lane 3C). Similar to changes in p27^{Kip}, p21^{WAF} increased dramatically in explants treated with bFGF for 7 days (Fig. 4A, compare lane 7F with the other lanes). Explants maintained in the absence of FGF for 7 days also had very low levels of p21^{WAF} (Figs. 4A, 4B, compare 3F and 3C). The accumulation of p21^{WAF} and p27^{Kip} in the bFGF-treated (vs. untreated explants) by 7 days is consistent with the role for these Cdk inhibitors in the withdrawal of cells from the cell cycle at this stage (Fig. 1E).

Changes of Levels of UPP Components during Lens Epithelial Cell Differentiation

Levels of p21^{WAF} and p27^{Kip} and of many other cell cycle regulators are controlled by UPP-mediated degradation in the lens epithelial cells as well as in other types of cells.^{19,20,27} The differentiation-associated accumulation of these cell cycle regulators suggests that some components of the UPP may also be downregulated during differentiation in lens explants. To test this hypothesis, we determined the expression of enzymes that are involved in ubiquitin conjugation. Western blot analysis showed that, as normalized with β -actin, levels of E1 were comparable between explants treated with or without bFGF (Fig. 5A) and that levels of E1 in explants cultured for 7 days in the presence or absence of bFGF were only slightly lower than those in explants cultured for only 3 days (Fig. 5A). This seems to indicate that levels of E1 are not altered with respect to progress through the cell cycle and differentiation and that E1 levels are adequate to catalyze ubiquitination in the explants whether or not differentiation is in progress.

In contrast to the absence of a relationship between bFGF treatment and E1 levels, protein levels and activities of several E2s were altered on stimulation of proliferation and differentiation by bFGF. To determine simultaneously the total levels of a specific E2 and the proportion of the E2 charged with ubiquitin (thiol ester form), the gels were run under nonreducing conditions. Both the free form and the thiol ester form of the E2 were quantified and compared between different treatments. Under nonreducing conditions, two bands (25 and 34 kDa) of Ubc1 were detected in lens explants (Fig. 5B). The upper band (~34 kDa) disappeared on treatment with DTT (data not shown), indicating that the upper band is the thiol ester form or "charged form." Levels of total Ubc1 (free form plus the thiol ester form) were upregulated in bFGF-treated lens explants, starting as early as 3 days of incubation. The levels of total Ubc1 in explants treated with bFGF for 3 and 7 days were 1.3 and 2.1 times higher, respectively, than those determined in explants not treated with bFGF (Fig. 5B, compare lane 3F with lane 3C, lane 7F with lane 7C). The levels of Ubc1 also increased with increasing time in culture, even without the presence of bFGF (Fig. 5B, compare lane 7C with lane 3C), and bFGF treatment enhanced even further the increase of Ubc1. Compared with explants cultured in the absence of bFGF for 3 days, the total Ubc1 level in explants treated with bFGF for 7 days was enhanced 3.5-fold (Fig. 5B, compare lane 7F with lane 3C). The proportion of Ubc1 in the thiol ester form also increased in the bFGF treated explants. Whereas in explants cultured without bFGF for 3 days, the thiol ester form of Ubc1 was barely detectable, this form of Ubc1 was observed in explants treated with bFGF for 3 days, and the proportion of the thiol ester form of Ubc1 reached approximately 20% of the total Ubc1 in explants treated with bFGF for 7 days.

Two bands of Ubc2 were observed in all the explants under nonreducing conditions (Fig. 5C). The upper band disappeared on reduction with DTT (data not shown), indicating that the upper band is the thiol ester form of Ubc2. The total amount of Ubc2 in the explants varied little in response to bFGF treatment. However, the proportion of thiol ester form of Ubc2 increased during bFGF-induced differentiation (Fig. 5C, compare lane 7F with lane 7C). Whereas less than 40% of Ubc2 was in thiol ester form in the explants not treated with bFGF (Fig. 5C), the thiol ester form of Ubc2 accounted for more than 60% of the total Ubc2 in the explants treated with bFGF for 7 days (Fig. 5C).

In many cells, Ubc3 is the E2 that is involved with regulating levels of Cdk inhibitors such as p21^{WAF} and p27^{Kip}. The total levels of Ubc3 decreased slightly with time of culture (Fig. 5D, compare lane 7C with lane 3C, lane 7F with lane 3F) but bFGF treatment attenuated the time-dependent decrease in the levels of total Ubc3 (Fig. 5D, compare 7F with 7C). In addition, the proportion of thiol ester form of Ubc3 increased significantly in the bFGF-treated explants (Fig. 5D, compare lane 3F with lane 3C, lane 7F with lane 7C).

The masses and the amino acid sequences of Ubc4 and -5 are almost identical. They are not separable on SDS-PAGE and the antibodies raised against Ubc5 react with both Ubc4 and -5. Therefore, we designated the band that reacted with antibodies to Ubc5 as Ubc4/5. In contrast to other E2s determined in this study, Ubc4/5 was not detectable under nonreducing conditions. Therefore, we could not determine the proportion of Ubc4/5 in thiol ester form. Under reducing conditions, a single band that corresponds to the uncharged form was detected in the explants (Fig. 5E). Levels of Ubc4/5 increased approximately twofold on treatment with bFGF for 3 days (Fig. 5E, compare lane 3F with lane 3C) and further increased as much as sixfold in the explants treated with bFGF for 7 days (Fig. 5E, compare lane 7F with lane 7C).

Dramatic cell-cycle-specific changes in Ubc7 levels in human lens cells in culture imply a role for this E2 in regulation of lens cell proliferation.²⁶ Consistent with crucial (but as yet unknown) roles for this enzyme in regulation of cell proliferation, most Ubc7 in lens explants were in the thiol ester form, even in explants not treated with bFGF (Fig. 5F). bFGF treatment for 7 days was associated with a limited increase in total levels of Ubc7, but it increased the proportion of the thiol ester form of Ubc7 to more than 95% of the total Ubc7 (Fig. 5F).

The total levels of Ubc9, the SUMO-conjugating enzyme, in the explants increased by approximately 12-fold after treatment with bFGF for 3 days (Fig. 5G, compare lane 3F with lane 3C). By 7 days of treatment with bFGF, the levels of total Ubc9 in the explants increased approximately 30-fold compared with those in explants not treated with bFGF. (Fig. 5G, compare lane 7F with lane 7C). Moreover, the bFGF-induced increase in levels of Ubc9 was mainly in the thiol ester form (Fig. 5G, compare lane 3F with lane 3C and lane 7F with lane 7C). In explants treated with bFGF for 7 days, more than 95% of the Ubc9 was in the thiol ester form.

To further characterize the ubiquitin conjugation system in the lens explants, we determined the expression of Cul1, the backbone of the SCF type of E3s. In contrast to the upregulation of several E2s, the levels of Cul1 in the explants decreased 47% and 75%, respectively, after 3 and 7 days of treatment with bFGF (Fig. 5H, compare lane 3F with lane 3C and lane 7F with lane 7C). The levels of Cul1 in explants cultured in the absence of bFGF for 7 days were also significantly lower (50%) than the levels observed in explants cultured for only 3 days (Fig. 5H, compare lane 7C with lane 3C). The levels of Cul1 in the explants treated with bFGF for 7 days were 88% lower than those detected in explants cultured without bFGF for 3 days. The dramatic downregulation of Cul1 during bFGF-induced differentiation is consistent with the observed increase in levels of p21^{WAF} and p27^{Kip} in bFGF-treated explants, because both p21^{WAF} and p27^{Kip} are substrates of the SCF type of E3s. The fact that little p21^{WAF} is observed in explants maintained without bFGF suggests that the lower level of Cul1 noted in these explants is sufficient to catalyze ubiquitination of this critical regulator.

Discussion

Temporally and spatially controlled proliferation and differentiation is required for lens formation and the maintenance of lens function.^{25,28–31} The initial proliferation provides enough of a mass of cells for terminal differentiation and tissue formation. This is essential, because once terminal differentiation begins, the cells lose their ability to proliferate. Therefore, it is reasonable to consider the initial proliferation as the first stage of the differentiation process. The UPP regulates cell proliferation and differentiation in most eukaryotic cells, though the mechanisms appear to differ among organisms and tissues. Based on our previous observation that ubiquitin conjugation activity increased during early stages of lens cell differentiation,¹¹ and that the UPP is essential for controlling the cell cycle in cultured lens epithelial cells and other types of cells,^{26,27,32,33} we hypothesized that the UPP plays a role in regulating lens cell proliferation and differentiation in vivo and in lens explants

in vitro. Consistent with our hypothesis, we found that inhibition of UPP proteasome activity delayed and diminished the initial bFGF-induced proliferation and subsequent differentiation of lens cells in a lens explant model (Table 1, Fig. 1). Thus, as in other types of cells, UPP activity is required for proper cell proliferation and differentiation to allow for organ formation.

p21^{WAF} and p27^{Kip} accumulated in bFGF-treated explants that had undergone the differentiation process (Fig. 4) as well as in explants not treated with bFGF but that had been exposed to proteasome inhibitor (Fig. 3). Accumulation of p27^{Kip} and p57^{Kip} was also observed during transition between proliferation and differentiation of lens epithelial cells, and this accumulation could be triggered by inhibition of Src family kinases.³⁴ Thus, it appears that degradation of cell cycle regulators, such as p21^{WAF} and p27^{Kip}, is regulated by the UPP. Our data are consistent with a role for these Cdk inhibitors in regulation of rapid proliferation and differentiation, such as is observed in the early days of explants treated with bFGF (Fig. 1E). That inhibition of the proteasome activity had little effect on the proliferative index in explants not treated with bFGF (Table 1) suggests that regulators other than or in addition to these two Cdk inhibitors are rate controlling for cell proliferation in the absence bFGF. Cell cycle control is a complex process, many steps of which remain poorly understood. The UPP is involved in many of aspects of signal transduction cascade and cell cycle regulation. An objective of our work is to identify substrates that are degraded by the UPP at the early and late stages of differentiation to further our understanding of the function of the UPP in these processes.

In recent work we attempted to separate functions of the UPP during early events such as the bFGF-induced proliferative burst, versus later events including the accumulation of crystallins and multilayering (Fig. 2). Using the proteasome inhibitor, we observed that the proteasome activity is not only required at the proliferative phase of lens cell differentiation, it is also required at a later phase of differentiation. If lactacystin was added at day 4, after most of the proliferation stopped, it reduced the production of β -crystallin and delayed the maturation of fibers cells (manuscript in preparation).

Several observations suggest that the downregulation of Cul1 (Fig. 5H), the backbone of the SCF type of E3s, which is involved in ubiquitination of p21^{WAF} and p27^{Kip},^{19,20} may account for the accumulation of these Cdk inhibitors and other molecules that must be degraded to facilitate progress through the cell cycle. First, protein levels of Cul1 are markedly diminished after withdrawal from the cell cycle and establishment of a differentiated phenotype. Second, levels of neither E1 nor Ubc3 (the E2 that works together with the SCF type of E3s to ubiquitinate p21^{WAF} and p27^{Kip}) were not significantly altered during the differentiation process. Third, a significant proportion of Ubc3 was in the thiol ester or active form, even in the explant that was treated with bFGF for 7 days (Fig. 5D). Taken together, these data indicate that there are sufficient levels of E1 and Ubc3 in lens cells. The levels of SCF type of E3s may determine the degradation rates of p21^{WAF} and p27^{Kip} and other regulators during proliferation and differentiation. The downregulation of this E3 may account for the accumulation of p21^{WAF} and p27^{Kip} in the differentiating lens cells. This is the first demonstration that alteration in levels of cell cycle regulators are associated with altered expression of ubiquitination machinery. Because withdrawal from the cell cycle is a prerequisite for the progress of the differentiation program,^{35,36} the downregulation of this E3 activity seems to be a mechanism by which the UPP regulates the differentiation program. If Cul1 is indeed shown to be rate limiting, then this also appears to suggest that other known substrates of the SCF may be important regulators of the lens cell cycle.

The downregulation of Cul1 may also account for the accumulation of the thiol ester form of Ubc3 in the bFGF-treated explants (Fig. 5D). This is because if E3 is rate limiting, decreased activity of the SCF type of E3s in the explants may block the transfer of ubiquitin from Ubc3 to its substrates, such as p21^{WAF} and p27^{Kip}. It is plausible that the downregulation of the SCF

type of E3s (or other types of E3s) also accounts for the increase in the proportions of the thiol ester form of Ubc2 and -7 in bFGF-treated lens explants. However, this does not exclude the possibility that some of these E2s become more active and are charged by E1 more efficiently during the bFGF-induced differentiation process.

In contrast to the downregulation of Cul1, several E2s (Ubc1, -2, -4/5, -7, and -9) were upregulated during bFGF-induced lens cell differentiation. bFGF treatment not only increased the total levels (free and thiol ester forms) of these E2s, but also increased the proportion of active or the thiol ester form of these E2s. The upregulation of these E2s may contribute to the increased conjugation activities during early stages of lens cell differentiation.¹¹ This is consistent with the upregulation of Ubc4 and increased ubiquitin conjugation activity in testis during the early stages of postnatal development.³⁷ Furthermore, support for this role of Ubc2 and -4 is found in data that showed that immunodepletion of Ubc2 or -4 from testis supernatants decreased rates of ubiquitin conjugation and supplementation of extracts with exogenous Ubc2 or -4, but not E1, can stimulate ubiquitin conjugation.³⁸

All E2s share a highly conserved core domain consisting of approximately 150 amino acids termed the Ubc domain, but each of them has a unique N- or C-terminal extension.³⁹ Although some of the E2s have overlapping functions, most of them interact with specific E3s and ubiquitinate specific substrates.⁴⁰ In yeast, Ubc1 is essential for survival in the absence of Ubc4 and -5. It is also required for growth after germination of spores⁴¹ and endocytosis of membrane proteins.⁴² However, the function of this E2 in rats or humans remains to be defined. It has been found that Ubc1 interacts with Huntingtin, possibly ubiquitinating it.⁴³ The upregulation of Ubc1 in bFGF-treated rat lens explants is similar to the upregulation of Ubc1 during transition of cornea keratocytes to the repair fibroblast phenotype.⁴⁴ Together with previous observations, this result suggests new roles for Ubc1 in regulation of mammalian cellular remodeling.

Ubc4 and -5 are closely related in sequence and complementary in function and have reported roles in targeting the abnormal or misfolded proteins for degradation.⁴⁵ We speculate that the increased levels of Ubc4/5 during bFGF-induced differentiation have a role in the removal of abnormal or obsolete proteins during cellular remodeling, such as removal of various organelles.

Ubc7 is an endoplasmic reticulum (ER)-associated protein that may have a role in ER-associated ubiquitination and degradation.⁴⁶ The finding that levels of Ubc7 increased during bFGF-induced differentiation and during G2/M transition of cultured human lens epithelial cells²⁸ suggests that Ubc7 also plays a role in regulation of the cell cycle. Identification of the substrates and the corresponding E3 for the proposed function for Ubc7 are in progress.

Unlike most Ubcs, Ubc9 mediates the conjugation of the ubiquitin-like protein SUMO to target proteins.^{47–49} We found that levels of Ubc9 increased dramatically with bFGF treatment and most of the increase of Ubc9 was in the thiol ester form. Indeed, by 7 days of bFGF treatment almost all the Ubc9 was in the thiol ester form. These data indicate that not only ubiquitin but also SUMO is involved in regulating the proliferation–differentiation program. It has been demonstrated that SUMO is involved in regulation of several transcription factors or regulators.^{50,51} AP-2 transcription factor is required for lens cell differentiation and formation,^{52,53} and it is also regulated by SUMO.⁵⁰ Roles for Ubc9 and SUMO during lens cell differentiation are under investigation.

The differential regulation of the components of the UPP during lens cell differentiation suggests that there is a reconfiguration of this pathway. The downregulation of Cul1 may facilitate the accumulation of p21^{WAF} and p27^{Kip} and other Cdk inhibitors, which in turn causes the withdrawing from cell cycle. Although the roles of Ubc1, -2, -4/5, -7, and -9 in lens cells

remain to be determined, it is tempting to speculate that the upregulation of these ubiquitin-conjugating enzymes may have function at later stages of the differentiation process, such as removal of nuclei and other organelles during maturation of lens fibers.

In summary, the dramatic increase in levels of several E2s and coincident decrease in levels of Cul1 indicate that there is a fundamental reconfiguration of the ubiquitin conjugation system during the lens cell differentiation process. The constant level of E1 and elevated thiol esters of E2s suggest that there is sufficient E1 activity to support the UPP in its various configurations in rat lens explants. This was different from what was observed in bovine lens epithelial cells, in which we found that E1 is rate limiting, particularly under stress conditions.¹³ Whereas the downregulation of the SCF type of E3s appears to contribute to the accumulation of Cdk inhibitors, withdrawal from the cell cycle, and establishment of a differentiated phenotype, the upregulation of Ubc4/5 and other ubiquitin-conjugating enzymes may contribute to the removal of obsolete proteins, such as various organelles. The upregulation of Ubc7, an ER-associated E2, may assure the quality of newly synthesized fiber-specific proteins, such as crystallins. To gain a more complete appreciation of the role of the UPP in and during lens cell proliferation and differentiation, further investigation of differentiation-related expression of other types of E3s, such as HECT-domain E3s, RING-finger domain E3s, and the APC type of E3s is warranted.

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Figure 1.

Lens cells differentiation was preceded by enhanced cell proliferation and followed by withdrawal from cell cycle. Lens capsules were peeled from 5-day-old rat lenses and cultured in the absence (A, D) or presence (B, E) of 100 ng/mL bFGF alone or 100 ng/mL bFGF with 10 μ M lactacystin- β -lactone (C, F) for 3 (A, B, C) and 7 (D, E, F) days. All the explants were cultured in medium 199 containing 1 μ g/mL insulin and 0.1% BSA. The explants were labeled with 100 μ M BrdU for 2 hours at day 3 or 7. The explants were then fixed with formaldehyde and sectioned. BrdU incorporation was detected with antibodies against BrdU. The yellow-brown nuclei indicate BrdU incorporation and cell proliferation.

Figure 2.

Expression of crystallin in explants indicated that bFGF promotes differentiation. *Lanes 1–4*: lens capsules were obtained from lenses of 5-day-old rats and cultured in the absence (3C or 7C) or presence (3F or 7F) of 100 ng/mL bFGF for 3 and 7 days in medium 199 containing 1 μ g/mL insulin and 0.1% BSA, collected at the indicated times and lysed in 1 $\times$ Laemmli buffer. *Lanes 5, 6*: samples were treated as in lane 4, except that 10 μ M lactacystin- β -lactone was (*lane 6*) or was not (*lane 5*) present for 7 days. Proteins in the lysate were resolved on 12% SDS-PAGE. The gel was scanned and the ratio of levels of crystallins (Crys.) to levels of cytoskeletal proteins (Cryo.) was calculated and presented in the text.

Figure 3.

Inhibition of proteasome activity resulted in accumulation of p21 and p27 in rat lens epithelial explants. Lens capsules were obtained from 5-day-old rat lenses and cultured in the absence of bFGF for 3 days and in the absence or presence of 10 μ M lactacystin- β -lactone. The explants were collected at day 3 and lysed in 1 $\times$ Laemmli buffer. Proteins in the lysate were resolved on 12% SDS-PAGE and transferred to nitrocellulose. Levels of p21 and p27 in the explants were determined by Western blot analysis and quantified by densitometry. (A) Typical Western blot result; (B, C) changes of relative levels of p21 and p27 after normalizing with levels of actin. The data are the mean of results in two independent experiments, $\pm$ SD.

Figure 4.

(A) Levels of p21 and p27 increased as lens fiber differentiation progressed. Lens capsules were obtained from 5-day-old rats and cultured in the absence (3C or 7C) or presence (3F or 7F) of 100 ng/mL bFGF for 3 and 7 days. The explants were cultured in medium 199 containing 1 μ g/mL insulin and 0.1% BSA. The explants were lysed in 1 $\times$ SDS loading buffer, and proteins in the lysate were resolved on SDS-PAGE and transferred to nitrocellulose membranes. Levels of p21^{WAF} and p27^{Kip} in the samples were determined with respective antibodies, and the blots were reprobbed with anti- β -actin antibodies to normalize levels of these substrates with respect to protein. (B) Densitometric quantification of p27^{Kip} levels from three experiments. The standard deviations of these assays were approximately 25%.

Figure 5.

Regulation of UPP enzymes and substrates during bFGF-induced lens fiber differentiation. Lens capsules were obtained from 5-day-old rat lenses and cultured in the absence (3C or 7C) or presence (3F or 7F) of 100 ng/mL bFGF for 3 and 7 days. The explants were cultured in medium 199 containing 1 μ g/mL insulin and 0.1% BSA. The explants were lysed in 1 $\times$ Laemmli buffer without β -mercaptoethanol, and proteins in the lysate were resolved on SDS-PAGE and transferred to nitrocellulose membrane. Levels of E1 (**A**), Ubc1 (**B**), Ubc2 (**C**), Ubc3 (**D**), Ubc4/5 (**E**), Ubc7 (**F**), Ubc9 (**G**), and Cul1 (**H**) in the samples were determined with respective antibodies and reprobed with anti- β -actin antibodies. Levels of these enzymes were normalized with levels of β -actin in these samples. The free form and ubiquitin thiol ester forms of Ubc1, -2, -3, -7, and -9 were quantified separately. The thiol ester form of Ubc4/5 is not detectable using this method; therefore, only the free form was reported. The data in the histograms are the average of three experiments and the standard deviations for these measurements were 25% to 30%. * $P < 0.05$; ** $P < 0.01$, when the total levels of the specific components were compared with those obtained from explants treated without bFGF for 3 days. #Statistically significant changes ($P < 0.05$) in the proportion of specific Ubcs in their thiol ester forms when compared with those obtained from explants treated without bFGF for 3 days.

Table 1
Effects of Proteasome Inhibitor and bFGF on Proliferative Index in Lens Explants Treated with or without bFGF

	Day 3		Day 7	
	-bFGF	+bFGF	-bFGF	+bFGF
-Lactacystin	3.6 ± 0.2	21.4 ± 10.7 [*]	4.1 ± 1.1	1.3 ± 1.2 [*]
+Lactacystin	3.7 ± 1.1	5.7 ± 3.2 [†]	5.2 ± 3.2	4.3 ± 1.3 [†]

^{*} Indicates that there was a statistically significant difference between explants with respect to BrdU incorporation in the presence or absence of bFGF ($P < 0.01$).

[†] The differences between explants treated with or without lactacystin are statistically significant ($P < 0.05$).

Lens capsules were peeled from 5-day-old rat lenses and cultured in M199 containing 1 µg/mL insulin and 0.1% BSA. Explants cultured in the absence or presence of 100 ng/mL bFGF were treated with 10 µM lactacystin-β-lactone for 3 and 7 days. The explants were labeled with 100 µM BrdU for 2 hours at day 3 or day 7. Then the explants were fixed with formaldehyde and sectioned. BrdU incorporation was detected with antibodies against BrdU. The percentage of BrdU-positive nuclei (yellow-brown) were counted under a microscope. Three explants from each group were evaluated and two sections from each explant were examined. Data presented are the mean ± SD.