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Upregulation and Maintenance of Gap Junctional Communication in Lens Cells

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Abstract

The cells of the lens are joined by an extensive network of gap junction intercellular channels consisting of connexins 43, 46, and 50. We have proposed, and experimentally supported, the hypothesis that fibroblast growth factor (FGF) signaling is required for upregulation of gap junction-mediated intercellular coupling (GJIC) at the lens equator. The ability of FGF to increase GJIC in cultured lens cells requires sustained activation of extracellular signal-regulated kinase (ERK). In other cell types, activation of ERK has been shown to block GJIC mediated by connexin43 (Cx43). Why ERK signaling does not block lens cell coupling is not known. Another unresolved issue in lens gap junction regulation is how connexins, synthesized before the loss of biosynthetic organelles in mature lens fiber cells, avoid degradation during formation of the organelle-free zone. We have addressed these questions using serum-free cultures (termed DCDMLs) of primary embryonic chick lens epithelial cells. We show that FGF stimulates ERK in DCDMLs via the canonical Ras/Raf1 pathway, and that the reason that neither basal or growth factor-stimulated GJIC is blocked by activation of ERK is because it is not mediated by Cx43. In fibroblastic cells, the normally rapid rate of degradation of Cx43 after its transport to the plasma membrane is reduced by treatments that either directly (ALLN; epoxomicin) or indirectly (generation of oxidatively un/mis-folded proteins by arsenic compounds) prevent the ubiquitin/proteasome system (UPS) from acting on its normal substrates. We show here that Cx45.6 and Cx56, the chick orthologs of mammalian Cx50 and Cx46, behave similarly in DCDMLs. When organelles lyse during the maturation of fiber cells, they release into the cytosol a large amount of new proteins that have the potential to saturate the capacity, and/or compromise the function, of the UPS. This would serve to spare gap junctions from degradation during formation of the organelle-free zone, thereby preserving GJIC between mature fiber cells despite the lack of de novo connexin synthesis.

Keywords

lens; gap junctions; connexin; proteasome; ERK

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INTRODUCTION

The lens consists of a monolayer of epithelial cells on the anterior side of the organ and layers of crystallin-rich, elongated secondary fiber cells arranged concentrically around a core of primary fibers. Differentiation of epithelial cells into secondary fibers is initiated near the anterior/posterior boundary of the organ, a region referred to as the lens equator (Piatigorsky, 1981). Because it lacks blood vessels, the lens must use non-vascular mechanisms to move nutrients into, and waste products out of, fiber cells in the lens center. This is achieved in part by an extensive network of gap junction channels that join the cells of the lens into what has been referred to as an ionic and metabolic syncytium (Goodenough, 1992). Gap junctions are clusters of intercellular channels that, when gated open, mediate the direct cell-to-cell transfer of low molecular mass (<1 kD) substances including ions, second messengers, and nutritional metabolites (Harris, 2001). They are composed of integral plasma membrane proteins known as connexins, three of which are present in the lenses of all species examined. Lens epithelial cells express connexin43 (Cx43), a highly evolutionarily conserved, widely distributed protein that is the most thoroughly studied member of the connexin family (Musil et al., 1990). Gap junctions in fiber cells are composed of connexin50 (Cx50) (White et al., 1992) and connexin46 (Cx46) (Paul et al., 1991), the chick orthologs of which are, respectively, Cx45.6 (Jiang et al., 1994) and Cx56 (Rup et al., 1993). Mature fiber cells possess an exceptionally large number of gap junctions, likely the highest concentration in any tissue in the body. Mice in which the genes for Cx46 (White et al., 1998) or Cx50 (Gong et al., 1997) have been deleted have defects in lens clarity and/or lens growth. Naturally occurring mutations in Cx46 or Cx50 that compromise their ability to mediate intercellular coupling lead to a variety of cataract phenotypes, both dominantly and (rarely) recessively inherited (Gerido and White, 2004; Ponnamm et al., 2007). Because of its essential role in proper vision, it is important to understand how gap junction function in lens cells is regulated.

We study lens gap junctions using serum-free cultures of primary embryonic chick lens epithelial cells, the cell type that more closely recapitulates the *in vivo* process of fiber-type gap junction formation than any mammalian system described (Menko et al., 1987; FitzGerald and Goodenough, 1986; TenBroek et al., 1994). These cultures, termed DCDMLs, include cells from the peripheral (i.e., pre-equatorial) and equatorial epithelium. Using this system, we have shown that gap junction-mediated dye coupling is greatly increased by fibroblast growth factor (FGF) at levels at which it diffuses out of intact vitreous bodies and would have access to cells at the lens equator (Le and Musil, 2001a; Le and Musil, 2001b). These findings led to a model in which vitreous-derived FGF is responsible for the higher levels of gap junction-mediated intercellular coupling (GJIC) measured in the lens cortex at the equator than at either pole, an asymmetry believed to be essential for lens transparency (Baldo and Mathias, 1992; Mathias et al., 1997; Mathias et al., 2007). The ability of FGF to increase GJIC in DCDMLs required sustained (≥ 12 h) activation of extracellular signal-regulated kinase (ERK) (Le and Musil, 2001b). This was a surprising and unexplained finding, given that activation of ERK had been shown to close Cx43 channels in several other cell types (Lampe and Lau, 2004; Warn-Cramer et al., 1998; Warn-Cramer et al., 1996; KEGG pathway map04540: gap junctions).

As important as understanding how GJIC is upregulated at the lens equator is understanding how it is maintained in mature lens fibers, a population of cells that do not turn over and that persist throughout the life span of the organism. A defining event in the terminal differentiation of epithelial cells into fibers is the destruction of intracellular organelles, which has been reported to take place in as few as 4 h (Bassnett and Beebe, 1992). In the absence of the endoplasmic reticulum and Golgi complex (Bassnett, 1995), synthesis of plasma membrane proteins is abolished. Consequently, the proteins found on the plasmalemma of mature fiber cells must have been synthesized prior to the formation of the organelle-free zone. Unlike the

great majority of integral plasma membrane proteins (Hare and Taylor, 1991), the half-life of connexins is only 1.5–4 h in vivo and in cells in culture (Traub et al., 1989; Beardslee et al., 1998; Musil and Goodenough, 1991; Fallon and Goodenough, 1981), including in lens DCDMLs (Le and Musil, 2001b). Importantly, this is also true of connexins after their assembly into functional gap junctions (Gaietta et al., 2002; Musil and Goodenough, 1991; VanSlyke and Musil, 2005). Thus during the ~4 h in which organelles are destroyed, >50% of each of the connexin species on the surface of lens cells could be degraded and be unable to be replaced. This does not, however, appear to occur given that mature fiber cells maintain high levels of gap junctional plaques (Dunia et al., 2006; Vrensen et al., 1992) that are essential for lens clarity (Gong et al., 1998); the decrease in GJIC in mature compared to cortical fiber cells can be accounted for by the functional inactivation of Cx50 within the lens nucleus (DeRosa et al., 2006; Baldo et al., 2001). Apparently, some mechanism exists that slows the degradation of gap junctions from lens cell surfaces during the period in which organelles are destroyed.

In this study, we use DCDMLs to address two related questions in lens physiology: (1) why doesn't ERK signaling block GJIC in lens cells? and (2) why don't gap junctions disappear during formation of the organelle-free zone? Based on our findings, we present a new model of how the levels of GJIC in the lens that are required for proper vision are established and then maintained.

MATERIALS AND METHODS

Materials

Recombinant bovine FGF-2 and human BMP4 were from R & D Systems (Minneapolis, MN). Cx43 was detected with a monoclonal mouse antibody (#C8093; Sigma-Aldrich), and Cx56 with a previously characterized polyclonal rabbit antibody (Jiang et al., 1994) kindly provided by Dr. Jean Jiang (University of Texas Health Science Center). The rabbit polyclonal antiserum against Cx45.6 (#505) was raised against a peptide encoding amino acids 117–129 of the cytoplasmic loop domain of Cx45.6 and recognizes the same species as the anti-Cx45.6 antiserum from Dr. Jiang used in our previous studies (Le and Musil, 2001b). N-cadherin was detected with a polyclonal pan-cadherin antibody (Sigma Aldrich), and B-galactosidase with a mouse IgG2a form Promega (#Z378). Brefeldin A (BFA; used at 6 ug/ml) was purchased from Epicentre Technologies, Madison, WI; ALLN (N-acetyl-leu-lue-norleucinal) and epoxomicin (used at 260 μ M and 10 μ M, respectively) were from Calbiochem (La Jolla, CA), and arsenic trioxide (ATO; used at 60 μ M) was from Sigma-Aldrich.

Cell culture and treatments

Cultures were prepared from E10 chick lenses and plated at 1.6×10^5 cells/well onto laminin-coated 96-well tissue culture plates as previously described in Le and Musil (1998). Cells were cultured in M199 medium plus BOTS (2.5 mg/ml bovine serum albumin, 25 mg/ml ovotransferrin, 30 nM selenium), penicillin G, and streptomycin (M199/BOTS), with or without additives at 37°C in a 5% CO₂ incubator. Cells were fed every 2 days with fresh medium. We refer to these cultures as dissociated cell-derived monolayers (DCDML) to distinguish them from related, but functionally distinct, systems such as central epithelial explants and immortalized lens-derived cell lines. Where indicated, DCDMLs were incubated with 15 μ M UO126 (Calbiochem; La Jolla, CA) for 45 min at 37°C prior to addition of FGF.

Plasmids and transient transfection of lens cells

One day after plating, DCDML cultures were transfected in M199 medium without BOTS or antibiotics using Lipofectamine PLUS (GibcoBRL) as specified by the manufacturer. Plasmids encoding wild type MEK1, a constitutively active mutant form of MEK1 (S217E/S221E) (Cowley et al., 1994; Yao et al., 1995), EGFP (BD Biosciences Clontech, Palo Alto, CA),

FLAG-ERK2, RasN17, RasV12, RapE63V12, the catalytic subunit of protein kinase A (Vossler et al., 1997; Mishra et al., 2005), or the lacZ gene product B-galactosidase (pCH110; Pharmacia) were used at a final concentration of 0.1 μ g DNA per well of a 96-well tissue culture plate as in our previous study (Le and Musil, 2001b). After incubation with DNA for 3 h, the transfection medium was supplemented with BOTS, penicillin G, and streptomycin. Unless otherwise specified, cells were cultured for an additional 48 h prior to analysis.

Immunoprecipitation of FLAG-ERK

Transiently transfected DCDML cultures were lysed in ice-cold 1 mM Tris base, 1 mM EDTA, 1 mM EGTA, 10 mM iodoacetamide, 2 mM PMSF, 0.6% SDS, pH 8.0. Lysates were boiled for 3 min and diluted with 4 volumes of immunoprecipitation buffer prior to immunoprecipitation with the M2 mouse anti-FLAG antibody (#F1804; Sigma-Aldrich) as previously described (Le and Musil, 1998). Immunoprecipitates from equal numbers of cells were analyzed for activated ERK by immunoblot analysis as described below.

Immunoblot analysis

For analysis of whole cell lysates, DCDML cultures were solubilized directly in SDS-PAGE sample buffer and boiled (Le and Musil, 2001a). Proteins were transferred to polyvinylidene fluoride membranes, and the blots probed with anti-phospho-p44/42 MAP kinase E10 monoclonal mouse antibody (specific for activated ERK) and/or anti-p44/42 MAP kinase polyclonal rabbit antibody (both purchased from Cell Signaling Technology, Danvers, MA). Immunoreactive proteins were detected as previously described (Le and Musil, 2001a). Unless otherwise indicated, all results shown are typical of at least three independent experiments.

Immunofluorescence Microscopy

DCDMLs grown on glass coverslips were fixed in 2% paraformaldehyde in PBS and processed for immunocytochemical detection of connexins 43, 45.6 and 56, N-cadherin, and B-galactosidase as previously described (Le and Musil, 1998; Le and Musil, 2001b). For detection of activated ERK, cells were fixed for 10 min on ice in 10% paraformaldehyde in PBS. After permeabilization with 100% methanol (10 min; -20°C), cells were incubated for 30 min at 25°C with PBS/1% bovine serum albumin and then overnight at 4°C with the E10 anti-phospho-p44/42 MAP kinase antibody. Cells were rinsed for 30 min in PBS/1% BSA/0.05% NP-40 detergent, after which the secondary antibody was applied in the same buffer and the rinse repeated. Immunofluorescence images were captured using a Leica DM LD photomicrography system and Scion Image 1.60 software.

Scrape-loading/dye transfer assay for gap junctional intercellular communication

DCDML cultures grown on laminin-coated coverslips were assessed for gap junction-mediated intercellular coupling as previously described (Le and Musil, 2001b; Boswell et al., 2008). In brief, the culture medium from a 3 day-old, confluent monolayer of lens cells was removed and saved. The cells were rinsed three times with Hank's balanced salt solution containing 1% bovine serum albumin (HBC), after which a 27-gauge needle was used to create two longitudinal scratches through the cell monolayer in the presence of a solution of Dulbecco's phosphate buffered saline containing one or more of the following: 0.75% rhodamine dextran ($M_r = 10$ kDa; Molecular Probes), 1% Lucifer yellow (Sigma-Aldrich), 0.25% Alexa594 (Molecular Probes), or 1% FITC-dextran (average molecular mass 40,000; Sigma-Aldrich). After exactly 2 min, the culture was quickly rinsed three times with HBC and then incubated for an additional 20 min in the saved culture medium to allow the loaded dye to transfer to adjoining cells (this is longer than in our previous studies to maximize the amount of dye detectable in coupled cells under control conditions). The culture was then rinsed three times with phosphate buffered saline and fixed. Dyes were visualized by fluorescence microscopy.

CHO cells (Fig 4), incubated for 4 h with ALLN to increase the level of GJIC to that of untreated DCDMLs (Musil et al., 2000), were assayed identically. Scrape-loading/dye transfer assays were measured using the averaged fluorescence plot profile method as previously described; similar results were obtained if the size of the total fluorescent surface area was assessed (Le and Musil, 2001b; Boswell et al., 2008). Because none of the compounds tested significantly altered the size of epithelial cells within the monolayer (data not shown), the distance of intercellular Lucifer yellow transfer was directly proportional to the number of coupled cells. The scrape-loading dye transfer assay has the advantage over microinjection techniques of allowing simultaneous monitoring of dye coupling in a large population of cells; its suitability for the assessment of gap junction-mediated intercellular communication has been well documented (el-Fouly et al., 1987; Venance et al., 1995; Opsahl and Rivedal, 2000).

RESULTS

FGF induces activation of ERK in lens cells by the canonical signaling pathway

The pathway by which FGF activates ERK in most cell types is via FGF receptor-induced phosphorylation of FRS2, which activates the small G protein Ras leading to the sequential downstream functional upregulation of the Raf1, MEK 1/2, and ERK 1/2 kinases (Schlessinger, 2004). Alternative mechanisms of ERK activation have, however, been described that utilize Rap1 instead of Ras, and/or B-Raf instead of Raf1 (Vossler et al., 1997; Kao et al., 2001; Busca et al., 2000). The G proteins and Rafs that are downstream of FGF receptors are known to have multiple, isoform-specific functions (O'Neill and Kolch, 2004; Hagemann and Rapp, 1999). One hypothetical explanation for why FGF-induced sustained activation of ERK increases rather than decreases GJIC in lens cells would be if ERK were activated via Rap1 and B-Raf, and if one or both proteins switches ERK from being an inhibitor of GJIC into a promoter of GJIC. Consistent with this possibility, Cushing et al. (2005) have reported that NGF increases Cx43-mediated GJIC in an ERK-dependent manner in PC12 cells, a cell type in which NGF and FGF induce sustained activation of ERK via Rap1 and B-Raf (Kao et al., 2001; York et al., 1998). Rap1 is ubiquitously expressed, and transcripts for B-Raf have been reported in the eye (Barnier et al., 1995).

To address this issue, we transiently transfected DCDMLs with plasmids encoding various signaling effectors. The success of this approach was dependent on: (1) the number of cells that expressed the plasmid-encoded protein at levels adequate to affect signaling cascades, and (2) the ability to efficiently co-transfect cells with two independent plasmids. In initial experiments, DCDMLs were transfected with a plasmid encoding EGFP. GFP-positive cells were detectable by immunofluorescence microscopy within 6 h of transfection, reaching a maximum of 70% of the total cell population within 48 h. Levels of expression were still > 50% for at least 5 days after transfection (Fig 1A). Next, we expressed a plasmid encoding a constitutively active form of MEK, the kinase immediately upstream of ERK in the MAPK cascade. We have previously shown that DCDMLs transfected with this construct have increased levels of activated (phospho) ERK compared to cells expressing wild type MEK or an irrelevant protein when assessed by anti-phosphoERK immunoblotting of whole cell lysates (Le and Musil, 2001b). We used the same antibody to show immunohistochemically that ~70% of the total cell population of DCDMLs transfected with the constitutively active MEK plasmid, but not with a plasmid encoding wild type MEK (or B-galactosidase; Fig 1C c'), had elevated levels of activated ERK (Fig 1B). As expected, pERK immunoreactivity was abolished in cells exposed to the highly specific MEK inhibitor UO126 (Favata et al., 1998; Le and Musil, 2001a). Finally, we transfected cells with the constitutively active MEK plasmid in the presence of one-third as much B-galactosidase -encoding plasmid. Virtually all of the cells that stained for B-galactosidase were also positive for pERK (Fig 1C, a' and b'). Control experiments verified that the number of cells expressing B-galactosidase was much greater at day 2–5 after

transfection when the amount of plasmid was increased, and that the anti-pERK antibodies did not cross-react with B-galactosidase (Fig 1C, c').

With this assurance that DCDMLs efficiently express exogenous proteins in the expected manner, we transiently transfected 1-day-old cultures with a plasmid encoding FLAG-tagged ERK, together with a two-fold excess of plasmids encoding constructs that either inhibit or enhance the function of Ras, Rap1, Raf-1, or B-Raf. Cell lysates were immunoprecipitated with anti-FLAG antibodies, and blots probed with anti-phosphoERK antibodies. Control experiments in which cells were cotransfected with an irrelevant plasmid (EGFP) demonstrated that, as expected, ERK-FLAG became phosphorylated in response to FGF stimulation (Fig 2, lanes 1 and 2). Expression of constitutively active Ras (RasV12) resulted in a massive increase in ERK-FLAG phosphorylation even in the absence of FGF (lane 3). RasV12 also specifically induced activation of endogenous ERK as assessed by anti-pERK immunoblotting of lysates of singly transfected cells (lane 8). In contrast, a dominant negative form of Ras (RasN17) abolished the ability of FGF to upregulate ERK-FLAG activation (compare lanes 2 and 5). Thus Ras is both sufficient for activation of ERK in unstimulated DCDMLs, and necessary for activation induced by FGF.

Prolonged elevation of Rap1 activity activates B-Raf, but has the opposite effect on Raf-1 by sequestering it in inactive Rap1/Raf-1 complexes (Vossler et al., 1997; Stork, 2003). We found that expression of a constitutively active form of Rap1 (RapV12) did not induce ERK-FLAG phosphorylation in the absence of FGF (Fig 3, lane 3), but rather inhibited ERK activation in response to the growth factor (lane 4; compare to top lane 2). Long-term activation of endogenous Rap1 can be achieved by high-level stimulation of protein kinase A signaling, for example by overexpression of the constitutively active catalytic subunit of PKA (Vossler et al., 1997). Similar to RapV12, cPKA prevented FGF from phosphorylating ERK-FLAG (lane 6). Taken together, these results indicate that FGF activation of ERK in lens cells is via the canonical Ras--> Raf1 signaling module.

Role of Connexin43 in gap junctional communication in lens cells

If activation of ERK by FGF in lens cells is mediated by same pathway as in most other cell types, then why is its effect on GJIC in DCDMLs so different? The ability of ERK to reduce GJIC was established using Cx43-expressing cells and shown to require direct binding of ERK to the cytoplasmic tail domain of Cx43 and its subsequent phosphorylation (Warn-Cramer et al., 1998; Warn-Cramer et al., 1996). The carboxyl terminal domains of Cx45.6, Cx56, and their human and murine homologs lack high probability binding sites for ERK (as assessed by the Scansite 2.0 Web server; Obenaus et al., 2003). GJIC mediated by these connexins would therefore be expected to be resistant to downregulation by ERK. We assess GJIC in DCDMLs and other adherent cell types using the scrape load/dye transfer assay (Le and Musil, 2001b; Boswell et al., 2008). A 27-g needle is used to make a vertical scratch through the center of a confluent monolayer of cells in the presence of one or more fluorescent dyes. Dyes incapable of penetrating gap junctions remain confined to the wounded cells bordering the scratch, whereas gap junction-permeable compounds are transferred to adjacent, unperturbed cells via open gap junctional channels.

To determine the role of Cx43 in lens cell GJIC, we took advantage of the finding by Dong et al. (2006) that gap junction channels composed of Cx43, but not of Cx45.6, are permeable to the dye Alexa594. We first conducted control experiments in CHO cells, which express endogenous Cx43 as their only connexin. As expected, the extent of Alexa594 transfer was comparable to that of co-loaded Lucifer yellow, a dye known to be permeable to gap junctions formed from most connexin species, including Cx43 (Fig 4A, top row). FITC-dextran (which at $M_r = 10$ kD is too large to pass through any gap junction channel) remained confined to cells at the edge of scrape (Fig 4A, bottom row). We then assessed the relative transfer of Lucifer

yellow and Alexa594 in DCDMLs. In contrast to the results shown in Fig 4A, intercellular transfer of Alexa594 was very low in DCDMLs (Fig 4B, top left), and the dye remained largely confined to cells that could be directly scrape-loaded with FITC-dextran (not shown). Culturing the cells with FGF, BMP4, or vitreous body conditioned medium (Le and Musil, 2001b; Boswell et al., 2008) had no effect on Alexa594 movement, despite robust upregulation of intercellular transfer of Lucifer yellow in the same cells (Fig 4B). We conclude that Cx43 plays a minor role in either basal or growth factor-stimulated GJIC in chick lens epithelial cells. The most likely reason ERK activation does not inhibit GJIC in lens cells is because the level of junctional coupling is controlled by a connexin that is not gated shut by this kinase (see Discussion)

Rapid turnover of gap junctions from lens cells requires a normally functioning ubiquitin-proteasome system

As in most cell types, a major means by which proteins in the cytosol of lens epithelial and fiber cells are destroyed is via the ubiquitin-proteasome system (UPS) (Huang et al., 1993; Pereira et al., 2003; Zandy and Bassnett, 2007). Degradation of proteins by the UPS is initiated by the enzymatic conjugation of chains of ubiquitin to the substrate, after which they are recognized by the 26S proteasome for proteolytic degradation within the 20S proteasome core (Hershko and Ciechanover, 1998). In several non-lens cell types, an acute increase in the level of novel UPS substrates in the cytosol leads to a decrease in proteasome and/or ubiquitin-mediated degradation of other, less abundant proteins (Bence et al., 2001). Although the mechanisms underlying these effects are unclear and may not be the same for all stimuli, a leading hypothesis is that the increase in the number of clients for the UPS saturates one or more limiting components of the ubiquitination or proteasomal machinery, thereby making it unavailable to mediate the degradation of its normal substrates (Kelly et al., 2007; VanSlyke and Musil, 2002; VanSlyke and Musil, 2005; Morimoto, 2008; Groothuis et al., 2006; Dantuma et al., 2006; Kaganovich et al., 2008). We have shown that small molecule inhibitors of the 20S proteasome reduce the degradation of Cx43 from the surface of non-lens cells (VanSlyke and Musil, 2005). In this regard, Cx43 is similar to several other integral membrane proteins that require the function of the proteasome and/or ubiquitination system to be transported from the plasma membrane to the lysosome for degradation (Longva et al., 2002; van Kerkhof et al., 2001; Melman et al., 2002). Turnover of Cx43 from the cell surface is also slowed by transient overexpression of a misfolded protein, or by subjecting the cells to oxidative or thermal stress (VanSlyke and Musil, 2005). Thus conditions that either directly (20S inhibitors) or indirectly (acutely increasing the level of UPS substrates in the cytosol) compromise the activity of the UPS system against its native clients stabilize Cx43 gap junctions from degradation.

Although not fully understood, an early stage in formation of the organelle-free zone is the permeabilization of organelle membranes, possibly involving 15-lipoxygenase (van Leyen et al., 1998), and fragmentation of organelles (Bassnett, 2002). Loss of membrane integrity would lead to an abrupt entry of organelle components into the cytosol, thereby flooding the cytoplasm with novel proteins that could act as competitive inhibitors of the UPS. It was therefore of interest to investigate whether degradation of lens gap junctions, like Cx43-containing gap junctions in other cell types, requires a normally functioning UPS. We first tested whether gap junctions in DCDMLs are stabilized by small molecule proteasome inhibitors. Brefeldin A (BFA) prevents the export of newly synthesized proteins from the ER and Golgi to the plasma membrane, but does not block endocytosis from the cell surface or subsequent degradation (Klausner et al., 1992). In the presence of BFA, connexins are removed from the cell surface but cannot be replaced with connexins from intracellular pools (VanSlyke and Musil, 2005). Hence the length of time required for connexins to disappear from surface of BFA-treated cells reflects their stability on the plasma membrane. As previously reported (Le and Musil,

2001b), a 3.5 h incubation of DCDMLs with BFA greatly reduced the cell surface immunolabeling of Cx43, Cx45.6, and Cx56 due to their rapid basal rate of degradation (Fig 5A; compare top and middle rows). Immunostaining at cell-cell borders of all three connexins was, however, preserved in cells treated with BFA plus ALLN, a commonly used inhibitor of 20S proteasome function (Fig 5A, bottom row). Under all conditions, the cultures maintained high levels of intercellular contact as assessed by cell-cell interface staining of the adherens junction protein N-cadherin. To determine if the gap junctions spared from degradation by ALLN were functional, cells treated with BFA and/or ALLN were subjected to scrape/load dye transfer analysis. Intracellular passage of Lucifer yellow was markedly greater in cells exposed to both compounds than to BFA alone (Fig 5B).

Although ALLN is widely used to assess the role of the proteasome in cellular processes, it also inhibits certain other proteolytic enzymes expressed in lens cells including calpains and some cathepsins. We therefore repeated the experiments with epoxomicin, a potent and highly specific, irreversible inhibitor of the proteasome that has no activity against such nonproteasomal enzymes (Meng et al., 1999; Marques et al., 2004). The results obtained were essentially identical to those with ALLN (Fig 6). Notably, Alexa594 did not spread from the cells at the wound edge, demonstrating that Cx43 did not significantly participate in gap junction coupling under these conditions. Epoxomicin and another proteasome inhibitor, lactacystin, have previously been reported to slow the degradation of total cellular pools of Cx45.6 in primary cultures of embryonic chick lens epithelial cells in the presence of cycloheximide (Yin et al., 2008).

Next, we examined if rapidly increasing the level of UPS substrates in the cytosol phenocopied the effects of small molecule proteasome inhibitors and reduced the degradation of gap junctions in DCDMLs. We acutely exposed DCDMLs to a heavy metal (arsenic) known to oxidatively denature proteins in the ordinarily reducing environment of the cytosol (Beckmann et al., 1992; Senisterra et al., 1997) without inactivating the 20S proteasome (Stanhill et al., 2006). We used ATO (arsenic trioxide), in current clinical use as a chemotherapeutic agent (Douer and Tallman, 2005; Gazitt and Akay, 2005). DCDMLs treated with BFA and ATO remained viable and maintained cell-cell contact. Intercellular staining for connexins 43, 45.6, and 56 was much greater than in cells treated with BFA alone. Moreover, intercellular transfer of Lucifer yellow was preserved by ATO to a similar extent as by proteasome inhibitors, and gap junctions remained impermeable to Alexa594 (Fig 7).

DISCUSSION

Although the importance of gap junctions for lens clarity is firmly established, how their function is regulated is still incompletely understood. Explanations for why some aspects of GJIC in lens cells are distinct from what has been described in other cell types fall in two general categories. First, such differences could be due to the types of connexins expressed in lens cells. It is well known that connexin family members differ from each other in terms of many functional properties, including their single channel parameters, permselectivities, and mechanisms of channel regulation (Harris, 2001). Second, the lens cell environment could exert an effect on connexins that is distinct from that experienced by connexins in other cell types. For example, both Cx46 and Cx50 (like most other connexins) are gated closed by low pH, which could affect their function in the acidic core of the lens.

That activation of ERK increases instead of blocks GJIC in lens cells seems to be a consequence of the first type of mechanism. Although the permeability of homomeric Cx43 intercellular channels has been well established to be inhibited by ERK activity in many cell types, we show here that such channels do not significantly contribute to Lucifer yellow dye coupling in DCDMLs (Fig 4). This implies that GJIC in lens cells must instead be mediated by a connexin

whose channel function is enhanced by ERK. Consistent with this possibility, a preliminary report by White and colleagues has shown that electrical coupling mediated by Cx50 (the mammalian ortholog of Cx45.6) between paired *Xenopus* oocytes is increased when ERK function is directly upregulated by coexpression of constitutively active MEK (Shakespeare et al., 2007). Transient expression of CA MEK also increases Lucifer yellow coupling in DCDMLs (Le and Musil, 2001b). In Cx50-expressing paired oocytes, as in DCDMLs (Le and Musil, 2001b), enhancement of GJIC downstream of ERK activation is not associated with a detectable increase in connexin protein levels. One possibility is that enhanced channel function is conferred by ERK-dependent post-translational modification of Cx50/45.6. They are, however, unlikely to be direct substrates for ERK given that neither Cx45.6 or its human and mouse counterparts contain high-probability sites for phosphorylation by, or binding of, ERK (as assessed by the Scansite 2.0 Web server; Obenauer et al., 2003).

Our results indicate that FGF increases activation of ERK in DCDMLs via the canonical pathway that includes Ras and Raf-1 (Fig 2 and 3). Xie et al. (2006) have reported that expression of dominant negative RasN17 in the lenses of transgenic mice greatly reduces the levels of activated, but not total, ERK in whole lens lysates. A similar result was observed in mice expressing a truncated form of FGF receptor that acts as a dominant negative inhibitor of FGF signaling (Govindarajan and Overbeek, 2001), consistent with an essential role for Ras in FGF-induced ERK activation in the mammalian lens in vivo. Zatechka and Lou (2002) have reported that FGF enhances the phosphorylation (activation) of Raf-1 and MEK1 in porcine lens cells, as expected if Raf-1 also participates in this pathway.

In contrast to their differing responses to ERK, both fiber connexins behave like Cx43 in terms of their rate of turnover from the plasma membrane in DCDMLs and their stabilization in the presence of proteasome inhibitors (Fig 5 and 6) or after increasing the level of UPS substrates in the cytosol by subjecting the cells to a mild, non-lethal oxidative stress (Fig 7). Thus the absence of Cx43 from mature fiber cells is unlikely to be due to preferential degradation of this connexin during formation of the organelle free zone, and is probably a result of transcriptional downregulation of Cx43 expression as epithelial cells transition into fibers. Based on our findings, we propose a possible model for how fiber connexins avoid degradation during formation of the organelle-free zone. Early in the process, the limiting membranes of the intracellular organelles lose their integrity, causing organellar contents to be released into the cytoplasm. One such organelle-derived protein, the inner mitochondrial membrane resident succinate-ubiquinone oxidoreductase, has been shown to be degraded by the UPS (Zandy and Bassnett, 2007). The fact that this enzyme is recovered in an intact state in the presence of lactacystin implies that it is a direct substrate of the proteasome. It is possible that at least some other organellar constituents are initially cleaved at specific sites by non-proteasome proteases known or suspected to be active in the cytosol during formation of the organelle-free zone (e.g., caspases; calpains). If the resulting protein fragments were stable, they would be expected to be detected in mass spectrometry analyses of mature fibers. This, however, is not what is observed: in the most complete proteome of the human lens compiled to date, at least four organellar proteins (the ER residents Erp29, endoplasmin, ER60, and TER ATPase) were detectable by 2D LC-MS/MS analysis of whole fetal lenses (which contain organelle-rich cells), but none were present in 6/6 samples prepared from nuclear cores of adult lenses (which lack cells with organelles) (Hains and Truscott, 2008). Instead, the available data indicate that organelle-derived proteins are completely destroyed during formation of the organelle-free zone. To our knowledge, the only proteolytic system capable of processively degrading intact proteins as well as protein fragments in a sequence-independent manner is the proteasome. The UPS appears to play a major role in organelle loss in reticulocytes during their transformation into erythrocytes (Haas, 1991). Autophagy, the process by which intracellular membranes and proteins are digested within secondary lysosomes, does not seem to be required for removal of organelles in either lens cells or reticulocytes (Matsui et al., 2006). Deletion of the genes

encoding the executioner caspases had no detectable effect on organelle loss in the lenses of transgenic mice (Zandy et al., 2005). We therefore hypothesize, but have not directly proven, that the permeabilization and fragmentation of organelle membranes causes an acute, massive increase in the number of UPS substrates in the cytosol. This increased load leads to a decrease in the ability of the ubiquitination and/or proteasome machinery to interact with their normal substrates, possibly by saturating the capacity of a widely used, limiting component of these systems. Candidates for this limiting component include the E1 and several members of the E2 (Ubc 2, 4, 5, and/or 7) family of ubiquitinating enzymes and/or the S7 subunit of the 26S proteasome, the levels of each of which are much lower in maturing fibers than in epithelial cells. Notably, Ubc4 was shown to be rate-limiting for de novo formation of polyubiquitinated conjugates in lens cell lysates (Pereira et al., 2003). In this manner, formation of the organelle-free zone phenocopies the effect of proteasome inhibitors on cell surface processes that require an active UPS system, including the rapid uptake of connexins into lysosomes for degradation. Gap junctions are therefore not cleared during formation of the organelle-free zone, after which they are stabilized due to the absence of the endosomal/lysosomal compartments necessary for their destruction (De Maria and Bassnett, 2007). In this view, it is the specialized features of the maturing fiber cell environment, and not the unique properties of the connexins expressed within these cells, that prevents them from being degraded during formation of the organelle-free zone. This preserves GJIC in the lens core, a requirement for maintaining lens clarity.

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Abbreviations

ATO	arsenic trioxide
BFA	brefeldin A
Cx	connexin
DCDMLs	dissociated cell-derived monolayers
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
GJIC	gap junction-mediated intercellular communication
UPS	ubiquitin/proteasome system

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Figure 1. DCDMLs can be efficiently transiently transfected and cotransfected

One-day-old DCDML cultures were transfected with plasmids encoding EGFP, a constitutively active form of MEK (CA MEK), wild type MEK (WT MEK), or B-galactosidase (B gal) as indicated using Lipofectamine. (A) At 20 h, 2 d, 3 d, and 5 d after transfection, expression of EGFP was monitored by fluorescence microscopy. (B) 48 h after transfection, cultures were analyzed for activated ERK using anti-phosphoThr202/Tyr204-specific ERK antibodies detected either immunohistochemically (top row) or by immunoblotting (bottom row). One set of CA MEK-expressing cultures were incubated with the MEK inhibitor UO126 (UO) for 45 min prior to analysis. Inset, level of anti-phosphoERK immunoreactivity in untransfected cultures after a 4 h treatment with 15 ng/ml FGF-2. (C) 48 h after transfection with 0.1 μ g/well CA MEK plasmid plus 0.03 μ g/well B-galactosidase plasmid (a' and b'), or 0.1 μ g/well B-galactosidase only (c'), cultures were double-stained with anti-B-galactosidase and anti-pERK antibodies. Arrows denote doubly-transfected cells. More than 50% of the cells continued to express CA MEK or B-galactosidase on day 5 after transfection with 0.1 μ g/well (not shown).

Figure 2. Role of Ras in ERK activation in DCDMLs

DCDMLs were transfected with the indicated plasmids, and 48 h later assessed for activation of either: ERK-FLAG by anti-FLAG immunoprecipitation followed by anti-pERK immunoblotting (lanes 1–5), or endogenous ERK by anti-pERK immunoblotting of whole cell lysates (lanes 6–8). In some cases, the cultures were treated with 15 ng/ml FGF-2 for 15 min immediately prior to lysis. Asterisk, heavy chain of the immunoprecipitating anti-FLAG immunoglobulin as detected by the secondary antibody used to visualize anti-pERK.

Figure 3. Role of Raf-1 in ERK activation in DCDMLs

DCDMLs were cotransfected with plasmids encoding ERK-FLAG and either RapV12 or the catalytic subunit of protein kinase A (cPKA), both known activators of Rap-1 and inhibitors of Raf-1. 48 h later, cells were treated with or without 15 ng/ml FGF for 15 min prior to lysis and immunoprecipitation of ERK-FLAG. Activation of ERK-FLAG was assessed by anti-pERK immunoblotting. Asterisk, heavy chain of the immunoprecipitating anti-FLAG immunoglobulin as detected by the secondary antibody used to visualize anti-pERK.

Figure 4. Cx43 mediates intracellular dye transfer in CHO cells, but not in DCDMLs

Scrape-load GJIC assays were conducted with a mix of 0.25% Alexa594 (594; permeable to Cx43 gap junctional channels) and either 1% Lucifer yellow (LY; permeable to Cx43, Cx45.6, and Cx56 channels), or 1% FITC-dextran (FITC-dex; impermeable to all gap junctional channels) in: (A) CHO cells or (B) DCDMLs as described in Material and Methods. High magnification views of Alexa594, Lucifer yellow, or FITC-dextran transfer, and their overlay. In (B), DCDMLs were incubated for 48 h with no additions (control), 15 ng/ml FGF-2, 10 ng/ml BMP4, or vitreous body conditioned medium (VBCM). Each panel depicts a portion of the right half of the scrape/load wound.

Figure 5. The proteasome inhibitor ALLN slows the normally rapid turnover of gap junctions in DCDMLs

DCDMLs were incubated for 3.5 h either with no additions (control), with 6 $\mu\text{g/ml}$ BFA, or with BFA plus 200 μM ALLN. The cultures were then immunostained for Cx45.6, Cx56, Cx43, or N-cadherin (A) or assayed for intercellular transfer of Lucifer yellow (B). Transfer of Lucifer yellow in BFA only-treated cells was 0.44 ± 0.55 fold that in untreated control cultures, and transfer of Lucifer yellow in BFA plus ALLN-treated cells was 1.02 ± 0.07 fold that in untreated control cultures (calculated from 12 measurements made in four independent experiments).

Figure 6. A mechanistically distinct, highly specific small molecular inhibitor of the proteasome mimics the effects of ALLN on DCMDL gap junctions

Following a 3.5 h incubation with BFA in the presence or absence of the irreversible proteasome inhibitor epoxomicin, DCDMLs were either assayed for intercellular transfer of Lucifer yellow (LY) and Alexa594 (594) after scrape-loading of the cells with a mixture of the two dyes, or immunostained for Cx45.6, Cx56, or Cx43.

Figure 7. Indirect perturbation of the ubiquitin-proteasome system by oxidative stress stabilizes gap junctions and GJIC in DCDMLs

DCDMLs were incubated for 3.5 h with BFA either in the absence or presence of 60 μ M arsenic trioxide (ATO), which disrupts the folding of oxidatively sensitive proteins within the cytosol. DCDMLs were either immunostained for Cx45.6, Cx56, Cx43, or N-cadherin, or assayed for intercellular transfer of Lucifer yellow and Alexa594 as in Fig 6. Transfer of Lucifer yellow in BFA only-treated cells was 0.35 ± 0.084 fold that in untreated control cultures, and transfer of Lucifer yellow in BFA plus ATO-treated cells was 0.94 ± 0.1 fold that in untreated control cultures (calculated from 11 measurements made in three independent experiments).