Phosphorylation of connexin 43 acts as a stimulus for proteasome-dependent degradation of the protein in lens epithelial cells

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Purpose: The lens is an avascular organ in which gap junctions provide a pathway for intercellular communication, which is vital to maintain lens transparency. Connexin43 (Cx43) is the main gap-junctional protein in lens epithelial cells. Phosphorylation of connexins is implicated in the regulation of intercellular communication. The objective of this report is to determine whether phosphorylation of Cx43 in lens epithelial cells alters its resistance to degradation by a proteasome dependent mechanism.

Methods: Primary cultures of LEC were incubated with protein kinase activator (TPA) and allowed to recover either in the presence or absence of proteasome or lysosome inhibitors. The contribution of the proteasome for the degradation of the phosphorylated form of Cx43 was further investigated by metabolic labeling with $^{32}$P or $^{35}$S-methionine. Subcellular distribution of Cx43 was evaluated by immunofluorescence using antibodies directed against Cx43. Gap junctional intercellular communication was evaluated by transfer of the dye Lucifer yellow.

Results: Inhibitors of proteasome and lysosome both stabilize Cx43, while proteasome inhibitors preferentially stabilize the phosphorylated form of the protein. Pulse chase experiments with $^{32}$P or $^{35}$S-methionine show that while phosphorylation destabilizes Cx43, proteasome inhibitors stabilize the phosphorylated form of the protein. Intercellular communication is inhibited by TPA and can be restored by proteasome inhibitors, probably by preventing loss of Cx43 from the plasma membrane following treatment with TPA.

Conclusions: Our observations support a model in which the combined action of phosphorylation and protein degradation by a proteasome dependent mechanism contribute to regulate Cx43 stability in plasma membrane and intercellular communication through gap junctions, thus adding a novel level of regulation to intercellular communication in lens epithelial cells.

Gap junctions are collections of transmembrane channels that serve as low resistance pathways for diffusion of low molecular mass substances [1]. Exchange of low molecular weight substances through gap junctions is an important way for cells to regulate homeostasis, proliferation and differentiation [2] and maintain homogeneous behavior and function [3,4]. Morphological studies show that the main component of gap-junctions in vertebrates is a family of proteins collectively called connexins. In various tissues, including the lens, adjacent cells communicate through gap junctions involving connexins. The vertebrate eye lens is one of the most important model systems used to study the function and regulation of gap junctions. Being an avascular organ, cells on inner layers of the lens fully depend on the metabolically active cells on the epithelial monolayer in a process that involves extensive networks of gap junctions. In the lens, connexin43 (Cx43) is only expressed in epithelial cells and is subsequently lost, and replaced by other connexins, during the differentiation of epithelial cells into fibers [5]. Lens epithelial monolayer is in direct contact with aqueous humor and contains many transporters and ion pumps. Thus, communication with adjacent cells is critical to regulate homeostasis throughout the lens [6]. The extent of gap junction intercellular communication, in lens as in other organs, is a direct result of the number and functionality of these channels [7,8].

In addition to regulation of connexin synthesis through transcriptional control, other and more rapid mechanisms are involved in regulation of gap junction intercellular communication such as altered subcellular localization and post-transcriptional modification [9,10]. One frequent post-transcriptional modification observed in connexins, including connexin43 (Cx43), consists on phosphorylation by various protein kinases [11]. One functional feature of phosphorylation involves either stabilization or destabilization of various connexins in different systems [12,13]. Connexin phosphorylation is also involved in protein trafficking and gap junction assembly [11].

Both the proteasome and lysosome have been implicated in Cx43 degradation [14]. Degradation of Cx43 by the lysosome is not surprising, given that the majority of membrane proteins are degraded in lysosomes. Although degradation of Cx43 by the proteasome in the secretory pathway has recently been demonstrated [15], the participation of the proteasome in degradation of Cx43 in plasma membrane comes more as a surprise. Proteasome consists of a multicatalytic protease complex that degrades rapid turnover proteins by a process that is...
generally dependent on ubiquitin and ATP [16]. The rate of Cx43 turnover has consistently been shown to be decreased by proteasome inhibitors in various cell types [14,17]. Additionally, proteasome inhibition is associated with an increase in gap junctional plaque assembly and intercellular dye transfer [18]. The role and functional implications of connexin phosphorylation in the lens is complex. For instance, activation of protein kinase C (PKC) in lens epithelial cells was shown to inhibit gap junction communication [8,19]. In contrast, phosphorylation of vertebrate lens chick fiber Cx56 is involved in stabilization of the protein [20].

The implications of Cx43 phosphorylation on the protein resistance to degradation by a proteasome dependent pathway have not been addressed before, nor have the functional implications of this mechanism been evaluated in lens epithelial cells.

**METHODS**

**Cell culture:** Eyeballs were removed from adult bovines and the anterior capsule of the lens, with the attached epithelium, was cut along the equator and cultured in a 24 well-plate containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL Life Technologies, Inchinnan, UK), 100 i.u./ml penicillin, 100 µg/ml streptomycin, at 37 °C with 5% CO₂. The epithelial cells were then allowed to spread out from the capsule into the plate. The cells were used until passage four.

**Antibodies and reagents:** The rabbit polyclonal anti-Cx43 antibodies were obtained from Zymed (San Francisco, CA, USA). The mouse monoclonal anti-Cx43 antibodies were obtained from Zymed (San Francisco, CA, USA) and BD Transduction Laboratories (San Diego, CA, USA). The mouse anti-Cx43 monoclonal antibody obtained from Zymed was raised against a peptide sequence that represents amino acid residues 360-376 of Cx43, while mouse anti-Cx43 monoclonal antibody obtained from Transduction Labs was raised against the peptide sequence corresponding to the amino acid residues 252-270 of Cx43. Unless otherwise noted, all other reagents were from Sigma, except MG132 that was obtained from Calbiochem (Darmstadt, Germany). TPA (12-O-tetradecanoylphorbol 13-acetate) and MG132 were dissolved from Calbiochem (Darmstadt, Germany). TPA (12-O-tetradecanoylphorbol 13-acetate) and MG132 were dissolved in dimethyl sulfoxide (DMSO).

**Metabolic labeling and immunoprecipitation:** Lens epithelial cells were rinsed with phosphate-free medium and subsequently labeled with 32 P (Amersham, Buckinghamshire, England) at 1.0 mCi/ml for 3 h in phosphate-deficient medium. Cells were treated with 50 ng/ml TPA during the final 30 min of pulse. The cells were then chased in DMEM supplemented with 0.5 mM unlabelled methionine, in the presence or absence of MG132, for 1 h. The samples were then processed and immunoprecipitated as described above.

**Western blotting and autoradiography:** Proteins were denatured by incubation for 30 min at 37 °C with Laemmli buffer and separated by SDS-PAGE either on 10% gel for western blotting or 12% gel for autoradiography. For western blot analysis the proteins were transferred to a PVDF membrane, and labeled with monoclonal antibodies to Cx43. Alternatively, for metabolic labeling experiments the gels were dried and autoradiographed. The dried gels were quantitated with a PhosphorImage analyser (STORM 860; Molecular Dynamics) and Image Quant 5.0 software (Molecular Dynamics). The intensity of the bands after Western blotting was determined by laser scanning of the films followed by quantitative densitometric analysis using NIH Image software. All results are representative of at least three experiments.

**Phosphatase treatments:** The dephosphorylation reactions were carried out in lysis buffer following overnight incubation at 37 °C in the presence of alkaline phosphatase (5 units) from *E. coli* (Sigma, St Louis, MO).

**Immunofluorescence:** Cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline solution (PBS). The samples were then washed with PBS, permeabilised with 1% v/v Triton X-100 in PBS, and blocked with goat serum (1:10) for 20 min prior to incubation with primary antibodies. Incubation with primary antibodies proceeded for 1 h at room temperature. The samples were then washed three times with PBS before incubation with the secondary antibody for 1 h at room temperature. The specimens were rinsed in PBS and mounted with Glycergel (Dako, Carpinteria, CA). All solutions were made up in 0.2% w/v BSA (Sigma) containing 0.02% sodium azide (Sigma) in PBS. For controls, primary antibodies were omitted.

**Dye transfer assay for gap junctional intercellular communication:** Lens epithelial cells grown on glass coverslips were assayed for gap junction-mediated intercellular coupling by dye transfer assay. Briefly, the culture medium from a confluent monolayer of lens epithelial cells (LEC) 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 multiple scrapes through the cell monolayer in the presence of Dulbecco’s phosphate buffered saline containing 0.5% rhodamine-dextran and 0.5% lucifer yellow. After exactly 1 min, the culture was rinsed three times with HBC and then incubated for an additional 8 min in the saved culture medium to allow the loaded dye to transfer to adjoining cells. The cells were then rinsed and fixed with 4% paraformaldehyde and imaged on a fluorescence microscope with a UV light source. The efficiency of intercellular communication through gap-junctions was quantified as the average distance traveled by the dye Lucifer yellow, 8 min after the dye loading.

Statistics: All results are representative of at least three experiments. Data are expressed as a sample mean±standard deviation (SD). Different samples were compared by using the Student’s t test and two-tailed probability (p). Values of p<0.05 were considered to be statistically significant.

RESULTS

In this study we have used primary cultures of bovine lens epithelial cells to investigate the role of phosphorylation on degradation of Cx43. Lens epithelial cells were treated with TPA (50 ng/ml), a known activator of PKC. Data represented in Figure 1A shows that Cx43 appears as two closely migrating bands. The slower migrating band, presumably corresponds to the phosphorylated form of the protein and represents 9±1.7% of the total Cx43 present in the cells. After 30 min of treatment with TPA there is a two-fold increase (19±3.8%) in the relative amount of the slower migrating band (Figure 1A, lane 2). On the other hand, densitometric analysis of Figure 1A show that the total amount of Cx43 decreases 19% (from 100 to 81±12.3%) following treatment with TPA. Treatment with alkaline phosphatase results in the total disappearance of the slower migrating band, with the concomitant increase of the faster migrating band, indicating that the slower migrating band corresponds to a phosphorylated form of Cx43 (Figure 1B). Moreover, the upper band cross-reacts with antibodies to phosphoserine residues (data not shown). To further investigate the mechanism responsible for the degradation of the phosphorylated forms of Cx43, lens epithelial cells were exposed to TPA for 30 min, in the presence of the proteasome inhibitor 40 µM MG132 (or 10 µM lactacystin) or the lysosomal inhibitor 10 mM NH₄Cl. Data represented in Figure 1C clearly shows the formation of a phosphorylated band following incubation with TPA either in the presence of MG132 (lane 1) or NH₄Cl (lane 5). In both cases the phosphorylated form of Cx43 corresponds to about 20% of the total amount of Cx43 present (21±3.4% for MG132 and 18±2.9% for NH₄Cl; Figure 1C). To further determine the involvement of the proteasome and the lysosome in degradation of the phosphorylated form of Cx43, the medium containing TPA was removed and the cells were allowed to “recover” for two h in DMEM in the presence of either MG132 (Figure 1C, lane 2) or NH₄Cl (Figure 1C, lane 4). Controls, consisting of cells incubated with TPA for 30 min, were allowed to “recover” in the absence of proteolytic inhibitors (Figure 1C, lane 3). The...
results obtained show that, when cells recover in the presence of the proteasome inhibitor the phosphorylated band is significantly stabilised (Figure 1C, lane 2) as compared to cells that “recovered” in the presence of lysosomal inhibitor NH4Cl (Figure 1C, lane 4) and as compared to controls that recovered in the absence of proteolytic inhibitors (Figure 1C, lane 3). Indeed, in control cells the phosphorylated band totally disappeared after 2 h, and lysosome inhibitors led to a decrease in the relative amount of the phosphorylated form of Cx43 from 18±2.9% to 6±0.9% (Figure 1C). However, when cells recovered in the presence of the proteasome inhibitor MG132 there was no detectable decrease in the relative amount of the phosphorylated form of Cx43. Similar data was obtained when MG132 was replaced by the more specific proteasome inhibitor lactacystin β-lactone and when a different monoclonal antibody to Cx43 was used (data not shown).

To further confirm that Cx43 is phosphorylated in vivo and that the phosphorylated forms of the protein accumulate preferentially in the presence of proteasome inhibitors, lens epithelial cells were pulse labeled with [32P]-orthophosphate for three h. For the last 30 min of incubation, TPA was added to the medium to activate PKC. The cells were then chased for 1 and 3 h with non-labeled medium either in the presence of MG132 (or lactacystin) or NH4Cl or in the absence of proteolytic inhibitors. Following immunoprecipitation with anti-Cx43 antibodies the samples were separated by SDS-PAGE and autoradiographed. Figure 2 shows that a specific band was immunoprecipitated that is attributed to phosphorylated form of Cx43. Following three h of chase with unlabelled medium in the absence of proteolytic inhibitors the phosphorylated form of Cx43 has dropped to undetectable levels (Figure 2A, lane 3). However, when cells were chased in the presence of proteasome inhibitors the phosphorylated form of Cx43 appears to be partially stabilized (Figure 2B, lane 3). In fact, after the 3 h of chase, 20±2.7% of the phosphorylated protein is still present in the cells. This data indicates that half life of the phosphorylated Cx43 is extended by proteasome inhibitors. Significantly, lysosome inhibitors do not stabilize the phosphorylated form of the protein. In fact, cells that were chased in the presence of NH4Cl present undetectable levels of phosphorylated Cx43 after 3 h (lane 3 of Figure 2C).

To evaluate the effect of proteasome inhibitor on the metabolic stability of Cx43, lens epithelial cells were pulse labeled with [35S]-methionine for 1 h, either in the presence or absence of TPA. The cells were then chased for 1 h in the presence or absence of the proteasome inhibitor MG132 and subsequently immunoprecipitated with anti-Cx43 antibodies. The data presented in Figure 3 shows that phosphorylation of Cx43 increases its susceptibility to degradation. In fact, phosphorylation results in a decrease of 73±6.1% in the levels of the protein after 1 h of chase, as compared to a decrease of 29±1.9% in controls (compare lane 2 with lane 6 in Figure 3).

However, when the cells were pulse labeled in the presence of TPA and chased in presence of proteasome inhibitor, Cx43 is stabilized (compare lane 2 with lane 4 in Figure 3). Controls incubated in the absence of TPA show that 29±1.9% of Cx43 is degraded after 1 h of chase (lanes 5 and 6 in Figure 3), whereas in the presence of MG132 there is no detectable degradation after 1 h of chase (lanes 7 and 8 in Figure 3). The functional implications of increased Cx43 degradation following phosphorylation was investigated in primary cultures of lens epithelial cells by scrape loading/dye transfer assays. The intercellular communication was quantified as the distance traveled by the dye Lucifer yellow 8 min following loading and the results obtained are presented in a histogram (Figure 4I). Incubation of the cells with proteasome inhibitors results in a 50±4.7% increase in intercellular communication through gap junctions (Figure 4D). This increase in intercellular com-

![Figure 2](http://www.molvis.org/molvis/v9/a5)
munication appears to be the direct result of the accumulation of functional Cx43 in the plasma membrane of epithelial cells. Indeed, Figure 4C shows an increased localization of Cx43 to plasma membrane following treatments with proteasome inhibitors. Not surprisingly, phosphorylation of Cx43, by stimulation of PKC with TPA, for 30 min, had the opposite effect, completely inhibiting dye transfer to adjacent cells (Figure 4F). This was concomitant with the decrease in the amount of Cx43 in plasma membrane as revealed by immunofluorescence (Figure 4E). The decrease in intercellular communication induced by TPA can be reverted by the proteasome inhibitor MG132. In fact, results reported in Figure 4 show that on the presence of proteasome inhibitors TPA induced only a 40±5.1% decrease in cell-cell communication (compare Figure 4F with Figure 4H). Consistently, proteasome inhibitors partly prevent disappearance of Cx43 from plasma membranes (Figure 4G) presumably by preventing the rapid degradation of its phosphorylated form, thus contributing to maintain intercellular communication.

**Figure 3.** Effect of proteasome inhibitors on the half life of Cx43 following phosphorylation. A: LEC were labeled with ^35^S for 1 h either in the presence or absence of 40 µM MG132. For the last 30 min of labeling, cells were treated with 50 ng/ml TPA. For controls, cells were metabolically labeled in the absence of TPA and MG132. The cells were chased for 1 h with non-labeled medium and Cx43 was immunoprecipitated using polyclonal antibodies against Cx43. Immunoprecipitated proteins were separated by SDS-PAGE and autoradiographed. B: Quantification of the amount of ^35^S labeled Cx43 after 1 h of chase using a PhosphorImage analyser, Storm 860. Data represents the average with standard deviation of three independent experiments.

**DISCUSSION**

Prior studies have shown that Cx43, as other connexins, is phosphorylated in various cells and tissues [11,21-24]. It is also established that the proteasome and lysosome participate in degradation of Cx43, at least in some types of cells [14]. The relationship between connexin phosphorylation and degradation still remains elusive for many cell systems and more so for the lens where no such information is available. Treatment of ovine lens epithelial cells with TPA has been demonstrated to induce a significant decrease in the amount of Cx43 [8]. Additionally, Lampe and Lau have suggested that phosphorylation can be involved on targeting Cx43 for degradation [11].

Recent data obtained with leptomeningeal cells showed that while the turnover of Cx43 is 2.7 h, the half-life of the phosphorylated form is reduced to 1.7 h [25], suggesting that phosphorylated Cx43 is a substrate for a rapid proteolytic pathway. These observations support the major conclusions of the present study by suggesting a close association between Cx43 phosphorylation and protein degradation.

It has been clearly demonstrated that TPA does not alter the levels of mRNA for Cx43 [8]. Therefore, the possibility that TPA leads to a decrease in Cx43 content by interfering with protein synthesis can be excluded. The decrease of Cx43 concentration following treatment with TPA observed in this study can be attributed to a higher rate of protein degradation. More importantly, data presented in this report indicates that phosphorylated Cx43 is degraded by a proteasome dependent pathway. It was not until recently that the ubiquitin-proteasome pathway was shown to participate in degradation of membrane proteins, although most of this degradation occurs in the secretory pathway. For example, it was recently demonstrated that misfolded connexins are substrates for the ER-associated degradation (ERAD), a proteolytic event that is carried out by the 26S proteasome [15]. Although phosphorylation of Cx43 occurs at multiple sites within the cell, the major phosphorylated forms of the protein are formed when the Cx43 reaches the cell membrane [26]. Therefore it is unlikely that phosphorylation has a role in ERAD of Cx43.

It has been reasonably established that activation of PKC results in decreased intercellular communication through gap junctions [8,27] and that inhibition of the proteasome leads to an accumulation of connexin in membrane and results in increased intercellular communication [18]. Our results support a model in which in vivo phosphorylation leads to an increased degradation of Cx43 by a mechanism involving the proteasome and that such degradation is responsible, at least in part, for the decreased intercellular communication observed in response to activation of PKC. Conversely, if the proteasome is inhibited, phosphorylated, and presumably some non-phosphorylated, Cx43 accumulates in membrane thus accounting for the increased intercellular communication observed in LEC in this study and by other authors in different cell systems [18]. It was reported years ago that PKC activator TPA, inhibits its communication in ovine lens epithelial cells through gap junctions and that this inhibition was followed by the gradual...
disappearance of Cx43 from cell interfaces [8]. More recently it was shown that activation or overexpression of PKCγ (but not other isoforms) results in a disassembly of gap junctions and a decrease in connexin 43 gap junctions at cell surface [28]. These observations support the results presented in this report by suggesting an association between PKC (presumably PKCγ) induced inhibition of intercellular communication, Cx43 phosphorylation and loss of connexin at cell surface. The results presented in this study show that proteasome inhibitors partly restore intercellular communication following inhibition by TPA. The higher levels of Cx43 present in cell interfaces may account for the increased intercellular communication observed under these circumstances.

Connexin phosphorylation is a common event in most cells with important implications in a variety of cellular processes such as trafficking, assembly/disassembly, gating of gap junction channels, and presumably altered susceptibility to degradation [11]. Degradation of phosphorylated Cx43 has been reported to be correlated with the rapid turnover/disassembly of gap junction plaques, with the consequent decrease in intercellular communication [29-31]. Therefore, we cannot exclude the possibility that Cx43 phosphorylation could be a “tag” to destabilize gap junction plaques, by a mechanism involving the proteasome. Indeed, degradation by the proteasome of a protein involved in gap junction assembly could be necessary for Cx43 internalisation and subsequent degradation.

Data presented in this report support a model in which rapid degradation of Cx43 in a proteasome-dependent step may constitute an additional regulatory mechanism for intercellular communication in the lens. Activation of one or more protein kinases would constitute a triggering signal targeting Cx43 for degradation in a process that is dependent on the proteasome. Degradation of membrane Cx43 and the resulting decrease in intercellular communication could, for example, provide the means for the rapid isolation of the cells

Figure 4. Effect of phosphorylation and proteasome inhibitors on intercellular communication and subcellular distribution of Cx43. LEC were either treated with 40 µM MG132 (C and D) for 1 h or 50 ng/ml TPA (E and F) for 30 min; simultaneous treatment with MG132 and TPA was performed by incubation with 40 µM MG132, for 1 h, and 50 ng/ml TPA was added for the last 30 min (G and H). Cells incubated in the absence of TPA and MG132 were used as controls (A and B). The cells were then either assayed for intercellular communication by Lucifer yellow dye transfer, after scrape loading (B, D, F, and H, magnification 200x), or fixed and stained with antibodies directed against Cx43 and imaged by confocal microscopy (A, C, E, and G). Intercellular communication was evaluated as the average distance traveled by the dye Lucifer yellow along the monolayer and is represented as a histogram (I). The values are the average of five individual experiments with a coefficient of variation typically inferior to 6%. 

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preventing the spread of potentially noxious effects to adjacent cells. The enzymes and intrinsic mechanisms whereby Cx43 eventually gets targeted for degradation in response to phosphorylation remain unknown.

ACKNOWLEDGEMENTS

This work was supported by grants from Portuguese Foundation for Science and Technology-FCT (Programme POCTI).

REFERENCES


The print version of this article was created on 30 Jan 2003. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.