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The Proteasome Regulates the Interaction Between Cx43 and ZO-1

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Abstract Gap junction (GJ) intercellular communication (GJIC) is vital to ensure proper cell and tissue function. GJ are multimeric structures composed of proteins called connexins. Modifications on stability or subcellular distribution of connexins have a direct impact on the extent of GJIC. In this study we have investigated the role of the proteasome in regulation of connexin 43 (Cx43) internalization. Although the participation of both the proteasome and lysosome has long been suggested in Cx43 degradation, the molecular mechanisms whereby proteasome contributes to regulate Cx43 internalization and intercellular communication are still unclear. The results presented in this study envision a new mechanism whereby proteasome regulates GJIC by modulating interaction between Cx43 and ZO-1. Immunoprecipitation experiments, in the presence of proteasome inhibitors, together with immunofluorescence data indicate that the proteasome regulates interaction between Cx43 and ZO-1. Overexpression of the PDZ2 domain of ZO-1 and the expression of Cx-43 fused in frame with a V5/HIS tag, suggest that interaction between the two proteins occurs through the PDZ2 domain of ZO-1 and the C-terminus of Cx43. When interaction between Cx43 and ZO-1 is reduced, as in the presence of proteasome inhibitors, Cx43 accumulates, forming large GJ plaques at plasma membrane. Data presented in this article suggest a new pathway whereby alterations in proteasome activity may impact on GJIC as well as on non-junctional communication with extracellular environment, contributing to cell and tissue dysfunction. J. Cell. Biochem. 102: 719–728, 2007. © 2007 Wiley-Liss, Inc.

Key words: connexin43; gap junction; proteasome; ubiquitin; ZO-1; endocytosis

Gap junctions (GJ) are intercellular channels that permit the passage of small molecules such as metabolites, ions, and second messengers [Kumar and Gilula, 1996]. Exchange of low molecular weight substances through gap junctions is an important mechanism for cells to regulate homeostasis, proliferation, differentiation and to ensure homogeneous tissue function [Simon and Goodenough, 1998; Matemba et al., 2006]. Gap junctions consist of two hemichannels, called connexons, that are located in the plasma membrane of two adjacent cells. Each connexon is composed of six subunits of a protein called connexin (Cx).

The extent of gap junction intercellular communication is a direct result of the number and functionality of these connexin-based pores. In addition to regulation by transcriptional control, other rapid mechanisms are also involved in regulation of gap junction intercellular communication. These mechanisms include altered subcellular localization and a variety of post-transcriptional modifications such as phosphorylation and rapid degradation of connexins. Both the proteasome [Laing and Beyer, 1995; Beardslee et al., 2000; Musil et al., 2000; Girao and Pereira, 2003; Fernandes et al., 2004 Laing et al., 2005] and lysosome [Laing et al., 1997; Musil et al., 2000] have been implicated in degradation of Cx43. Whereas degradation of Cx43 by the lysosome has long been established, the role of the proteasome in Cx43 turnover is still poorly understood. Initial studies suggested that the proteasome could be directly involved in connexin degradation [Laing and Beyer, 1995]. However, more recently, it has been hypothesized that proteasome acts by regulating the stability of Cx43 at the plasma membrane, most likely by degrading a connexin-interacting

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protein [Musil et al., 2000; Girao and Pereira, 2003]. Indeed, it has been demonstrated that proteasome inhibition is associated with an increase in gap junctional plaque assembly and intercellular dye transfer, suggesting that this proteolytic complex is somehow involved in internalization of Cx43.

Cx43 is known to interact with several proteins, including cytoskeletal proteins and anchoring proteins, such as E-cadherin [Fujimoto et al., 1997], caveolin-1 [Schubert et al., 2002], and ZO-1 [Giepmans and Moolenaar, 1998; Toyofuku et al., 1998; Wu et al., 2003] that may regulate channel assembly and/or the stability of Cx43 at the plasma membrane. The interaction of Cx43 with zonula occludens (ZO-1) has been studied in great detail and several studies have focused on the mechanisms and functional implications of such interaction. ZO-1 is a member of the membrane-associated guanylate kinase (MAGUK) family, that is, important in regulation of signal transduction, protein targeting and control of cell polarity [Gonzalez-Mariscal et al., 2000; Harhaj and Antonetti, 2004]. Immunoprecipitation and yeast-two-hybrid experiments have demonstrated that interaction between ZO-1 and Cx43 occurs through the last five aminoacids (DDLEI) of the C-terminus (CT) of Cx43 and the second PDZ domain (PDZ2) of ZO-1 [Giepmans and Moolenaar, 1998; Giepmans et al., 2001; Singh and Lampe, 2003; Jin et al., 2004; Sorgen et al., 2004]. Moreover, studies using mutant connexin peptides that lack the CT binding domain or in which the CT is blocked, revealed that formation of GJ can occur without interaction of Cx43 with ZO-1 [Jordan et al., 1999; Bukauskas et al., 2000; Toyofuku et al., 2001b]. However, the GJ formed under such conditions are abnormally large and some of the channels are inactive, suggesting that ZO-1 is somehow involved in regulation of GJ function [Hunter et al., 2003; 2005; Jin et al., 2004]. The implications and the physiological role of the interaction between Cx43 and ZO-1 remain controversial. Early studies hypothesized that ZO-1 could act as an anchor stabilizing GJ at plasma membrane, most likely through cytoskeletal anchoring [Toyofuku et al., 1998, 2001b]. More recently, it was demonstrated that interaction between Cx43 and ZO-1 is a dynamic process that is involved in the regulation of Cx43 endocytosis [Barker et al., 2002; Duffy et al., 2004; Segretain et al., 2004]. However, the precise role of ZO-1 in Cx43 endocytosis is still controversial. Indeed, studies carried out in Sertoli cells and cardiac myocytes showed that Cx43 interacts more strongly with ZO-1 during endocytosis of GJ [Barker et al., 2002; Segretain et al., 2004]. Conversely, data obtained by Duffy suggested that internalization of GJs, induced by intracellular acidification, is facilitated by dissociation of Cx43 from ZO-1 [Duffy et al., 2004].

More recently it was demonstrated that interaction between Cx43 and ZO-1 might act as a regulator of the size of GJ, by controlling the rate of channel accretion at plaque periphery [Hunter et al., 2005]. Indeed, when interaction between Cx43 and ZO-1 is reduced, such as by the attachment of a tag to the C-terminus of Cx43 or by the use of a peptide inhibitor that contains the Cx43 PDZ-binding domain, there is a reduction of ZO-1 peripherally associated with GJ and a significant increase in plaque size.

However, the regulation of the interaction between ZO-1 and Cx43 still remains to be elucidated. In this study we provide evidence that the proteasome plays an important role in regulating interaction between Cx43 and ZO-1. Indeed, the data presented in this study show that proteasome inhibition results in a decreased interaction between Cx43 and ZO-1. Moreover, the presence of a tag in the C-terminus of Cx43, that prevents interaction with ZO-1, reverts the MG132-induced accumulation of large Cx-43 plaques at the plasma membrane. These data suggest that efficient interaction between Cx43 and ZO-1 is regulated by the proteasome, presumably by involving degradation of a putative Cx43 binding protein.

MATERIALS AND METHODS

Antibodies

The rabbit anti-Cx43 polyclonal antibody, obtained from Zymed was raised against a peptide corresponding to a segment of the third cytoplasmic domain (C-terminal portion) of rat Cx43 (Cat No 71-0700). The mouse anti-Cx43 monoclonal antibody obtained from Zymed was raised against a cytoplasmic sequence located near the C-terminus of rat Cx43 (Cat No 13-8300). The mouse anti-Cx43 monoclonal antibody obtained from BD Tranduction Laboratories was raised against a peptide sequence that represents amino acid residues 252–270 of

rat Cx43. Polyclonal antibodies against ZO-1 were obtained from Zymed.

Culture and Treatment of Cells

NRK cells or HEK293 cells were cultured in Dulbecco's Modified of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. To determine the role of the proteasome on Cx43 interaction with ZO-1, cells were incubated, for 2 h, in the presence of the proteasome inhibitors MG132 (40 μM) or Lactacystin (10 μM).

Cell Transfection

Transient transfections of HEK 293 cells were performed by incubating the cells with Lipofect-AMINE reagent in low serum medium (Life Technologies), during 6 h. Plasmids encoding full length Cx43 or HIS-V5-tagged Cx43 were cloned into the pcDNA3.1/V5/His vector (Invitrogene).

Immunoprecipitation and Western Blotting

NRK cells or HEK293 transfected cells were rinsed with PBS at 4°C, ressuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3) supplemented with protease inhibitor cocktail (Roche), 2 mM PMSF, 10 mM iodacetamide, 50 mM NaF, 500 μM $NaVO_4$, and incubated on ice during 30 min. The samples were then centrifuged at 10,000*g*, during 10 min, and the supernatants used for immunoprecipitation. Briefly, protein A was incubated with polyclonal antibodies directed against Cx43 (Zymed, Cat No 71-0700), for 1 h, at 4°C or nonimmune rabbit serum, followed by incubation with supernatants, for 3 h at 4°C. The samples were then centrifuged and the protein A-sepharose sediments ressuspended in Laemmli buffer and denatured at 37°C, for 30 min.

For Western blot analysis of the immunoprecipitated proteins, samples were separated by SDS–PAGE, transferred to a PVDF membrane, and labeled with monoclonal antibodies to Cx43 (Zymed, Cat No 13-8300) or polyclonal antibodies to ZO-1.

Immunofluorescence

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline PBS. The samples were then washed with PBS, permeabilised with 0.2% 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 against Cx43 (Transduction Lab) and ZO-1 (Zymed) 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). 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. The images were collected by confocal microscopy using a Carl Zeiss LSM410 system and Bio-Rad MRC-600.

RESULTS

Previous studies showed that inhibition of Cx43-ZO-1 interaction results in a significant increase in the size of GJ plaques [Hunter et al., 2003; 2005; Jin et al., 2004]. On the other hand, it was shown that proteasome inhibition leads to the accumulation of Cx43 at the plasma membrane, with the formation of large plaques [Musil et al., 2000; Girao and Pereira, 2003; Fernandes et al., 2004]. This led us to hypothesize that an active proteasome is required to regulate the interaction of Cx43 with ZO-1.

To investigate the role of the proteasome in Cx43-ZO-1 interaction, NRK cells, that endogenously express Cx43 and ZO-1, were incubated either in the presence or absence of the proteasome inhibitor MG132 (or lactacystin), for 2 h. To further evaluate the association of Cx43 and ZO-1, Cx43 or ZO-1 was selectively immunoprecipitated using polyclonal antibodies against each of these proteins, followed by Western blot and probing with antibodies

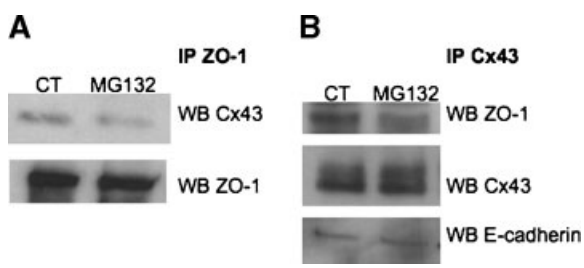

Fig. 1. Proteasome inhibitors reduce the interaction between Cx43 and ZO-1. Endogenous Cx43 from NRK cells was immunoprecipitated using polyclonal antibodies against ZO-1 (**A**) or Cx43 (**B**). Immunoprecipitated material was resolved by SDS–PAGE and immunoblotted with antibodies against Cx43 (A) or ZO-1 and E-cadherin (B).

against ZO-1 or Cx43, respectively (Fig. 1). The results obtained clearly show that, in the cells incubated with MG132 (or lactacystin), the amount of Cx43 immunoprecipitated with anti-ZO-1 antibodies (Fig. 1A), and the amount of ZO-1 immunoprecipitated with anti-Cx43 antibodies (Fig. 1B), is lower than in control cells. These results demonstrate, for the first time, that proteasome inhibition leads to a decreased interaction between Cx43 and ZO-1.

To further investigate if the effect of proteasome inhibitors in binding of Cx43 to ZO-1 is specific, cell lyzates were immunoprecipitated with anti-Cx43 antibodies and were subsequently Western blotted with antibodies against E-cadherin, which is known to interact with Cx43. The results presented in Figure 1B show that interaction between Cx43 and E-cadherin is not affected by proteasome inhibitors. This suggests that it is not likely that proteasome inhibitors are unspecifically disrupting interaction between Cx43 and its binding partners. To verify that the effect of MG132 on the amount of immunoprecipitated proteins is not due to variations in the initial amount of proteins, we reprobed the same membranes with the antibodies used in the immunoprecipitation assays. The results show that the amount of Cx43 and ZO-1 are not significantly altered following incubation with MG132. In the immunoprecipitated Cx43 there is an accumulation of slower migrating bands that correspond to the phosphorylated forms of the protein (Fig. 1B, middle panel). Additional controls were generated by probing the membranes, containing extracts before immunoprecipitation with Ponceau S or with antibodies against actin. In both cases the staining for the different samples was comparable, indicating equal sample loading (data not shown).

To further confirm the decrease of Cx43-ZO-1 interaction induced by proteasome inhibitors, NRK cells, that endogenously express Cx43, were incubated either in the presence or absence of MG132 (or lactacystin), and then fixed and stained with monoclonal antibodies directed against Cx43 and polyclonal antibodies against ZO-1. Cells were subsequently imaged by immunofluorescence confocal microscopy. The results show that, in controls, the majority of Cx43 colocalizes with ZO-1 (Fig. 2A a–c) at the plasma membrane, while in cells incubated with MG132 it is possible to observe the formation of large punctate staining (Fig. 2A d–f; see arrows) corresponding to GJ plaques that do not colocalize with ZO-1 (Fig. 2A f; see arrows). To confirm that this does not result from the fixation procedure, in addition to PFA, cells were also fixed with methanol/acetone (Fig. 2B). Taken together, these results suggest that efficient interaction between Cx43 and ZO-1 requires an active proteasome. Based on this observation and previous reports it is possible to speculate that proteasome inhibitors may prevent degradation of a putative Cx43 interacting protein, thus preventing an efficient association between Cx43 and ZO-1.

To confirm that the binding of a protein to the C-terminus of Cx43 is sufficient to disrupt or weaken its interaction with ZO-1, we added a tag to the C-terminus of Cx43. HEK239 cells, that do not endogenously express Cx43 [Toyofuku et al., 1998, 2001a,b], were transiently transfected with a plasmid containing the cDNA for the full length of Cx43 with or without a V5/His tag at the C-terminus. Cx43 was subsequently immunoprecipitated and the amount of ZO-1 that co-precipitated was assessed by Western blot. The results presented in Figure 3 show that the amount of ZO-1 that co-precipitates with Cx43 significantly decreases when a tag is added to the C-terminus of Cx43. The observation that a tag in the C-terminus of Cx43 reduces but not completely abolishes the interaction with ZO-1, can reflect either the residual expression of connexins with a PDZ binding motif in HEK293 cells or that a week interaction with ZO-1 still occurs in the absence of a PDZ binding domain in Cx43. To confirm that the levels of expression of both forms (tagged or untagged) of Cx43 were similar, the membranes were reprobed with antibodies to Cx43.

To further confirm that interaction of Cx43 with ZO-1 occurs through the PDZ2 domain of ZO-1, HEK293 cells were co-transfected with the untagged form of Cx43 and the PDZ2 domain of ZO-1. The PDZ2 domain of ZO-1 is the portion of the protein that interacts with the last 5 aminoacids of the C-terminus of Cx43. Thus, overexpression of the PDZ2 domain is likely to act as a competitive inhibitor for the binding of ZO-1 to the C-terminus of Cx43. As shown in Figure 4A the amount of ZO-1 that coprecipitates with Cx43 is lower in cells that overexpress the PDZ2 domain as compared to controls. These results were further confirmed by immunofluorescence. Indeed, overexpres-

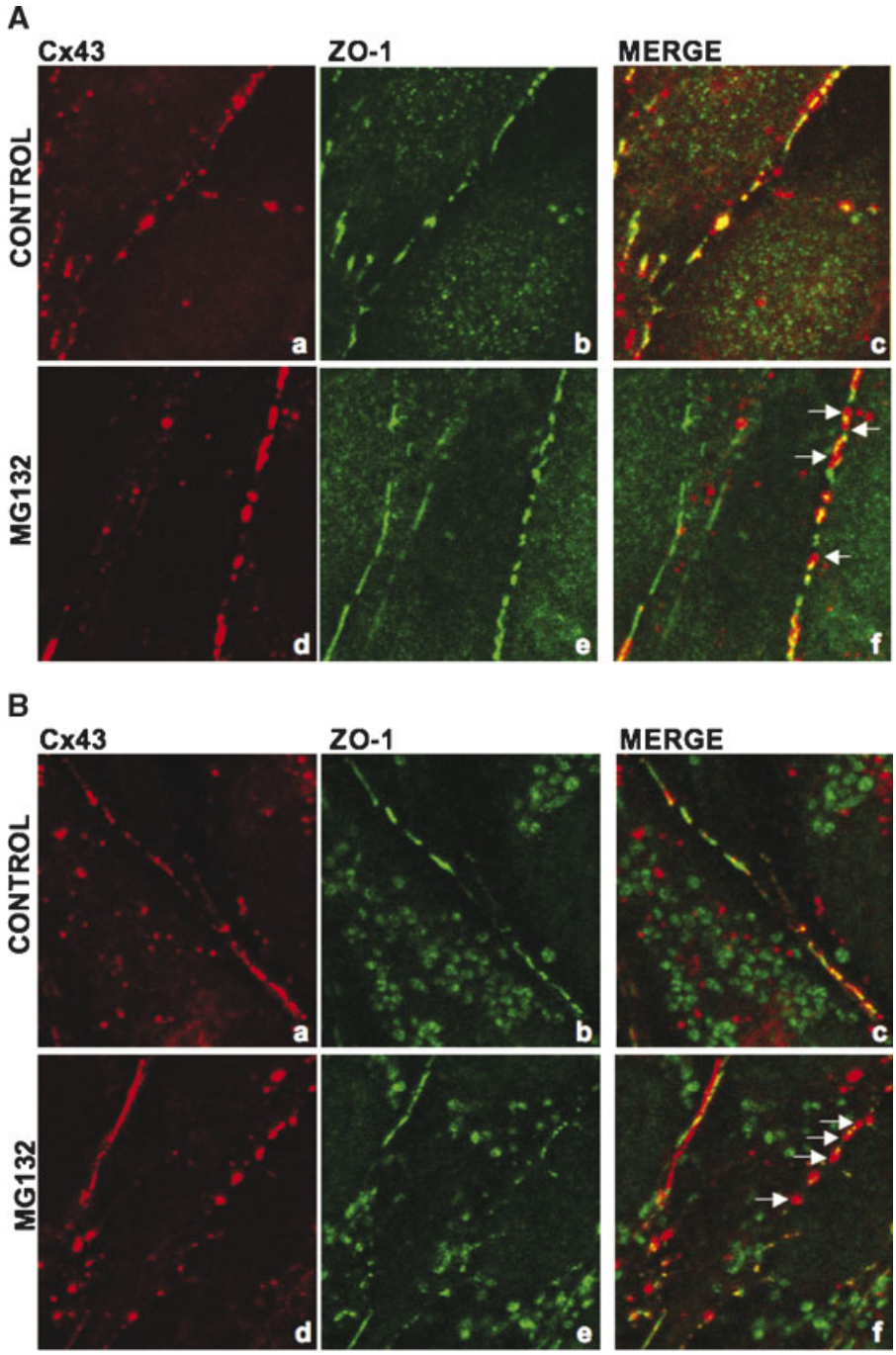

Fig. 2. Proteasome inhibitors reduce the colocalization of Cx43 with ZO-1. NRK cells were incubated with 20 μM MG132, for 2 h, and then fixed either with PFA 4% (**A**) or methanol/acetone (**B**) and double labeled with monoclonal antibodies directed against Cx43 (red) and polyclonal antibodies directed against ZO-1 (green) and imaged by confocal microscopy. Incubation with MG132 results in the accumulation of Cx43 at the plasma membrane and in a decrease in the extent of colocalization between Cx43 and ZO-1 (see arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

sion of the PDZ2 domain results in a decreased co-localization between Cx43 and endogenous ZO-1 (Fig. 4B c, f; see arrows). These results show that overexpression of the PDZ2 domain of ZO-1 disrupts the interaction between Cx43 and ZO-1.

We do propose a model in which stabilization of Cx43, at the plasma membrane, depends on

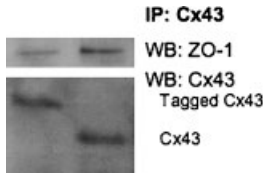

Fig. 3. The presence of a tag in the carboxyl terminal of Cx43 leads to a decreased interaction with ZO-1. HEK293 cells were transfected with Cx43 or V5-His tagged Cx43. Cx43 was immunoprecipitated using polyclonal antibodies against Cx43. Immunoprecipitated material was resolved by SDS–PAGE and immunoblotted with antibodies against ZO-1 and Cx43 as indicate in the picture.

the interaction of its C-terminus with the PDZ domain of ZO-1. We hypothesize that one mechanism by which the Cx43/ZO-1 interaction is regulated could be by degradation of an as yet unknown putative protein that binds to the C-terminus of Cx43. If this were the case, then binding of a tag to the C-terminus of Cx43 would, make the tagged Cx43 insensitive to the effects of proteasome inhibitors.

To verify this hypothesis, HEK293 cells were transfected either with a tagged or untagged Cx43. The transfected cells were then incubated in the presence or absence of the proteasome inhibitors for 3 h, after which the cells were fixed and stained with antibodies directed against Cx43 and ZO-1. As shown in Figure 5A, when cells overexpressing the untagged form of Cx43, are incubated with MG132, a large punctate staining is revealed at

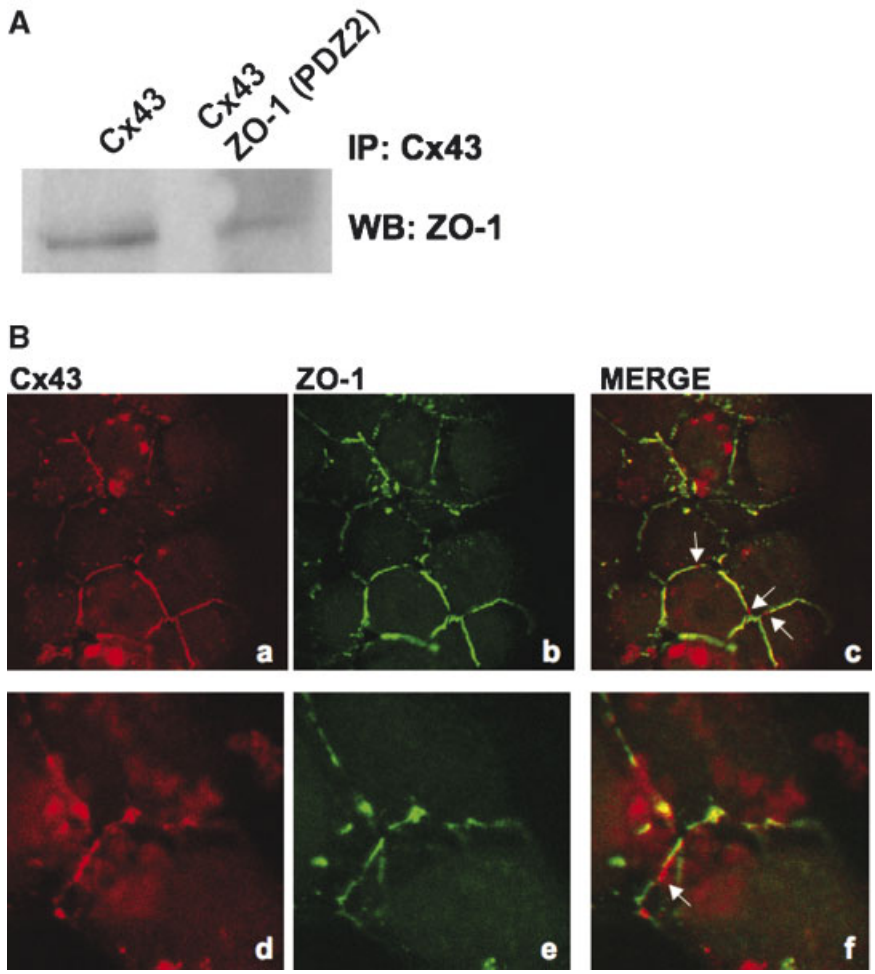

Fig. 4. Overexpression of the PDZ2 domain of ZO-1 disrupts interaction between Cx43 and ZO-1. HEK293 cells were transfected either with Cx43 alone or cotransfected withCx43 and the PDZ2 domain of ZO-1. Cx43 was immunoprecipitated using polyclonal antibodies against Cx43. Immunoprecipitated material was resolved by SDS–PAGE and immunoblotted with antibodies against ZO-1 (**A**). Alternatively, the cells were fixed and double labeled with monoclonal antibodies directed against Cx43 (red) and polyclonal antibodies directed against ZO-1 (green) and imaged by confocal microscopy (**B**). Overexpression of the PDZ2 domain of ZO-1 results in a decrease of colocalization of Cx43 and ZO-1 (arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

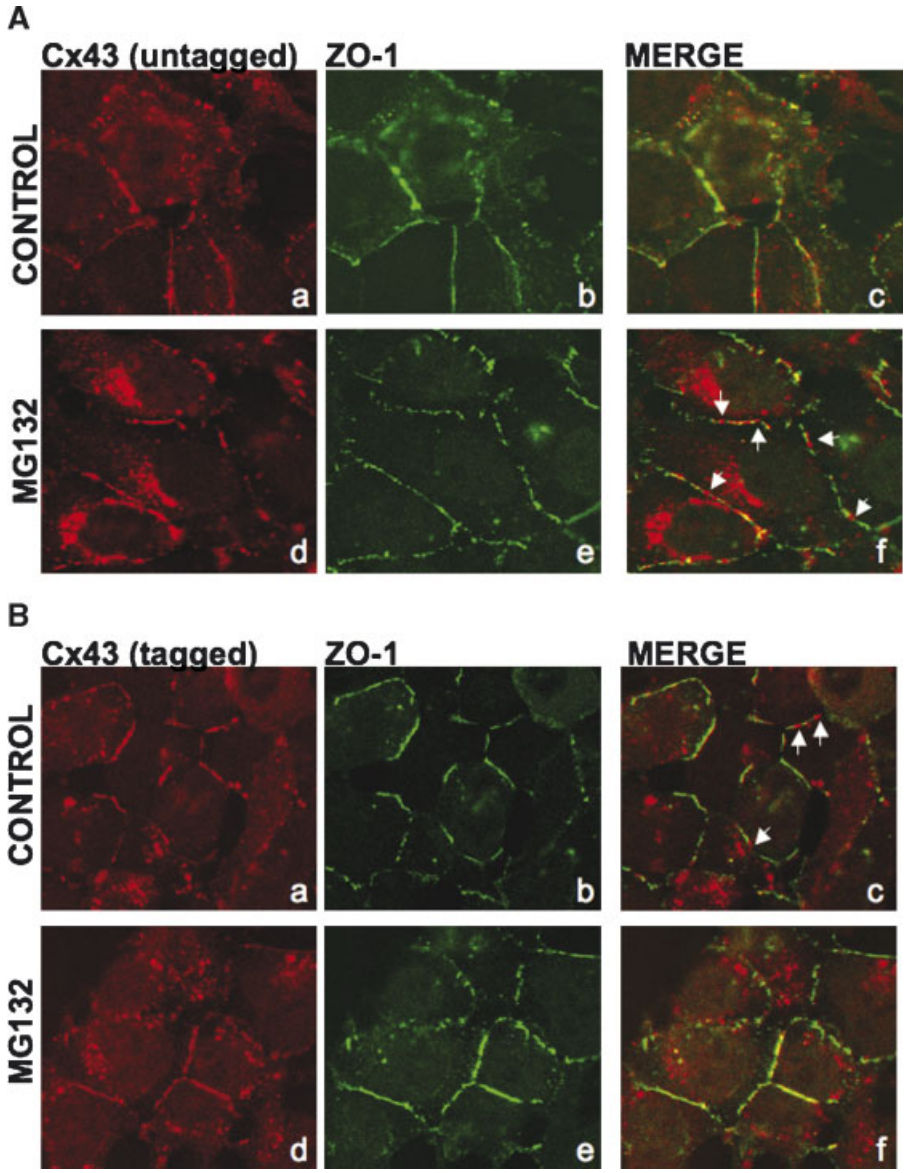

Fig. 5. The effect of proteasome inhibitors is abolished when Cx43 has a tag in the C-terminus. HEK293 cells transfected with untagged Cx43 (**A**) or V5-His tagged Cx43 (**B**) were incubated with MG132, for 3 h. The cells were then fixed and double labeled with monoclonal antibodies directed against Cx43 (red) and polyclonal antibodies directed against ZO-1 (green) and imaged by confocal microscopy. Proteasome inhibition does not result in a decreased colocalization between Cx43 and ZO-1 when a tag is present in the C-terminus of Cx43. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the plasma membrane (Fig. 5A d), as described before for distribution of endogenous Cx43 NRK cells. On the other hand, proteasome inhibition has no effect on cells that overexpress the tagged form of Cx43 (Fig. 5B d). Additionally, cells overexpressing the tagged form of Cx43 present larger punctate staining that do not colocalize with ZO-1 (Fig. 5B a–c, arrows), as compared to cells overexpressing the untagged form of Cx43 (Fig. 5A a–c).

DISCUSSION

The results presented in this study demonstrate that the proteasome regulates the interaction between Cx43 and ZO-1, as revealed by coprecipitation experiments and immunofluorescence analysis. Indeed, incubation of the Cx43 expressing NRK cells with the proteasome inhibitor MG132 (or lactacystin), results both in a decrease in the amount of ZO-1 that

coprecipitates with Cx43 and in a diminished colocalization of the two proteins at the plasma membrane. The reduction of Cx43-ZO-1 association is not accompanied by a reduction in the association between Cx43 and E-cadherin, suggesting that the proteasome is specifically affecting the interaction between Cx43 and ZO-1. Moreover, the presence of a tag in the C-terminus of Cx43, that prevents interaction with ZO-1, reverts the MG132-induced accumulation of large Cx43 plaques at the plasma membrane. Taken together, these findings suggest that interaction between Cx43 and ZO-1 can be regulated by the proteasome. We and others have previously shown that the proteasome can regulate GJIC [Musil et al., 2000; Girao and Pereira, 2003; Fernandes et al., 2004]. Indeed, it is well established that proteasome inhibition leads to an accumulation of large plaques of GJ and, as a consequence, to an increase of GJIC. However, the mechanism whereby proteasome increases intercellular communication is still unknown. Recently, Hunter et al. [2005] proposed a model in which ZO-1 controls the rate of Cx43 channel accretion at the periphery of GJ. The authors demonstrate that reduction of the interaction between ZO-1 and Cx43 leads to the formation of abnormally large GJ plaques, with the concomitant accumulation of the phosphorylated forms of Cx43. This observation is consistent with the model proposed in the present study. For example, by preventing degradation of a putative Cx43-interacting protein, proteasome inhibitors could also prevent interaction between Cx43 and ZO-1 resulting in the formation of large plaques of GJ. Moreover, we have shown before that phosphorylation acts as a signal targeting Cx43 for internalization by a proteasome-dependent mechanism [Girao and Pereira, 2003; Fernandes et al., 2004]. Indeed, it is conceivable phosphorylation plays an important role in proteasome-dependent regulation of the interaction between ZO-1 and Cx43. Consistently, it was hypothesized that interaction between Cx43 and ZO-1 is associated with increased internalization of Cx43 [Barker et al., 2002; Segretain et al., 2004] and that phosphorylation is a prerequisite for such Cx43-ZO-1 interaction and subsequent Cx43 endocytosis [Barker et al., 2002; Segretain et al., 2004]. These observations are consistent with the model proposed in this study for the proteasome-dependent internalization of Cx43. That is, proteasome regulates association between Cx43 and ZO-1 and increased association between both proteins leads internalization of Cx43 and reduction of GJIC. According to this hypothesis, proteasome inhibition would result in the accumulation of Cx43 at the plasma membrane. This is likely to be relevant in conditions where the proteasome activity is inhibited or reduced, such as under oxidative stress. Indeed, it was previously shown that oxidative stress leads to the accumulation of Cx43 at the plasma membrane and, as a consequence, to a deregulation of GJIC [VanSlyke and Musil, 2002, 2005; Girao et al., 2004]. The results presented in this study envision an alternative mechanism that may associate deregulation of intercellular communication to oxidative stress. It is suggested that oxidative stress inhibits the proteasome, which, in turn leads to a poor interaction between Cx43 and ZO-1, thus enhancing GJ plaques formation and intercellular communication. In addition to its role in GJIC, connexins are also involved in cell communication with the extracellular environment via hemichannels. Cx43-based hemichannels are implicated in various processes in cell physiology and pathology including volume regulation [Quist et al., 2000], efflux of NAD^+ and ATP [Cotrina et al., 1998; Stout et al., 2002; Goodenough and Paul, 2003] and acceleration of cell death during metabolic inhibition [Contreras et al., 2002]. A previous study indicated that interaction of Cx43 with ZO-1 could further contribute to regulate the pool of Cx43 that is involved in GJIC versus the pool that is involved in the non-junctional communication mediated by Cx43 hemichannels [Hunter et al., 2005]. Thus, it is conceivable that the changes in proteasome activity, may modulate, not only GJIC but also non-junctional communication through connexons, regulating other cellular processes such as apoptosis. Taken together, the results presented in this study suggest a new role for the proteasome in controlling GJIC, by regulating the association between Cx43 and ZO-1.

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