Erratum

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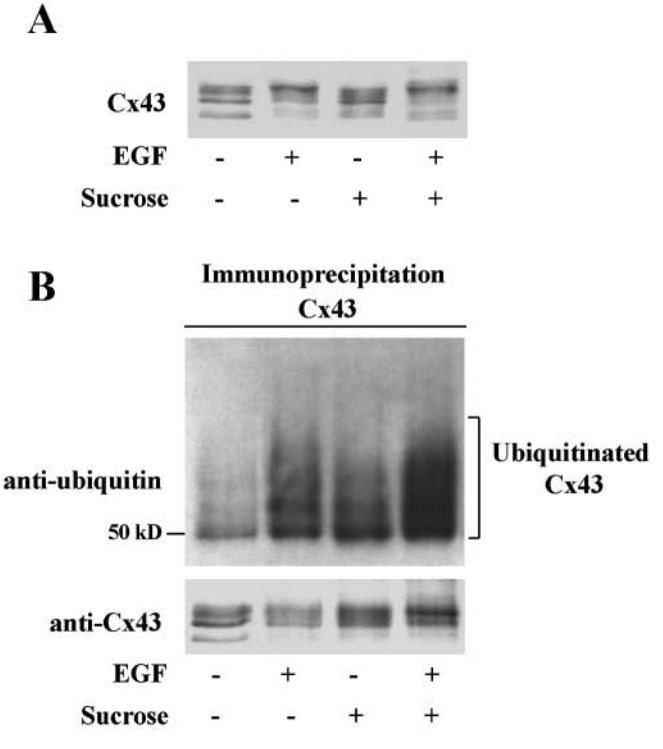
In the online version of this article the bottom line of Fig. 8B was missing. The correct figure is shown below. We apologise for any inconvenience caused.



Epidermal growth factor regulates ubiquitination, internalization and proteasome-dependent degradation of connexin43

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Summary

Connexins are membrane-spanning proteins that form gap junction channels between adjacent cells. Connexin43 (Cx43), the most widely expressed member of the connexin family in tissues and cell lines, has a rapid turnover rate and its degradation involves both the lysosomal and ubiquitin-proteasome pathway. It was previously shown that the proteasome is involved in regulating the number of functional gap junctions at the plasma membrane. However, little is known about how proteasome-dependent turnover of Cx43 is controlled. Epidermal growth factor (EGF) induces hyperphosphorylation of Cx43 and a rapid, transient decrease in gap junctional intercellular communication. In this study, we show that, along with inhibition of gap junctional intercellular communication, EGF induces disorganization, internalization and degradation of Cx43 gap junction plaques in IAR20 rat liver epithelial cells. These EGF-induced modifications of Cx43 were counteracted by the MEK1 inhibitor PD98059, indicating that the effects were mediated by the mitogen-activated protein kinase pathway. The EGF-induced destruction of Cx43 was proteasome-dependent, because the loss of Cx43 protein was counteracted by the proteasome inhibitor MG132 but not the lysosomal inhibitor leupeptin. Furthermore, EGF induced ubiquitination of Cx43, which was associated with the Cx43 hyperphosphorylation. The EGF-induced Cx43 ubiquitination was counteracted by PD98059. The EGF-induced internalization of Cx43 was blocked by hypertonic sucrose treatment, indicating that EGF mediates internalization of Cx43 via a clathrin-dependent mechanism. Our results indicate that ubiquitination of Cx43 occurs at the plasma membrane before Cx43 internalization. Taken together, these data provide the first evidence that EGF-induced phosphorylation of Cx43 induces binding of ubiquitin and targets Cx43 for internalization and degradation in a proteasome-dependent manner.

Key words: Connexin43, Epidermal growth factor, Mitogen-activated protein kinase, Ubiquitin, Proteasome

Introduction

Gap junctions are specialized domains in the plasma membrane containing intercellular channels between neighbouring cells. Gap junction channels are formed by the docking of two hemichannels of connexin proteins contributed by each adjacent cell. These channels are found in most animal tissues and enable cells to exchange cytoplasmic components (<1 kDa) directly, including second messengers, nucleotides and ions (Goodenough et al., 1996). Gap junctions are thought to be important in embryonic development, cellular growth control and differentiation (Guthrie and Gilula, 1989; Loewenstein, 1979; Mehta et al., 1986). The most widely expressed member of the connexin family in tissues and cell lines, connexin43 (Cx43), has been reported to behave as a classical tumour suppressor gene both in cell culture and animal tests, and restores the growth regulatory and differentiation properties of carcinoma cells (Hirschi et al., 1996; Huang et al., 1998; Omori and Yamasaki, 1998; Qin et al., 2002; Rose et al., 1993). Connexins have four hydrophobic membrane-spanning domains, and phosphorylation of the cytoplasmic C-terminal region is an important way to modulate the function of gap junctions (Lampe and Lau, 2000). Phosphorylation of connexins might directly affect gap junction channel gating or modulate connexin intracellular trafficking, channel assembly and connexin turnover (Crow et al., 1990; Lampe, 1994; Lampe et al., 2000; Lau et al., 1991; Musil and Goodenough, 1991; Oh et al., 1991; Puranam et al., 1993; TenBroek et al., 2001). Thus, an important step towards understanding the molecular mechanisms underlying the regulation of gap junctional intercellular communication (GJIC) is to identify the signalling pathways involved in the diverse aspects of the life cycle of connexins.

Gap junction endocytosis is a unique process in which the entire gap junction or a fragment of it is internalized into one of the apposing cells. The internalized gap junction, termed an annular gap junction, is then degraded or possibly reused (Gaietta et al., 2002; Jordan et al., 2001; Larsen et al., 1979; Naus et al., 1993). Degradation of Cx43 involves both the lysosome and the ubiquitin-proteasome system (Laing and Beyer, 1995; Laing et al., 1997; Larsen and Hai, 1978; Musil et al., 2000; Qin et al., 2003; Thomas et al., 2003). In the ubiquitin-proteasome system, proteins are marked for

degradation by covalent linkage to a polyubiquitin chain. The protein is then recognized and degraded by the 26S proteasome, a 2000 kDa ATP-dependent proteolytic complex (Ciechanover, 1998). In contrast to most other transmembrane proteins, gap junctions are dynamic plasma membrane domains with rapid turnover rates. Cx43 has been reported to have a half-life of only 1.5-5 hours (Fallon and Goodenough, 1981; Musil et al., 1990; Traub et al., 1989). The instability of Cx43 indicates that gap junction assembly or disassembly might be important factors in modifying GJIC. It was recently reported that GJIC can be regulated by modulating the level of Cx43 turnover by proteasomal inhibitors (Musil et al., 2000). Nevertheless, the control of the various stages of the Cx43 life cycle with respect to its ubiquitination, internalization and degradation is poorly understood.

The epidermal growth factor (EGF) receptor is the prototypical member of the Erb-B family of receptor tyrosine kinases (Gullick, 1998). The EGF-receptor signal transduction pathways have been correlated with various processes that contribute to the development of malignancies, such as effects on cell cycle progression, inhibition of apoptosis, angiogenesis, tumour cell motility and metastasis. EGF has been shown to induce serine phosphorylation on Cx43 mediated by the mitogen-activated protein kinase (MAPK) pathway (Cameron et al., 2003; Rivedal and Opsahl, 2001; Warn-Cramer et al., 1996; Warn-Cramer et al., 1998). The MAPK-mediated phosphorylation of Cx43 correlates with a rapid, transient decrease in GJIC (Cottrell et al., 2003; Kanemitsu and Lau, 1993; Lau et al., 1992). The mechanisms underlying this decrease are poorly understood but have been shown to involve changes in the gap junction channel open probability or permeability (Kim et al., 1999; Cottrell et al., 2003).

In the present study, we show that, along with modulation of the gating properties of Cx43 gap junction channels, EGF regulates the ubiquitination, internalization and destruction of Cx43. These Cx43 modifications are mediated by the MAPK pathway. We provide evidence that the EGF-induced acceleration of internalization and degradation of Cx43 depend on the proteasomal system. Furthermore, our results indicate that the EGF-induced Cx43 internalization is a clathrin-mediated process and that ubiquitination of Cx43 occurs at the plasma membrane before its internalization. Taken together, our results show that EGF regulates multiple levels of the Cx43 gap junction life cycle and provide new insights into the control of Cx43 stability.

Materials and Methods

Reagents and antibodies

EGF, PD98059, MG132, leupeptin, cycloheximide and Alexa-488-conjugated transferrin were obtained from Sigma (St Louis, MO, USA). Protein A-Sepharose was from Amersham Biosciences (Piscataway, NJ, USA). The anti-Cx43 antiserum was made in rabbits injected with a synthetic peptide consisting of the 20 C-terminal amino acids of Cx43 (Rivedal et al., 1996). Anti-actin antibodies were obtained from Sigma. Anti-ubiquitin antibodies were obtained from Babco (Covance, CA, USA). Alexa-488-conjugated goat anti-rabbit IgG antibodies were from Sigma. Goat anti-rabbit IgG and donkey anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase were from Bio-Rad (Hercules, CA, USA) and Jackson Immunoresearch Laboratories (West Grove, PA, USA), respectively.

Cell culture and treatment

The rat liver epithelial cell line IAR20 was obtained from the International Agency for Research on Cancer (Lyon, France). The cells were originally isolated from normal inbred BD-IV rats and express endogenous Cx43 (Asamoto et al., 1991; Montesano et al., 1977). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco BRL Life Technologies, Inchinnan, UK). IAR20 cells were plated onto 60 mm (10×10^5) or 100 mm (27×10^5) Petri dishes (Costar, Cambridge, MA, USA) 48 hours before experiments. The growth medium was replaced with DMEM with 1% FBS after 24 hours. In some experiments, cells were incubated in a hypertonic sucrose solution to block clathrin-mediated endocytosis. Cells were rinsed twice with DMEM supplemented with 20 mM Hepes and incubated in the same medium for 30 minutes at 37°C. The cells were then washed twice with 0.45 M sucrose in Hepes-buffered DMEM before incubation for 45 minutes at 37°C in the same medium (Hansen et al., 1993; Heuser and Anderson, 1989; Wu et al., 2001).

Determination of GJIC by scrape loading

Quantitative scrape loading was performed as previously described (Leithe et al., 2003; Opsahl and Rivedal, 2000). Briefly, the confluent cell layer was washed twice with PBS and 0.05% (w/v) Lucifer Yellow (Sigma) dissolved in PBS without Ca^{2+} and Mg^{2+} was added; the layer was then cut with a surgical scalpel. After 3.5 minutes, the Lucifer Yellow solution was removed, the dishes rinsed four times with PBS, fixed in 4% formaldehyde in PBS and mounted with a glass coverslip. Digital monochrome images were acquired by a COHU 4912 CCD camera (COHU, San Diego, CA, USA) and a Scion LG-3 frame grabber card (Scion, Frederick, MD, USA). Analysis was done using NIH Image software. The levels of GJIC were determined as the relative area of dye coupled cells. Exposing the cells to 30 μM chlordane for 1 hour resulted in a complete block of GJIC. Thus, the fluorescent cells following such exposure have obtained the dye directly through the scrape process and were used to define zero GJIC. The data are presented as mean±s.d. relative to control.

Immunofluorescence microscopy

The IAR20 cell monolayer was fixed with 4% formalin in PBS for 30 minutes at room temperature, rinsed with PBS and permeabilized with 0.1% Triton. Cells were incubated with PBS containing 5% (w/v) dried milk and 0.1% Tween for 1 hour and subsequently with anti-Cx43 antibodies (diluted 1:500) for 1 hour, washed with PBS and incubated with Alexa-488-conjugated goat anti-rabbit IgG antibodies for 1 hour. The cells were mounted with Mowiol. Immunofluorescence images were captured using a Nikon E800 microscope with a Spot-2 camera.

Transferrin internalization assay

For Alexa-488-transferrin internalization, cells were rinsed with DMEM supplemented with 20 mM Hepes and incubated in the same medium for 30 minutes at 37°C. The cells were then rinsed with Hepes-buffered DMEM with or without 0.45 M sucrose, and incubated with Alexa-488-transferrin (50 μg ml^{-1}) in the presence or absence of 0.45 M sucrose for 45 minutes at 37°C. Cells were rinsed with PBS and then fixed and mounted. Immunofluorescence images were captured as described above.

Western blotting

IAR20 cells were washed with PBS and scraped in 500 μl of sodium dodecyl sulfate (SDS) electrophoresis sample buffer (10 mM Tris, pH 6.8, 15% w/v glycerol, 3% w/v SDS, 0.01% w/v bromophenol blue and 5% v/v 2-mercaptoethanol). The cell lysates were sonicated and

heated for 5 minutes at 95°C. Samples were separated by SDS / 8%-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as described (Rivedal et al., 1996). The blotting membranes were developed with 4-chloro-1-naphthol (Bio-Rad) for the anti-Cx43 antiserum and with chemiluminescence (Cell Signaling Technology, Beverly, MA, USA) for the anti-actin and anti-ubiquitin antibodies. The intensity of Cx43 bands was quantified using Scion Image (Scion).

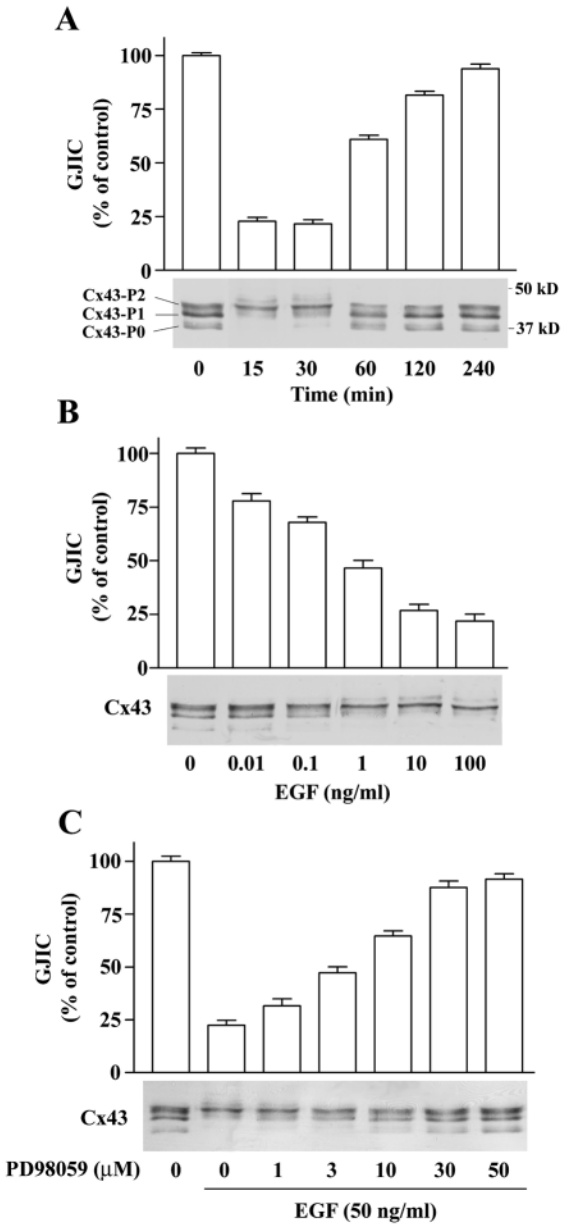

Fig. 1. Effects of EGF on GJIC and the Cx43 phosphorylation pattern in IAR20 cells. GJIC was measured by quantitative scrape loading. For Cx43 band-pattern examination, cell lysates were prepared as indicated and subjected to SDS-PAGE and western blotting with anti-Cx43 antibodies. (A) Cells were exposed to 50 ng ml^{-1} EGF for different lengths of time. (B) Cells were exposed to increasing concentrations of EGF for 30 minutes. (C) Cells were exposed to increasing concentrations of PD98059 for 30 minutes, then to EGF at a final concentration of 50 ng ml^{-1} and incubated for 30 minutes, in the sustained presence of PD98059.

Immunoprecipitation

IAR20 cells grown in 100 mm Petri dishes were treated as indicated, and washed once with ice-cold PBS before treatment with lysis buffer [PBS, 10% glycerol, 0.25% sodium deoxycholate, 0.45% sodium lauroyl sarcosine, protease and phosphatase inhibitor cocktails (Sigma), and 2mM EDTA] for 10 minutes on ice. Cell lysates were sonicated and precleared by incubation with protein A-Sepharose beads at 4°C for 30 minutes with shaking. Beads were pelleted by centrifugation at 1000 ***g*** for 5 minutes at 4°C and supernatant was collected. Each aliquot was added anti-Cx43 antibodies and protein A-Sepharose beads. Preimmune serum from the same animal was used as negative controls. The reaction mixture was incubated at 4°C for 2 hours with shaking. The pellet was collected by centrifugation at 1000 ***g*** for 5 minutes at 4°C and washed five times with ice-cold lysis buffer. After the final wash, the pellet was resuspended in 30 μl of 1× western sample buffer and heated to 95°C for 5 minutes before protein separation by 8% SDS-PAGE electrophoresis. Western blot analysis was performed as described above.

Results

EGF induces transient hyperphosphorylation of Cx43 and inhibition of GJIC

Cx43 usually exists in several phosphorylated forms, which are seen in western blots as different bands. The conversion of the fastest migrating band, termed Cx43-P0, to the two major slower migrating bands, termed Cx43-P1 and Cx43-P2, is associated with changes in Cx43 localization and function (Laird et al., 1991; Solan et al., 2003; Musil et al., 1990; Musil et al., 2000). Confluent IAR20 cells were incubated with 50 ng ml^{-1} EGF and the phosphorylation pattern of Cx43 was examined by western blotting at different time points. EGF caused an increase in the P2 band after 15 minutes and 30 minutes, concomitant with a decrease in the P0 and P1 bands (Fig. 1A). After 1 hour of EGF treatment, the intensity of the P2 band was reduced and, after 4 hours, the phosphorylation pattern of Cx43 was similar to untreated cells. The effect of EGF on cell-cell communication through gap junction channels was measured by quantitative scrape loading. In agreement with previous studies on other cell types, EGF induced transient inhibition of GJIC (Kanemitsu and Lau, 1993; Lau et al., 1992; Warn-Cramer et al., 1996; Warn-Cramer et al., 1998). After 15 minutes of 50 ng ml^{-1} EGF treatment, GJIC had decreased to 25% of that in untreated cells; at 4 hours, GJIC had returned to control levels (Fig. 1A). The EGF-induced hyperphosphorylation of Cx43 and inhibition of GJIC was concentration dependent (Fig. 1B). The hyperphosphorylation of Cx43 and the inhibition of GJIC in response to EGF were specific to MAPK activation, because the Cx43 band pattern shift and inhibition of GJIC were counteracted in cells preincubated with the MEK1 inhibitor PD98059 before EGF treatment (Fig. 1C).

EGF induces internalization of Cx43

We next investigated whether the EGF-induced hyperphosphorylation of Cx43 was associated with alterations in the localization of Cx43. By immunofluorescence microscopy, untreated cells were found to have most of their Cx43 organized as gap junction plaques at the plasma membrane. In addition, faint immunostaining of Cx43 was found in the Golgi area (Fig. 2A). Treatment with 50 ng ml^{-1}

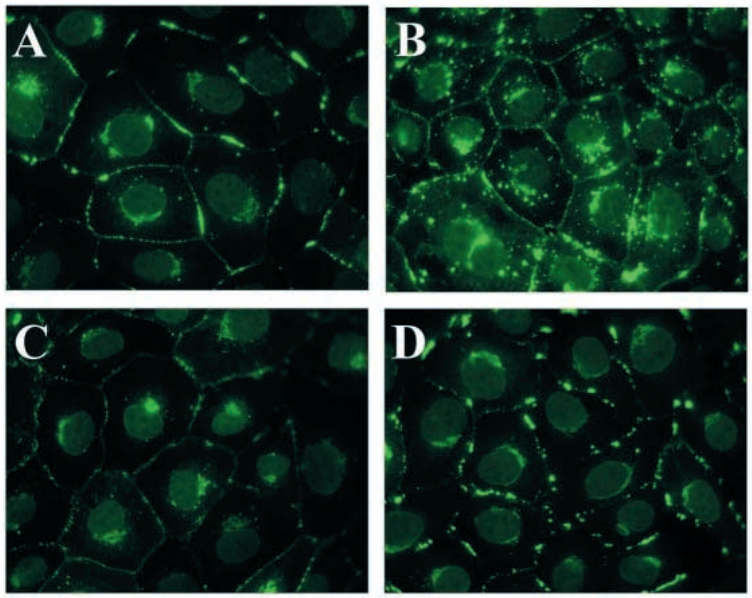

Fig. 2. Effect of EGF on Cx43 localization in IAR20 cells. IAR20 cells were either left untreated (A) or treated with 50 ng ml^{-1} EGF for 30 minutes (B) or 60 minutes (C). (D) IAR20 cells were preincubated with 50 μM PD98059 for 30 minutes, then incubated with EGF at a final concentration of 50 ng ml^{-1} and incubated for 30 minutes in the sustained presence of PD98059. The cells were fixed, immunostained with anti-Cx43 antibodies and visualized using fluorescence microscopy.

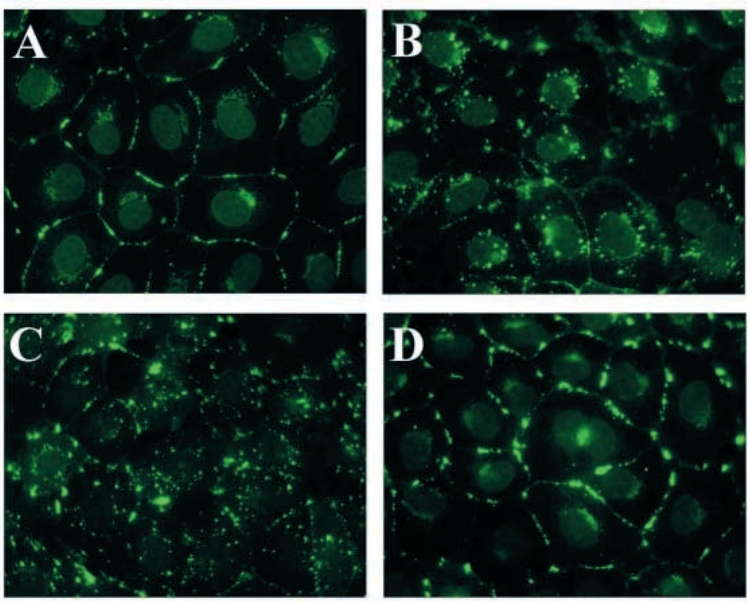

Fig. 3. Effects of cycloheximide and MG132 on EGF-induced relocalization of Cx43. IAR20 cells were either left untreated (A) or treated with 50 ng ml^{-1} EGF for 30 minutes (B). (C) Cells were preincubated with 10 μg ml^{-1} cycloheximide for 2 hours, then exposed to EGF at a final concentration of 50 ng ml^{-1} and incubated for 30 minutes in the sustained presence of cycloheximide. (D) Cells were preincubated with 10 μM MG132 for 30 minutes, then exposed to EGF at a final concentration of 50 ng ml^{-1} and incubated for 30 minutes in the sustained presence of MG132. Cells were fixed, immunostained with anti-Cx43 antibodies and visualized using fluorescence microscopy.

EGF for 30 minutes resulted in disorganization of gap junctions and large amounts of Cx43 became localized in intracellular punctuate structures (Fig. 2B). EGF treatment for 1 hour resulted in reduced immunofluorescence staining of Cx43 both at the plasma membrane and in vesicular compartments (Fig. 2C). The role of the MAPK pathway in the EGF-induced increase of Cx43 in intracellular vesicular compartments was studied using the MEK1 inhibitor PD98059. Preincubating IAR20 cells with PD98059 for 30 minutes counteracted the EGF-induced delocalization of Cx43 (Fig. 2D). These results indicate that the EGF-induced increase of Cx43 staining in intracellular vesicles requires MAPK activity.

Because the EGF-induced increase of Cx43 in intracellular vesicular compartments was associated with loss of plasma membrane plaques, the increase in immunofluorescence staining of Cx43 in vesicular compartments could be the result of either accelerated Cx43 internalization or a block in Cx43 trafficking to the plasma membrane. To distinguish between these two possibilities, cells were incubated with cycloheximide to deplete intracellular stores of Cx43 in the Golgi area. Cycloheximide treatment for 2 hours caused a loss of Cx43 labelling in the Golgi region (not shown) and loss of the Cx43-P0 band on western blots (see below). The Cx43-P0 band has previously been reported to be localized in the Golgi region (Musil and Goodenough, 1991). Thus, under these conditions, most Cx43 is in a phosphorylated form and localized to the plasma membrane. When cells were treated with EGF after preincubation with cycloheximide for 2 hours, EGF still induced an increase of Cx43 staining in vesicular compartments (Fig. 3C). These data suggest that the increase was due to accelerated Cx43 internalization rather than a block in Cx43 trafficking to the plasma membrane.

Gap junction internalization has previously been shown to require proteasomal activity (Laing et al., 1997; Qin et al., 2003). Thus, to provide further evidence that the appearance of Cx43 in intracellular vesicles was due to accelerated internalization, cells were preincubated with the proteasomal inhibitor MG132 for 30 minutes before EGF treatment. Under these conditions, the increase of Cx43 in vesicular compartments was completely counteracted (Fig. 3D). Similar results were obtained with the highly specific proteasomal inhibitor lactacystin (not shown). The endocytosis of transferrin was not inhibited by MG132, indicating that proteasomal inhibition did not induce pleiotropic effects on endocytosis in IAR20 cells (not shown). These results show that the proteasome is essential in the EGF-stimulated delocalization of Cx43 and provide further evidence that the increased intracellular labelling of Cx43 in response to EGF is due to increased internalization. The effect of EGF on the localization of another plasma membrane protein, the tight junction protein ZO-1, was also investigated. The cellular distribution of ZO-1 was not altered in response to EGF, indicating

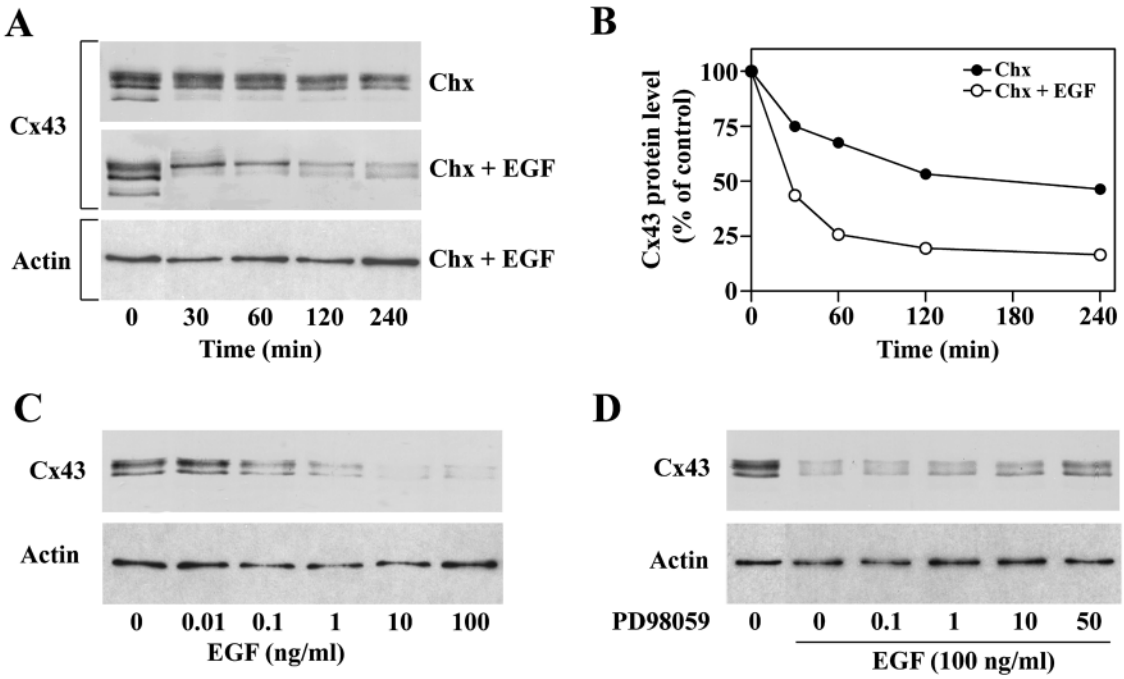

Fig. 4. EGF induces degradation of Cx43. Cell lysates were prepared as indicated and equal amounts of total cell protein were subjected to SDS-PAGE and western blotting with anti-Cx43 antibodies. As gel loading controls, the blots were stripped and reprobed with anti-actin antibodies. (A) IAR20 cells were treated with 10 μg ml^{-1} cycloheximide (chx) alone or in combination with 100 ng ml^{-1} EGF. (B) The Cx43 band intensities on gels shown in (A) were measured using Scion Image. The Cx43 band intensities were plotted relative to the intensity of Cx43 bands in untreated cells. (C) Cells were treated with 10 μg ml^{-1} cycloheximide and increasing concentrations of EGF for 4 hours. (D) Cells were preincubated with increasing concentrations of PD98059 (μm) for 30 minutes before treatment with 10 μg ml^{-1} cycloheximide and 100 ng ml^{-1} EGF for 4 hours in the sustained presence of PD98059.

that EGF selectively accelerates Cx43 internalization, rather than generally affecting plasma membrane trafficking (not shown).

EGF induces proteasome-dependent degradation of Cx43

We next performed western blotting to determine the effect of EGF on the Cx43 protein level. Cells were incubated with cycloheximide to block protein synthesis, in combination with EGF for different time points. As expected, cycloheximide alone caused a gradually reduction in the amount Cx43 protein (Fig. 4A,B). When cycloheximide was incubated with EGF, there was a strong increase in the P2 band at 15 minutes, indicating hyperphosphorylation of Cx43, as described above. This hyperphosphorylation of Cx43 was transient and, after 2 hours, there was a distinct reduction in the Cx43-P2 band. At this time, the total Cx43 protein level was strongly reduced compared with cells incubated with cycloheximide alone. After 4 hours of incubation with cycloheximide and EGF, Cx43 protein was barely visible (Fig. 4A,B). The effect of EGF on Cx43 stability was concentration dependent (Fig. 4C). Because the MAPK pathway appeared to be involved in the EGF-induced Cx43 phosphorylation and endocytosis, we investigated the role of this pathway in Cx43 degradation. When cells were preincubated with the MEK1 inhibitor PD98059, the EGF-induced degradation of Cx43 was reduced, indicating that the EGF-induced decrease in Cx43 protein level is mediated via the MAPK pathway (Fig. 4D).

The rapid reduction of Cx43 protein level induced by EGF was probably caused by proteolytic degradation rather than by inhibition of transcription or translation. To confirm that proteolytic degradation was involved, we used different protease inhibitors. IAR20 cells were incubated with cycloheximide, EGF and the proteasomal inhibitor MG132. MG132 did not affect the EGF-induced band pattern shift of Cx43 (Fig. 5A). MG132 did, however, stabilize the Cx43-P2 band, causing it to remain strong at all time points investigated. Thus, under these conditions, the EGF-induced decrease in Cx43 protein level was counteracted, indicating that the EGF-induced degradation of Cx43 is proteasome dependent (Fig. 5A,B). Similarly, the EGF-induced degradation of Cx43 was inhibited by the proteasomal inhibitor lactacystin (not shown). The lysosomal inhibitors leupeptin (Fig. 5A,B) and E-64 (not shown) did not counteract the EGF-induced reduction in the Cx43 protein level.

EGF increases Cx43 ubiquitination

Ubiquitination plays an important role in the internalization and degradation of many plasma membrane proteins (Strous and Govers, 1999). We therefore investigated whether EGF affected the ubiquitination level of Cx43. IAR20 cells were treated with 100 ng ml^{-1} EGF for different times and Cx43 protein was immunoprecipitated. The presence of ubiquitin-conjugated Cx43 was determined by western blot using anti-ubiquitin antibodies (Fig. 6). As a control, ubiquitin was not detected in immunocomplexes isolated with preimmune serum.

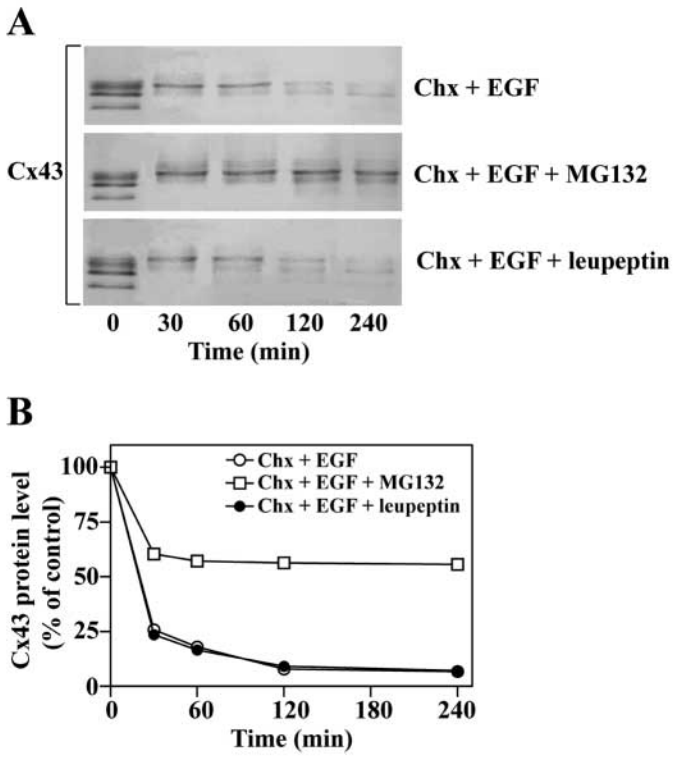

Fig. 5. Effect of MG132 and leupeptin on EGF-induced Cx43 degradation. (A) IAR20 cells were treated with 10 μg ml^{-1} cycloheximide (chx) in combination with either 100 ng ml^{-1} EGF, 100 ng ml^{-1} EGF and 10 μM MG132, or 100 ng ml^{-1} EGF and 100 μM leupeptin. Cell lysates were prepared at the indicated time points and equal amounts of total cell protein were subjected to SDS-PAGE. Cx43 was detected by western blotting with anti-Cx43 antibodies. (B) The Cx43 band intensities on gels shown in (A) were measured using Scion Image. The Cx43 band intensities were plotted relative to the intensity of Cx43 bands in untreated cells.

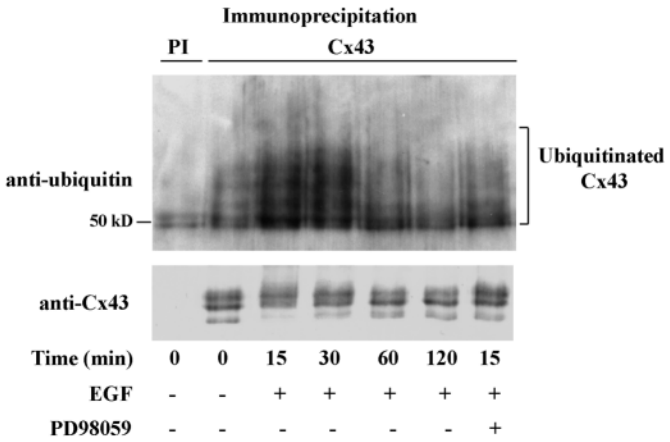

Fig. 6. Effect of EGF on ubiquitination of Cx43. IAR20 cells were treated with 100 ng ml^{-1} EGF for different lengths of time or preincubated with 50 μM PD98059 for 15 minutes before addition of 100 ng ml^{-1} EGF. Cell lysates were subjected to immunoprecipitation with anti-Cx43 antibodies or preimmune serum (PI) as control. Equal amounts of total cell protein were subjected to SDS-PAGE and western blotting using affinity-purified anti-ubiquitin antibodies (top) or anti-Cx43 antibodies (bottom).

In untreated cells, a faint series of bands formed a smear, indicating that ubiquitination of Cx43 is part of the normal life cycle of Cx43 in IAR20 cells. Following EGF treatment for 15 minutes and 30 minutes, there was a strong increase in the ubiquitination of Cx43 compared with untreated cells. The increase in Cx43 ubiquitination was transient and, after 120 minutes of EGF treatment, the amount of ubiquitinated Cx43 had decreased to levels below that of untreated cells. When cells were incubated with PD98059 before EGF treatment for 15 minutes, the increase in ubiquitination of Cx43 was counteracted. Importantly, the EGF-induced ubiquitination of Cx43 occurred concomitantly with the induced hyperphosphorylation of Cx43. Together, these data strongly suggest that EGF-induced phosphorylation of Cx43 accelerates binding of ubiquitin, and that this modification occurs via the MAPK pathway.

Evidence that the EGF-induced internalization of Cx43 is clathrin dependent

The mechanisms underlying gap junction endocytosis are poorly understood, but electron microscopic studies have indicated that gap junctions are associated with clathrin coats (Larsen et al., 1979). To examine the mechanisms underlying the EGF-induced endocytosis of Cx43, the Cx43 delocalization in response to EGF treatment was determined under conditions in which clathrin-mediated endocytosis was blocked. Hypertonic conditions have previously been shown to inhibit clathrin-mediated endocytosis by preventing budding of clathrin-coated vesicles (Hansen et al., 1993; Heuser and Anderson, 1989; Wu et al., 2001). As shown in Fig. 7B, the EGF-induced Cx43 endocytosis was inhibited by hypertonic sucrose medium. Hypertonic medium also inhibited Cx43 internalization induced by 12-*O*-tetradecanoylphorbol 13-acetate (not shown). To verify the inhibitory effects of hypertonic medium on clathrin-mediated endocytosis, we examined whether hypertonic medium was able to prevent the internalization of transferrin. Transferrin has previously been shown to be internalized exclusively via clathrin-coated pits (Mellman, 1996). As shown in Fig. 7C, Alexa-488-conjugated transferrin accumulated in intracellular vesicles in IAR20 cells incubated in the absence of sucrose. By contrast, little transferrin was internalized in cells incubated in hypertonic sucrose medium (Fig. 7D). Importantly, hypertonic conditions did not affect the EGF-induced activation of extracellular signal-regulated kinases 1 and 2, indicating that signalling from the EGF receptor was not inhibited under these conditions (not shown). To investigate further the role of clathrin in EGF-induced endocytosis of Cx43, we used methyl-β-cyclodextrin (MβCD), which causes acute cholesterol depletion from the plasma membrane and inhibits clathrin-mediated endocytosis (Rodal et al., 1999). Preincubating IAR20 cells with 10 mM MβCD strongly counteracted the EGF-induced internalization of Cx43 endocytosis (not shown). Together, these results indicate that the EGF-induced endocytosis of Cx43 is a clathrin-mediated process. As shown in Fig. 8, EGF induced hyperphosphorylation and ubiquitination of Cx43 even when Cx43 internalization was inhibited by hypertonic medium. These results indicate that ubiquitination of Cx43 is an early event in the internalization process of Cx43 and

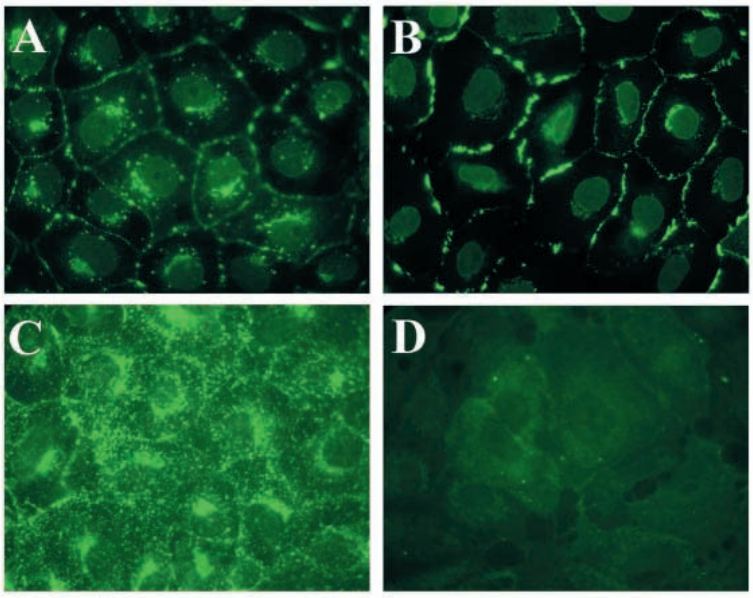

Fig. 7. Effect of hypertonic sucrose medium on EGF-induced internalization of Cx43. IAR20 cells were incubated with 50 ng ml^{-1} EGF for 45 minutes at 37°C in Hepes-buffered DMEM without (A) or with (B) 0.45 M sucrose. The cells were fixed, immunostained with anti-Cx43 antibodies and visualized using fluorescence microscopy. Cells were incubated with 50 μg ml^{-1} Alexa-488-transferrin in the absence (C) or presence (D) of 0.45 M sucrose for 45 minutes at 37°C. The cells were fixed and transferrin was visualized using fluorescence microscopy.

occurs at the plasma membrane before its clathrin-mediated endocytosis. Interestingly, the hyperphosphorylation and ubiquitination of Cx43 appeared to be more prominent in the absence than in the presence of endocytosis, probably owing to accumulation of these modified forms of Cx43 at the plasma membrane.

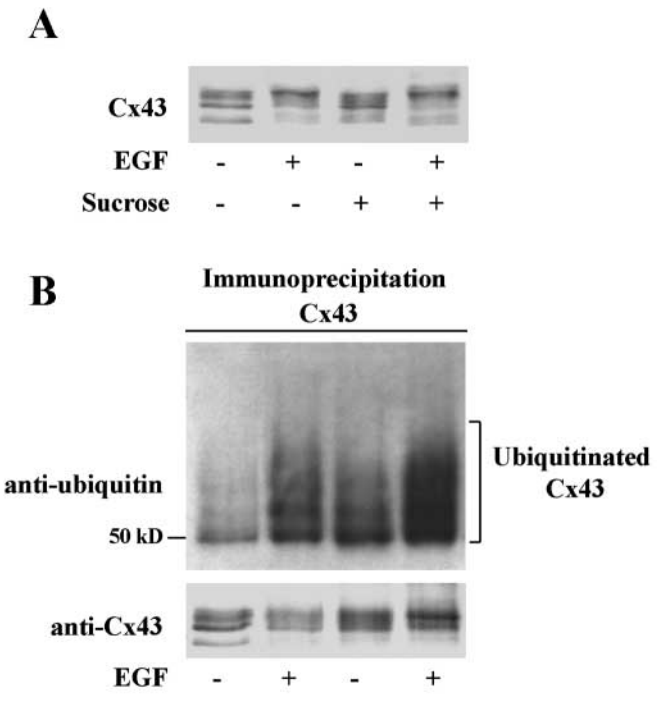

Fig. 8. Cx43 is hyperphosphorylated and ubiquitinated in the presence of hypertonic sucrose medium. (A) Cells were either left untreated or treated with 50 ng ml^{-1} EGF for 15 minutes in the presence or absence of 0.45 M sucrose as indicated. Cell lysates were prepared and equal amounts of total cell protein were subjected to SDS-PAGE. Cx43 was detected by western blotting with anti-Cx43 antibodies. (B) Cells were either left untreated or treated with 50 ng ml^{-1} EGF for 15 minutes in the presence or absence of 0.45 M sucrose as indicated. Cell lysates were subjected to immunoprecipitation with anti-Cx43 antibodies. Equal amounts of total cell protein were subjected to SDS-PAGE and ubiquitin and Cx43 were detected by western blotting using affinity-purified anti-ubiquitin antibodies (top) and anti-Cx43 antibodies (bottom).

Discussion

Gap junctions are dynamic plasma membrane domains with high turnover rates (Goodenough et al., 1996). Degradation of Cx43 involves both the lysosomal and the ubiquitin-proteasome pathway, and modulation of proteasome activity has been shown to regulate the number of gap junction plaques at the plasma membrane (Laing and Beyer, 1995; Laing et al., 1997; Larsen and Hai, 1978; Musil et al., 2000; Qin et al., 2003; Thomas et al., 2003). However, little is known about how ubiquitination, internalization and proteasome-dependent degradation of Cx43 are controlled. In this study, we have used a cell line that endogenously expresses Cx43, which is largely localized to the plasma membrane as gap junction plaques. Thus, this cell line provides an excellent model for investigating the regulation and the molecular mechanisms underlying the internalization of Cx43 gap junctions in a native system. EGF has previously been shown to reduce GJIC transiently by altering the gating properties of Cx43 gap junction channels (Cottrell et al., 2003; Kanemitsu and Lau, 1993; Lau et al., 1992). Here, we report that EGF has several modes of influence on Cx43 gap junctions. To the best of our knowledge, the present data represent the first analysis demonstrating that EGF regulates ubiquitination, internalization and proteasome-dependent degradation of Cx43.

Using immunofluorescence microscopy, we have shown that EGF induces an increase of Cx43 in intracellular vesicular compartments, which is associated with loss of gap junction plaques at the plasma membrane. This EGF-induced subcellular localization of Cx43 was observed even after the intracellular stores of Cx43 in the Golgi apparatus were depleted with cycloheximide, indicating that the increase of Cx43 in intracellular vesicles was not due to a block in transport of Cx43 to the plasma membrane. Moreover, when the proteasome inhibitors MG132 or lactacystin were added before EGF, the increase of Cx43 in intracellular vesicles was counteracted and Cx43 remained organized in gap junctions at the plasma membrane. Proteasome activity was previously shown to be required for gap junction internalization (Laing et al., 1997; Qin et al., 2003). Together, these data suggest that EGF-induced phosphorylation of Cx43 accelerates internalization of Cx43 gap junction plaques. The EGF-induced internalization of Cx43 was counteracted by the MEK1 inhibitor PD98059, indicating that EGF exerts its effect on Cx43 localization via the MAPK pathway. Previous studies

failed to note disassembly and endocytosis of Cx43 gap junctions after EGF treatment (Lau et al., 1992). The discrepancy with our results might be due to differences in cell type or cell culture conditions.

Little is known about how changes in the phosphorylation state of Cx43 contribute to protein stability. The observation that EGF induced Cx43 endocytosis led us to investigate whether EGF also induced Cx43 degradation. Western blot studies indicated that EGF strongly accelerated Cx43 degradation and that this increase in Cx43 proteolysis was associated with hyperphosphorylation of Cx43. When cells were treated with the specific MEK1 inhibitor PD98059, the EGF-induced degradation of Cx43 was strongly counteracted. Together, these results indicate that EGF-induced internalization of Cx43 is followed by Cx43 degradation. To elucidate the relative importance of the lysosomal and proteasomal proteolytic systems in the EGF-induced degradation of Cx43, cells were incubated with different cell-permeable protease inhibitors. The proteasome contains multiple proteolytic sites that function together in protein degradation (Ciechanover, 1998). MG132 is a substrate analogue and a potent inhibitor primarily of the chymotrypsin-like activity of the proteasome. The highly specific proteasome inhibitor lactacystin links covalently to the hydroxyl groups on active threonine residues, thereby inactivating the chymotrypsin- and trypsin-like activities of the proteasome (Lee et al., 1996). Both MG132 and lactacystin counteracted the EGF-induced degradation of Cx43. By contrast, leupeptin and E64, two inhibitors of cathepsins, did not affect the EGF-induced degradation of Cx43. These data suggest that the proteasome plays a dominant role in EGF-induced degradation of Cx43 in IAR20 cells. Further studies are required to determine the involvement of lysosomes in this process. MG132 did not counteract the rapid inhibition of GJIC induced by EGF, indicating that proteasome inhibition does not affect the EGF-induced modulation of the gap junction gating properties (E.L. and E.R., unpublished).

Many plasma membrane proteins have been shown to be ubiquitinated (Strous and Govers, 1999). Different ubiquitin protein ligases recognize specific motifs in the target protein and catalyse ubiquitination. To what extent these sites are recognized by the ligases might depend on modifications of the motifs, including serine phosphorylation. This study indicates that EGF-induced phosphorylation of Cx43 increases ubiquitination of Cx43, which is associated with accelerated Cx43 internalization and degradation. When MEK1 was inhibited, the EGF-induced ubiquitination, internalization and degradation of Cx43 were strongly counteracted, suggesting that MAPK-mediated serine phosphorylation of Cx43 is involved in the increased ubiquitin binding and turnover of Cx43 in response to EGF. What role ubiquitin might play in Cx43 endocytosis remains to be elucidated. Moreover, it is currently unknown whether the EGF-induced internalization of Cx43 is sufficient to trigger Cx43 degradation or whether EGF also affects postendocytic steps of Cx43 required for its degradation. It is also currently unclear whether Cx43 is a direct substrate for proteasomal degradation. It has been suggested that the proteasome might play other, indirect roles in Cx43 endocytosis, for instance by regulating the stability of unknown proteins involved in Cx43 internalization (Qin et al., 2003). Further work is required to elucidate the roles of ubiquitin and the proteasome in Cx43 turnover.

Despite the rapid turnover rate of gap junctions, the molecular mechanisms underlying gap junction endocytosis are still poorly understood. It was recently shown using multicolour fluorescence microscopy that, under steady state conditions of Cx43 expression, newly synthesized connexons are continually being taken to the cell surface and assembled into channels specifically at the edges of the gap junction. From there, they migrate toward the centre of the plaque, from where they are removed by endocytosis (Gaietta et al., 2002). Gap junction endocytosis is a unique process in which the entire gap junction or a fragment of it is internalized into one of the two contacting cells (Jordan et al., 2001). Previous electron microscopy studies reported that gap junction invaginations in granulosa cells were associated with patches of clathrin-like bristles (Larsen et al., 1979). In light of these previous observations, we examined whether EGF-induced Cx43 endocytosis was clathrin dependent. We show that EGF-induced Cx43 endocytosis is blocked by hypertonic medium, in which clathrin-mediated endocytosis is inhibited (Hansen et al., 1993; Heuser and Anderson, 1989; Wu et al., 2001). Receptor-mediated endocytosis of transferrin, a clathrin-dependent process, was also abolished under these conditions. Moreover, the EGF-induced internalization of Cx43 was strongly counteracted by the cholesterol-extracting drug MβCD, which previously has been shown to inhibit clathrin-mediated endocytosis (Rodal et al., 1999). Together, these results suggest that the EGF-induced endocytosis of Cx43 gap junctions is a clathrin-dependent process. However, it should be realized that both hypertonic sucrose and MβCD have global effects, and we cannot rule out the possibility that other mechanisms than inhibition of clathrin function are involved in the block in Cx43 internalization observed under these conditions. Our results indicate that ubiquitination of Cx43 is an early event in Cx43 internalization that occurs at the plasma membrane before its internalization.

Inhibition of GJIC is considered to be an important step in multistage carcinogenesis. This hypothesis is based on studies showing that most tumour cells have dysfunctional GJIC, which is often associated with aberrant connexin localization and reduced connexin protein levels (Holder et al., 1993; Loewenstein, 1979; Yamasaki and Naus, 1996). Moreover, several tumour-promoting agents and oncogenes have been reported to inhibit GJIC. It is thought that post-translational modulation of connexins, including increased internalization and degradation, could be a major mechanism of dysfunctional GJIC induced by carcinogens (Yamasaki and Naus, 1996). Other studies indicate that Cx43 suppresses growth in the absence of functional channels (Huang et al., 1998; Omori and Yamasaki, 1998; Qin et al., 2002), suggesting that the tumour-suppressing effect of Cx43 could involve other functions than forming functional cell-cell communication. Regardless of the actual tumour-suppressing mechanisms of Cx43, increased Cx43 degradation will abrogate these functions. EGF receptor expression contributes to the growth and survival of cancer cells and is overexpressed in many epithelial cancers, including head, neck, breast, colon and prostate cancer (Salomon et al., 1995; Spaulding and Spaulding, 2002). Based on these studies, it is possible that increased signalling from EGF receptor is involved in reducing Cx43 protein levels in tumours by

targeting Cx43 for proteasome-dependent degradation. A clearer understanding of how cellular levels of Cx43 are regulated by the proteasome might be an important step in elucidating the role of Cx43 as a tumour suppressor gene.

In conclusion, this study shows that EGF has several modes of influence on Cx43 gap junctions. Along with a rapid inhibition of cell-cell communication via gap junctions, EGF accelerates ubiquitination, internalization and proteasome-dependent degradation of Cx43. These modifications of Cx43 are mediated by the MAPK pathway. Furthermore, our results indicate that the EGF-induced internalization of Cx43 is a clathrin-mediated process and that ubiquitination of Cx43 occurs at the plasma membrane before its internalization.

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