The origin of annular junctions: a mechanism of gap junction internalization

Karen Jordan, Rochelle Chodock, Art R. Hand* and Dale W. Laird‡
Department of Anatomy and Cell Biology, Medical Sciences Building, M465, University of Western Ontario, London, Ontario, Canada N6A 5C1
*Present address: Department of Pediatric Dentistry, University of Connecticut Health Center, Farmington, CT 06030, USA
‡Author for correspondence (e-mail: dwlaird@julian.uwo.ca)

Accepted 30 November 2000
Journal of Cell Science 114, 763-773 © The Company of Biologists Ltd

SUMMARY

Gap junctional intercellular communication is established when connexin proteins oligomerize into connexon hemichannels, which then pair at the cell surface with connexons from neighboring cells to form functional gap junction channels. Gap junction channels routinely cluster into gap junction plaques, which can exhibit dynamic characteristics while under the frequent processes of formation and removal from the cell surface. We have three lines of evidence to suggest that one mechanism of gap junction removal occurs when one of two contacting cells internalizes the gap junction contribution from both cells. First, in coculture experiments, green fluorescent protein-tagged connexin43 (Cx43-GFP) expressed in normal rat kidney (NRK) cells can be internalized into contacting cells that do not express Cx43-GFP, and the incidences of identifying these internalized structures increase in the presence of lysosomal inhibitors. Secondly, time-lapse imaging of live NRK cells revealed that large areas of gap junction plaques containing Cx43-GFP were internalized as vesicular-like structures into one of two adjacent cells. Finally, when live NRK cells that express endogenous Cx43 were microinjected with anti-Cx43 antibodies, antibody-tagged gap junctions were visualized in cells that contacted the microinjected cell within 3-6.5 hours. Together our results strongly suggest that one mechanism of gap junction removal from the cell surface involves a unique process in which the entire gap junction or a fragment of it is internalized into one of the two contacting cells as an annular junction.

Key words: Connexin43, Gap junction, Internalization, Endocytosis, Confocal microscopy

INTRODUCTION

Gap junctions are intercellular channels found at sites of close cell-cell apposition. Gap junction channels allow for the passage of small molecules and ions between adjacent cells (Flagg-Newton and Loewenstein, 1979; Beyer et al., 1990; Elfgang et al., 1995). Interestingly, gap junction channels cluster on the cell surface into tightly packed arrays commonly referred to as gap junction plaques (Goodenough et al., 1996). It has been determined that gap junctions in the liver have a half-life of approximately 5 hours (Fallon and Goodenough, 1981) and cardiac gap junctions have a half-life of 1.3 hours (Beardslee et al., 1998) in vivo. Likewise, the half-life of connexins in vitro ranges from 1-3.5 hours (Traub et al., 1987; Musil et al., 1990a; Laird et al., 1991; Laird et al., 1995; Darrow et al., 1995; Musil et al., 2000). These results suggest that gap junctions are constantly being renewed, but little information exists as to how gap junctions are removed from the cell surface.

In several electron microscopic studies, double-membrane, intracellular structures termed annular junctions or gap junction profiles were identified (Larsen et al., 1979; Larsen et al., 1981; Mazet et al., 1985). Immunogold labeling for connexins confirmed that these double-membrane structures were connexin-rich (Dermietzel et al., 1991; Risley et al., 1992; Naus et al., 1993). In some cells, annular junctions have been reported to be clathrin-coated (Larsen et al., 1979), associated with the actin cytoskeleton (Larsen et al., 1979), associated with phagolysosomes (Ginzberg and Gilula, 1979), or constituents of multivesicular structures (Severs et al., 1989). Despite such findings, there has been no direct evidence indicating the origin of annular junctions. Once internalized, gap junction proteins have been reported to be degraded by both lysosomes (Larsen and Tung, 1978; Ginzberg and Gilula, 1979; Murray et al., 1981; Larsen and Risinger, 1985; Vaughan and Lasater, 1990; Vaughan and Lasater, 1992; Rahman et al., 1993; Naus et al., 1993; Musil et al., 2000) and proteasomes (Laing and Beyer, 1995; Musil et al., 2000).

Using time-lapse imaging of live MDCK cells expressing a connexin43-green fluorescent protein (Cx43-GFP) chimera, we previously found that the majority of gap junctions in the membrane exhibited restricted mobility. Occasionally, however, fluorescent gap junction plaques coalesced and portions of gap junctions containing Cx43-GFP rapidly internalized (Jordan et al., 1999). There was also considerable heterogeneity in the intermediates used to transport Cx43-GFP (Jordan et al., 1999), suggesting that some of these transport intermediates may represent internalized gap junction en route to degradation. Together, these studies suggest that the dynamic properties of connexins and gap junctions contribute to the regulation and extent of gap junctional intracellular communication (GJIC) between contacting cells.

To identify the mechanism(s) of gap junction internalization we followed the fate of GFP-tagged and antibody-tagged Cx43
in living cells. Cocultures of Cx43-GFP expressing NRK cells and endogenous Cx43 expressing wild-type cells revealed that Cx43-GFP was internalized into contacting cells that lacked the GFP-tagged connexin. Also, time-lapse imaging of live NRK cells expressing Cx43-GFP revealed that gap junctions were rapidly internalized into only one of the two cells participating in the formation of the gap junction plaque. Finally, antibody-tagged gap junction plaques were endocytosed by NRK cells contacting the antibody microinjected cells. Therefore, although both contacting cell membranes participate in the establishment of a gap junction plaque, we provide evidence that one cell may internalize the Cx43 or Cx43-GFP gap junction. This mechanism of gap junction removal from the cell surface involves the internalization of connexin-rich structures referred to as annular junctions.

MATERIALS AND METHODS

Cells, reagents and cDNA constructs

All media, sera and reagents were obtained from Gibco BRL (Burlington, ON, USA), Becton Dickinson (St Laurent, QC, USA) or Sigma Chemical Company (St Louis, MO, USA). LipofectAMINE Plus reagent was obtained from Gibco BRL. Normal rat kidney (NRK-52E) cells (American Type Culture Collection, Rockville Maryland, USA; 1571-CRL) were grown in DME medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Trypsin solution (0.25%, 1 mM EDTA) was purchased from Gibco BRL. Engineering of the connexin43-green fluorescent protein (Cx43-GFP) was performed as described previously (Jordan et al., 1999). Red fluorescent nanobeads (20-30 nm) were purchased from Molecular Probes (Eugene, OR, USA).

Antibodies

The mouse anti-E-cadherin monoclonal antibody was purchased from Transduction Labs (Mississauga, ON, Canada). The rabbit anti-Cx43 polyclonal antibody (CT-360) was raised against a peptide sequence that represents amino acid residues 360-382 of rat Cx43 (Laird and Revel, 1990). The antibody was affinity purified against the peptide as previously described (Laird and Revel, 1990). Mouse anti-Cx43 monoclonal antibody was purchased from Chemicon International Inc. (Temecula, CA, USA), and rabbit anti-GFP polyclonal antibody was purchased from ClonTech (Palo Alto, CA, USA). The following secondary antibodies were all purchased from Jackson ImmunoResearch Laboratories Inc., PA, USA: goat anti-mouse IgG conjugated to Texas Red, donkey anti-mouse IgG conjugated to 7-amino-4-methylcoumarin-3-acetic acid (AMCA), goat anti-rat IgG conjugated to fluorescein or Texas Red, goat anti-rabbit IgG conjugated to Texas Red, and goat anti-mouse IgG conjugated to fluorescein.

Immunolabeling of NRK cocultures and microinjections

NRK cells stably transfected with cDNA encoding Cx43-GFP were trypsinized and plated on 12 mm glass coverslips with wild-type NRK cells. The wild-type NRK cells were previously incubated in the presence of 20-30 nm red fluorescent nanobeads at a 1:25,000 dilution for 24 hours at 37°C. The NRK cocultures were allowed to grow for 24-48 hours following by incubation for 3-6.5 hours in the presence or absence of 50 mM NH₄Cl or 100 μM leupeptin. Cells were rinsed three times with phosphate-buffered saline (PBS) and fixed with 80% methanol/20% acetone at −20°C and immunolabeled as previously described (Laird et al., 1995). For indirect immunofluorescence studies, cocultures were labeled with anti-E-cadherin monoclonal antibody, washed six times over 30 minutes in PBS and incubated in either goat anti-mouse IgG conjugated to Texas Red or goat anti-mouse IgG conjugated to AMCA. Coverslips were rinsed again in PBS six times over 30 minutes, rinsed in distilled water and mounted on glass slides with Airvol mounting medium followed by analysis on a Zeiss LSM 410 inverted confocal microscope as previously described (Laird et al., 1995). The number of Cx43-GFP positive structures per wild-type NRK cell were counted, starting with cells located at the top of the Cx43-GFP-containing cell and proceeding in a clockwise fashion, progressing to all other adjacent, labeled parental cells. Identification of NRK cells transiently transfected with Cx43-GFP was done when NRK cells were fixed, permeabilized and labeled as stated above with anti-GFP antibody followed by incubation with donkey anti-rabbit IgG conjugated to Texas Red. Identification of cell boundaries was done when NRK cells were fixed, permeabilized, and labeled as stated above with anti-ZO-1 antibody followed by incubation with goat anti-rabbit IgG conjugated to Texas Red.

NRK cells prepared for microinjection were grown on glass coverslips and pressure-microinjected with affinity-purified anti-Cx43 antibody (ranging in concentration from 310 μg/ml to as high as 2350 μg/ml; see individual experiments) in the presence or absence of 800 μg/ml synthetic peptide (amino acids 360-382) (Beyer et al., 1987) that correspond to the region of Cx43 used to generate the antibody. After approx. 10 minutes in culture medium or 70 minutes in 100 μM leupeptin-supplemented culture medium the cells were fixed in 3.7% formaldehyde and permeabilized in 0.5% Triton X-100 before labeling with goat anti-rabbit IgG conjugated to Texas Red. To identify the injected cell, 2-4 mg/ml of rat IgG was often comicroinjected with the anti-Cx43 antibody and localized after cell fixation and permeabilization with an additional incubation with goat anti-rat IgG conjugated to fluorescein. In other experiments, NRK cells were comicroinjected with non-specific rat IgG (2-4 mg/ml) and mouse anti-Cx32 monoclonal antibody (3 mg/ml, Fujikura et al., 1993). After fixation and permeabilization, the two microinjected antibodies were localized using goat anti-mouse IgG conjugated to Texas Red and goat anti-rat IgG conjugated to fluorescein.

In other experiments, NRK cells incubated for 3.0-6.5 hours in the presence and absence of 10 mM NH₄Cl were pressure-microinjected with 940-2350 μg/ml anti-Cx43 antibody and cells were fixed, permeabilized and immunolabeled for the microinjected antibody as described above. The same cells were treated with a mouse anti-Cx43 monoclonal antibody followed by goat anti-mouse IgG conjugated to fluorescein.

Transfection of NRK cells with cDNA encoding Cx43-GFP

For transient transfections, NRK cells were plated on glass coverslips in 60 mm culture dishes to reach 50-60% confluency just prior to transfection. NRK cells were transfected in Opti-MEM1 medium (Canadian Life Technologies) containing LipofectAMINE Plus reagents and 1-2 μg of plasmid DNA. After a 3 hour incubation at 37°C, an equal volume of DME medium containing 20% FBS was added to the DNA/LipofectAMINE Plus suspension, and cells were incubated for an additional 24-48 hours at 37°C. NRK cells were then fixed and prepared for indirect immunofluorescence labeling as previously stated.

Imaging of Cx43-GFP in living cells

NRK cells stably transfected with cDNA encoding Cx43-GFP were trypsinized and plated in 35 mm glass bottom tissue culture dishes (MatTek Corporation, MA, USA) with or without wild-type NRK cells previously incubated in the presence of 20-30 nm red fluorescent nanobeads. Cocultured cells were incubated at 37°C for 24-48 hours. For live cell imaging, culture medium was replaced with 2 ml of Hanks balanced salt solution supplemented with 2 mM glutamine, 100 μ/ml penicillin, 100 μg/ml streptomycin, 300 μ/ml calcium chloride, 100 μg/ml magnesium sulfate, and 10 mM Hepes buffer, pH 7.2 (reagents purchased from Gibco BRL or Sigma Chemical
Gap junction internalization

Company). Live cells were then placed on a 20/20 Technology (Mississauga, ON, Canada) temperature-controlled stage that maintained cells at 37°C for the duration of live cell imaging. Living NRK cells expressing Cx43-GFP were cocultured with NRK cells containing red fluorescent nanobeads. The cells were imaged using either a single 488-nm argon/krypton laser line or simultaneously imaged using both 488-nm and 568-nm argon/krypton laser lines on a Zeiss LSM 410 inverted confocal microscope equipped with a 63× oil immersion (1.4 numerical aperture) objective. Optical sections were line scanned at a speed of 32 seconds/image and collected continuously for up to 10.5 minutes. Focus, contrast and brightness settings remained constant during the course of imaging acquisition. Final time-lapse images were developed using both Paint Shop Pro5 and Adobe Premiere software.

Immunoelectron microscopy

For EM studies, albino rat irises were dissected from eyes enucleated from anesthetized animals and fixed in 3% paraformaldehyde/0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer. Small pieces of the irises were dehydrated in methanol and embedded at low temperature in Lowicryl K4M. Sections were labeled with 10-20 μg/ml anti-Cx43 antibody using the procedure described (Murphy et al., 1994). Briefly, grids were blocked with 1% BSA/1% instant milk for 1 hour. Subsequently, the sections were incubated with primary antibody overnight at 4°C followed by secondary goat anti-rabbit IgG conjugated to gold particles (10 nm, Amersham) for 1 hour. All sections were counterstained with uranyl acetate for 1 minute, followed by lead citrate for 1 minute. Electron micrographs were taken on a Philips CM10 transmission electron microscope.

RESULTS

Distribution of Cx43 and Cx43-GFP in NRK cells

To examine the internalization of gap junctions we chose to use Cx43 expressing NRK cells that have well defined sites of cell-cell apposition. Double-immunolabeling for Cx43 (Fig. 1A,C) and ZO-1 (Fig. 1B,C) revealed clearly defined areas of NRK cell-cell contact. To further examine Cx43 gap junction internalization we constructed a Cx43-GFP fusion protein (Jordan et al., 1999). When the cDNA encoding this Cx43-GFP chimera was stably expressed in NRK cells, it was transported and assembled into fluorescent gap junction plaques (Fig. 1D). Our previous studies demonstrated that Cx43-GFP assembled into functional gap junction channels with many wild-type Cx43 characteristics (Jordan et al., 1999). These results show that NRK cells have defined lateral borders and Cx43-GFP has intracellular and plasma membrane distribution patterns that mimic those of endogenous Cx43.

Cx43-GFP gap junction internalization

Since the Cx43-GFP chimera was found to exhibit both phenotypic and functional characteristics (Jordan et al., 1999) similar to wild-type Cx43, we used this construct to investigate the internalization of gap junction plaques containing Cx43-GFP. When NRK cells were transiently transfected with Cx43-GFP, we observed Cx43-GFP-containing intracellular structures in nontransfected adjacent cells (Fig. 2). To confirm that the fluorescent structures found in the non-transfected NRK cells were due to Cx43-GFP and to denote the cell borders, NRK cells were double-labeled for GFP (red) and E-cadherin (blue) (Fig. 2). In all cases, the fluorescent structures observed in cells contacting the Cx43-GFP expressing cells were labeled with anti-GFP antibodies (Fig. 2 insert, arrows). These results suggested that Cx43-GFP was being internalized into a contacting cell that had never been transfected with cDNA encoding Cx43-GFP.

To examine this possibility, we established an NRK cell line that stably expressed Cx43-GFP (Jordan et al., 1999). Cx43-GFP expressing NRK cells were cocultured with wild-type NRK cells that had ingested red fluorescent nanobeads. After a 24 hour incubation, cell cultures were imaged live for the presence of Cx43-GFP and red nanobeads (Fig. 3A) or fixed, permeabilized and immunolabeled with anti-E-cadherin antibody to denote the cell boundaries (Fig. 3B-D). Cx43-GFP fluorescence was found in red nanobead-positive wild-type NRK cells (Fig. 3A-D, see inserts and arrows). Cx43-GFP fluorescent structures found in red nanobead-positive cells were few in number and frequently located juxtaposed to the plasma membrane (Fig. 3A,B; see inserts and arrows), suggesting that...
their formation was infrequent or a possible rapid degradation of internalized Cx43-GFP gap junctions. It had been shown previously that internalized gap junctions were associated with lysosomes in several cell types (Larsen and Tung, 1978; Ginzberg and Gilula, 1979; Murray et al., 1981; Larsen and Risinger, 1985; Vaughan and Lasater, 1990; Vaughan and Lasater, 1992; Rahman et al., 1993; Naus et al., 1993) and Cx43 degradation was prolonged by threefold in the presence of lysosomal inhibitors in NRK cells (Musil et al., 2000). Thus, we incubated the NRK cocultures for 3 hours in the presence of the lysosomal inhibitors NH₄Cl (Fig. 3C) or leupeptin (Fig. 3D) in an attempt to delay or inhibit lysosomal degradation and possibly increase the number of Cx43-GFP structures found in red nanobead-positive cells. Semi-quantitative analysis revealed that red nanobead-positive NRK cells contained an average of 0.58±0.33 (n=50 contacting cells) Cx43-GFP structures, which increased to 4.29±1.32 (n=50 contracting cells) when cocultures were treated with leupeptin or NH₄Cl for 3-6 hours. In addition, approximately 73% of the Cx43-GFP structures found in the red nanobead-positive NRK cells had a diameter of greater than 0.5 μm (n=187 red nanobead-positive NRK cells). Interestingly, both leupeptin and NH₄Cl treatments resulted in the finding of many more intracellular Cx43-GFP structures in the Cx43-GFP expressing NRK cells and there consistently appeared to be a loss of gap junctions at the cell surface (Fig. 3C,D).

**Time-lapse imaging of Cx43-GFP internalization and annular junction formation**

To examine the fate of Cx43-GFP gap junctions present at the cell surface in live cells, we used time-lapse imaging over a period of 10.3 minutes (Fig. 4A-I). In the course of only 2 minutes, an area of cell surface gap junctions appeared to bud and completely separate from the row of preexisting gap junction plaques and enter only one of the two cells expressing Cx43-GFP (Fig. 4D-H, inserts and arrows). It was also observed that when the Cx43-GFP had been removed from the cell surface, there was a rapid redistribution of the cell surface Cx43-GFP. The internalization of Cx43-GFP gap junctions into only one of two contacting cells suggests that the entire gap junction or a fragment of a gap junction is pinched off and

---

**Fig. 2.** Colocalization of Cx43-GFP fluorescence with immunofluorescently labeled GFP. NRK cells were transiently transfected with Cx43-GFP, fixed and double-immunolabeled for GFP (red) and E-cadherin (blue). Overlay of Cx43-GFP fluorescence and the GFP labeling pattern reveal complete colocalization of GFP and Cx43-GFP (pink/yellow). Anti-E-cadherin labeling (blue) indicates cell boundaries and arrows in insert denote intracellular Cx43-GFP structures. Bars, 10 μm.

**Fig. 3.** Internalization of Cx43-GFP. NRK wild-type cells were incubated with red fluorescent nanobeads for 24 hours and cocultured for 24 hours with NRK cells that stably express Cx43-GFP. Cells were imaged live (A) or fixed, permeabilized and labeled for E-cadherin (blue) to denote the cell boundaries (B-D). Scattered Cx43-GFP punctate structures were identified in red nanobead-positive NRK cells when imaged under live conditions (A, arrows) as well as fixed conditions (B). In an attempt to increase the number of Cx43-GFP structures observed in red nanobead-positive NRK cells, cocultures were incubated for 3 hours in the presence of 50 mM NH₄Cl (C) or 100 μM leupeptin (D). Note the increase in intracellular Cx43-GFP positive structures in the red nanobead-positive NRK cells (C,D; see insert and arrows). Bars, 10 μm.
internalized. It is likely that either one of the two contacting cells would be capable of internalizing any given gap junction (see Fig. 5A).

**Antibody-tagged Cx43 gap junctions internalize into contacting cells**

To further examine the initial stages of Cx43 gap junction internalization, we investigated the hypothesis that microinjected anti-Cx43 antibodies that specifically recognize the cytoplasmic domain of Cx43 could be used not only to label gap junctions in live NRK cells but also to follow their fate (Fig. 5B). In the first set of experiments, live NRK cells were comicroinjected with anti-Cx43 antibody and non-specific rat IgG (Fig. 5B) and incubated at 37°C for 70 minutes. After fixation and labeling with appropriate fluorescent secondary antibodies, numerous gap junction plaques were observed at the cell surface of the microinjected cell (Fig. 6A,C red; arrows). Moreover, the binding of the antibody to the gap junction plaques did not induce their rapid removal, as fluorescent punctate structures were rarely observed in the microinjected or neighboring cells during this incubation time. The microinjected cell was easily identified by the presence of rat IgG (Fig. 6B,C green/yellow). The specificity of this anti-Cx43 antibody binding was demonstrated by the lack of anti-Cx43 gap junction plaque labeling in cells comicroinjected with anti-Cx43 antibody and excess Cx43 peptide antigen (amino acids 360-382) (Fig. 6D,F yellow) and rat IgG (Fig. 6E,F, green/yellow).

Once it was established that anti-Cx43 antibodies could bind to gap junctions in live NRK cells, experiments were performed to follow the fate of anti-Cx43 IgG-labeled gap junctions. NRK cells microinjected with excess rabbit anti-Cx43 antibodies were incubated for 6 hours in the presence of NH₄Cl prior to fixation, permeabilization and treatment with secondary antibodies conjugated to fluorescent dyes (Fig. 7A,Aa,B). Excess anti-Cx43 antibody (2.3 mg/ml) was used in these experiments such that microinjected cells could be found and imaged without the necessity of comicroinjecting the non-specific rat IgG antibody. Furthermore, the presence of excess anti-Cx43 antibodies was designed to ensure that a population of gap junctions would be adequately labeled. In these studies, the cells that contact the
microinjected cell were observed to have anti-Cx43 antibody labeled structures (Fig. 7Aa,B; arrows) while cells that did not contact the microinjected cell were essentially devoid of annular junctions (Fig. 7Aa,B). In other experiments where less concentrated anti-Cx43 antibody (818 μg/ml) was microinjected into NRK cells and incubated at 37°C in the absence of NH4Cl, fewer immunolabeled structures were identified in the cells that contact the microinjected cell (Fig. 7C; arrows). Interestingly, internalized antibody-tagged gap junctions were not observed in all cells that contacted the microinjected cell nor did they receive an equal number, suggesting that there may be some undefined predisposition as to which cells receive a gap junction that is destined for internalization.

As a control, NRK cells that lack Cx32 were comicroinjected with high concentrations of non-specific rat IgG (3.7 mg/ml) (Fig. 8a,B) and anti-Cx32 antibody (3.0 mg/ml) and were incubated for 6 hours at 37°C (Fig. 8b). The color overlay (Fig. 8c) demonstrates that neither rat IgG nor anti-Cx32 antibodies, which are not targeted to NRK gap junctions, were internalized into cells that contact the microinjected cell (Fig. 8d, asterisk).

To demonstrate independently and confirm that annular junctions present in the cells contacting the microinjected cell were positive for Cx43, NRK cells were microinjected with a rabbit anti-Cx43 antibody and incubated for 3-3.5 hours at 37°C (Fig. 9). These cells were subsequently fixed, permeabilized and labeled with a mouse anti-Cx43 antibody and fluorescent anti-rabbit (Fig. 9a) and anti-mouse (Fig. 9b) secondary antibodies. Annular junctions were clearly identified in neighboring cells that double-labeled with both anti-Cx43 antibodies (Fig. 9c, yellow). When the microinjected cell was re-imaged under lower contrast and brightness conditions, punctate structures within the cytoplasm labeled with both anti-Cx43 antibodies (Fig. 9 inserts; arrows) indicating that gap junction removal from the cell surface is probably bidirectional (Fig. 5B).

In semi-quantitative studies where several microinjected cells incubated for 3-3.5 hours were examined, an average of three Cx43-positive annular junctions were found per contacting cell (Table 1). Microinjected cells incubated for 6-6.5 hours had an average of four antibody-labeled annular junctions per contacting cell. On the other hand, control cells comicroinjected with anti-Cx43 antibodies and peptide used to generate the antibody, had only 0.1 fluorescent structures per contacting cell.

**Table 1. Accumulation of anti-Cx43 labeled annular junctions in cells that contact the microinjected cell**

<table>
<thead>
<tr>
<th>Microinjected reagents</th>
<th>Post microinjection incubation time (hours)</th>
<th>Number of Cx43-positive annular junctions in cells contacting the microinjected cell</th>
<th>Average number of Cx43-positive annular junctions in cells contacting the microinjected cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>3.0-3.5</td>
<td>33</td>
<td>3.1±0.37</td>
</tr>
<tr>
<td>Antibody</td>
<td>6.0-6.5</td>
<td>32</td>
<td>4.2±0.51</td>
</tr>
<tr>
<td>Antibody + peptide</td>
<td>3.0-3.5; 6.0-6.5</td>
<td>10</td>
<td>0.1±0.03</td>
</tr>
</tbody>
</table>

Affinity-purified anti-Cx43 antibody (940-2350 μg/ml) was microinjected into NRK cells in the presence or absence of 800 μg/ml synthetic peptide and the cells were incubated for 3-6.5 hours at 37°C in the presence of NH4Cl. Punctate structures in cells neighboring the microinjected cell had diameters of 0.4-1.0 μm. Each microinjected cell was surrounded by 5-8 contacting cells. A non-specific background of 0.2 particles/cell was observed by counting 157 cells over five independent experiments using equivalent contrast, brightness and magnification microscopic settings, as used in the collection of data from microinjected cells. This non-specific background was due to the occasional precipitation of the secondary antibody.

Values are means ± s.e.m.

In vivo identification of annular junctions in rat iris epithelial cells

Since our results strongly suggest that cultured NRK cells are capable of internalizing gap junctions as annular junctions, we proposed to determine whether these structures were a common occurrence in vivo. To this end, immunoelectron microscopy was used to ascertain if under steady-state conditions gap junctions are internalized in rat iris epithelial cells in vivo as annular junctions. We chose to examine epithelial tissue of the eye, since annular junctions were previously identified in ciliary epithelium in vivo (Raviola et al., 1980; Tenkova and Chaldakov, 1990). Many Cx43 immunogold-labeled annular junctions were routinely observed in rat iris epithelial cells (Fig. 10; asterisks) and
were clearly composed of a double membrane (Fig. 10; inserts).

DISCUSSION

Although gap junctions are structures that rapidly turnover (Fallon and Goodenough, 1981; Laird et al., 1995; Beardslee et al., 1998), the mechanism of gap junction internalization remains poorly understood. In previous experiments on fixed cells, cytoplasmic bimembranous vesicles commonly referred to as gap junction profiles or annular gap junctions were identified by electron microscopy in vitro (Larsen et al., 1979; Mazet et al., 1985; Severs et al., 1989) and in vivo (Larsen et al., 1979; Raviola et al., 1980; Tenkova and Chalakov, 1990). In more recent years, immunogold-labeling studies have identified intracellular double membrane structures that label for connexins (Dermietzel et al., 1991; Risley et al., 1992; Naus et al., 1993). These structures were speculated to derive from the invagination and internalization of gap junctions, although no direct evidence for this mechanism of gap junction internalization was provided.

Our experiments are the first to specifically address a mechanism of gap junction internalization. We have three lines of evidence to suggest that complete or portions of gap junctions are internalized into one of the two opposing cells, resulting in the formation of the annular junction. First, Cx43-GFP expressing NRK cells cocultured with wild-type parental NRK cells revealed that Cx43-GFP was internalized into neighboring cells devoid of the GFP-tagged Cx43. In addition, time-lapse imaging of live NRK cells provided evidence that large areas of Cx43-GFP gap junction plaques were internalized as vesicular-like structures into only one of two adjacent cells. The third body of evidence for the origin of the annular junction was provided when antibody-tagged Cx43 gap junctions in NRK cells were clearly identified in cells that contacted the antibody-microinjected cell.

The Cx43-GFP fusion protein was a good candidate for investigating Cx43 internalization since we had previously shown that GFP-tagged Cx43 exhibited phenotypic and functional properties that were both much like those of wild-type Cx43 (Jordan et al., 1999; Bukauskas et al., 2000). In coculture experiments in which nanobead-labeled NRK cells were grown with Cx43-GFP expressing NRK cells, we observed annular junctions in the NRK wild-type cells. The internalized products were found at an average of 0.6 annular junctions per neighboring parental cell, while the average number of annular junctions increased to four per contacting cell with the introduction of lysosomal inhibitors. Both proteasomal and lysosomal degradation

Fig. 6. Specific binding of microinjected anti-Cx43 antibodies to gap junctions in live NRK cells. A live NRK cell with apposing cells was comicroinjected with anti-Cx43 antibody (300 μg/ml) (A-C; yellow) and non-specific rat IgG (B; yellow) antibodies followed by a 1.2 hour incubation at 37°C. The cells were fixed, permeabilized and labeled with appropriate secondary antibodies. The arrows (A-C) denote immunolabeled punctate structures at the cell periphery that are not labeled with non-specific rat IgG (B). As a control, NRK cells were comicroinjected with anti-Cx43 antibody and molar excess synthetic peptide (800 μg/ml), which represented the region of Cx43 used to generate the anti-Cx43 antibody (D-F; yellow). Non-specific rat IgG and ZO-1 immunolabeling were used to denote the microinjected cell and localize the boundaries of neighboring cells, respectively (E,F; green). Bars, 10 μm.
pathways have been proposed to be responsible for the degradation of Cx43 (Larsen and Tung, 1978; Ginzberg and Gilula, 1979; Murray et al., 1981; Larsen and Risinger, 1985; Naus et al., 1993; Laing and Beyer, 1995; Musil et al., 2000). The increase in number of undegraded annular junctions in the presence of lysosomal inhibitors was not surprising as Musil and colleagues showed that the degradation of Cx43 in NRK cells was delayed by threefold in the presence of the lysosomal inhibitor chloroquine (Musil et al., 2000). While the inhibitor leupeptin is reasonably specific for lysosomal proteases, NH₄Cl affects the pH of a number of intracellular compartments, including endosomes/lysosomes, and may have more general effects on intracellular function. Consequently, the accumulation of intracellular Cx43-GFP fluorescent structures in cells treated with lysosomal inhibitors may be a combination of internalized annular junctions and swollen

Fig. 7. Internalization of anti-Cx43 antibody labeled gap junctions into adjacent cells. NRK cells were microinjected with anti-Cx43 antibody (2350 µg/ml) and incubated at 37°C for 6-6.5 hours in the presence of 10 mM NH₄Cl (A,Aa,B). Note the presence of antibody-labeled annular junctions only in cells that contact the microinjected cell (Aa,B; arrows). In additional experiments where NRK cells were microinjected with 818 µg/ml of anti-Cx43 antibody and incubated for 6 hours in culture medium in the absence of lysosomal inhibitors, antibody-labeled annular junctions were observed but typically fewer in number (C; arrow). The asterisks denote the microinjected cells. Bars, 10 µm.

Fig. 8. Comicroinjection of anti-Cx32 antibodies and rat IgG in NRK cells. NRK cells comicroinjected with high concentrations of anti-Cx32 antibody and rat IgG were fixed, permeabilized and immunolabeled with appropriate secondary antibodies conjugated to Texas Red (A) and FITC (B), respectively. Overlay of independent signals (C) reveals that the rat IgG and the anti-Cx32 antibody remained inside the injected cell (C,D; asterisks). (D) Transmitted light image of the same field as in A-C. Bar, 10 µm.
transport intermediates that are en route to or from the cell surface.

We had previously identified two distinct populations of Cx43-GFP transport intermediates in live time-lapse images based upon the size of the GFP fluorescent vesicles; <0.5 μm and ≥0.5 μm (Jordan et al., 1999). We found that 73% of annular junctions identified in NRK wild-type cells were ≥0.5 μm in size. Fluorescent structures <0.5μm may reflect smaller annular junctions or, most likely, they may represent Cx43-GFP transport intermediates en route to the cell surface. Cx43-GFP annular junctions were found to completely colocalize with the immunofluorescence localization patterns of GFP and Cx43 suggesting, as expected, that these annular junctions contain both the GFP and Cx43 moieties (results not shown). Time-lapse imaging revealed that in the course of 2 minutes, a portion of a gap junction containing the Cx43-GFP appeared to bud from the membrane, and upon internalization into only one of the two cells, it exhibited vesicular-like and luminal properties.

In another approach to determine the fate of cell surface gap junctions we microinjected affinity-purified anti-Cx43 antibodies that specifically recognized the cytoplasmic carboxy-terminal domain of Cx43 (Laird and Revel, 1990). Although anti-connexin antibodies have been microinjected into mammalian cells to examine their effect on cell-cell communication (Hertzberg et al., 1985; Yancey et al., 1989; Lal et al., 1993), binding to the gap junction plaques was not previously demonstrated. We employed the confocal microscope to show that anti-Cx43 antibodies specifically bind to gap junction plaques in live NRK cells. The mere binding of the antibody to the gap junction did not induce a rapid and large-scale removal of gap junctions, as few fluorescently labeled structures were observed in adjacent cells when microinjected cells were incubated at 37°C for less than 1.5 hours. Since the half-life of Cx43 in NRK cells is 2-2.5 hours (Musil et al., 1990b), we incubated the microinjected cells for up to 6.5 hours in order to allow sufficient time for antibody-tagged gap junctions to be removed from the cell surface. The fact that antibody-labeled gap junctions were removed into a cell that contacted the microinjected cell provided compelling evidence that live NRK cells removed gap junctions from the cell surface such that one of the two apposing cells internalized large numbers of channels from its contacting neighbor to form annular junctions. Thus, one mechanism of gap junction downregulation involves direct participation of apposing cells. It is possible that annular junctions are protected from rapid degradation by the binding of the anti-Cx43 antibody or by the presence of a lysosomal...

**Fig. 9.** Double anti-Cx43 immunolabeling of annular junctions. NRK cells were microinjected with rabbit anti-Cx43 antibody (2350 μg/ml) and incubated at 37°C for 3.5 hours. Cells were fixed, permeabilized and immunolabeled with fluorescent antibodies for microinjected anti-Cx43 antibody (A,C; yellow) or re-labeled for Cx43 using a mouse anti-Cx43 monoclonal antibody followed by fluorescent anti-mouse IgG (B,C; green/yellow). The arrows denote several structures that reside outside the microinjected cell and carry the antibody from the microinjected cell, which double-label with a second anti-Cx43 antibody. On occasion, the steric inhibition of the rabbit anti-Cx43 polyclonal antibody binding to Cx43 at residues 360-382 may impair the binding of the mouse monoclonal anti-Cx43 antibody binding to Cx43 at residues 252-270, resulting in differential intensity of annular junction immunostaining. The inserts in A-C represent the microinjected cell that has been re-imaged under lower contrast and brightness settings. Note that the punctate array seen inside the microinjected cell (A-C, inserts; arrows) has a similar pattern to that observed when the same cell is labeled with a second anti-Cx43 antibody (B,C, inserts; arrows). Bars, 10 μm.
inhibitor. The decision as to which of the apposing cells will receive the internalizing gap junction is unclear.

The removal of integral membrane proteins into a neighboring cell is not without precedent. It was shown in the developing Drosophila retina that the integral membrane ligand, bride of sevenless, from R8 photoreceptor neurons binds the sevenless tyrosine kinase receptor on a R7 cell and is internalized (Cagan et al., 1992). It was suggested that the ligand partitioned out of the R8 cell membrane into the membrane of the R7 cell or that the R7 endocytosed the ligand to form a double-membrane structure. It is difficult to envisage how the polytopic membrane protein, Cx43, and attached antibodies or GFP could partition into the neighboring cell membrane. Consequently, we propose that one mechanism of gap junction internalization in NRK cells involves the removal of the entire gap junction into one of the two contacting cells. A remaining question from this study is how prevalent is this mechanism of gap junction internalization. Even in the presence of protease inhibitors, GFP- or antibody-tagged Cx43 internalized gap junctions are not frequently found in NRK cells. In some in vivo tissues like the rat iris epithelium, annular junctions are numerous and easy to identify, while in other tissues such as the liver, internalized gap junctions are rare or absent. Given the fact that both proteasomes and lysosomes have been reported to degrade connexins, with the level of their involvement being cell-type specific (Laing and Beyer, 1995; Musil et al., 2000), it is reasonable to propose that other routes of presenting connexins for degradation may exist and the level of involvement of such mechanisms may also be somewhat cell-type specific. Consequently, given the rarity of annular junctions in some cells it is unlikely that annular junctions are the only mechanism responsible for clearing gap junctions from the cell surface. This process would require considerable energy and necessitates the coordinated involvement and membrane repair of both contacting cells. It is also possible that there are both constitutive and regulatory mechanisms for removing gap junctions from the cell surface depending upon the physiological need of the tissue. The lack of readily identified annular junctions in a number of cells suggests that gap junctions may also be capable of disassembling en route to degradation. Such a mechanism has been proposed in regenerating hepatocytes (Fujimoto et al., 1997), where electron microscopic studies suggest that gap junction plaques are broken into smaller aggregates before they are removed from the cell surface.

In summary, we have provided data showing that annular junctions are in fact internalized gap junctions, and this unique and well-coordinated process provides a mechanism for downregulating several hundred gap junction channels at one time. It remains to be determined if other junctional complexes can be internalized in a similar manner.

The authors would like to thank Drs J.-P. Revel, S. Fraser, J. Bergeron, P. Lampe, T. Woodward and M. Sia for their insightful comments. We also thank Drs Y. Wang and J. O-Rourke for providing the rat irises. Drs N. Gonatas and Y. Fujikura generously provided the anti-MG-160 and anti-Cx32 (HAM8) antibodies, respectively. The hybridoma R26.4C (anti-ZO-1) developed by D. Goodenough was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, The John Hopkins University School of Medicine.
REFERENCES


