Intracellular Trafficking Pathways in the Assembly of Connexins into Gap Junctions*

(Received for publication, October 30, 1998, and in revised form, January 19, 1999)

Christopher H. George‡, Jonathan M. Kendall§, and W. Howard Evans

From the Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, Wales, United Kingdom

Trafficking pathways underlying the assembly of connexins into gap junctions were examined using living COS-7 cells expressing a range of connexin-aequorin (Cx-Aeq) chimeras. By measuring the chemiluminescence of the aequorin fusion partner, the translocation of oligomerized connexins from intracellular stores to the plasma membrane was shown to occur at different rates that depended on the connexin isoform. Treatment of COS-7 cells expressing Cx32-Aeq and Cx43-Aeq with brefeldin A inhibited the movement of these chimeras to the plasma membrane by 84 ± 4 and 88 ± 4%, respectively. Nocodazole treatment of the cells expressing Cx32-Aeq and Cx43-Aeq produced 29 ± 16 and 4 ± 7% inhibition, respectively. In contrast, the transport of Cx26 to the plasma membrane, studied using a construct (Cx26/43T-Aeq) in which the short cytoplasmic carboxy-terminal tail of Cx26 was replaced with the extended carboxy terminus of Cx43, was inhibited 89 ± 5% by nocodazole and was minimally affected by exposure of cells to brefeldin A (17 ±11%). The transfer of lucifer yellow across gap junctions between cells expressing wild-type Cx32, Cx43, and the corresponding Cx32-Aeq and Cx43-Aeq chimeras was reduced by nocodazole treatment and abolished by brefeldin A treatment. However, the extent of dye coupling between cells expressing wild-type Cx26 or the Cx26/43T-Aeq chimeras was not significantly affected by brefeldin A treatment, but after nocodazole treatment, transfer of dye to neighboring cells was greatly reduced. These contrasting effects of brefeldin A and nocodazole on the trafficking properties and intercellular dye transfer are interpreted to suggest that two pathways contribute to the routing of connexins to the gap junction.

Gap junctions provide a direct intercellular route allowing adjacent cells in tissues and organs to communicate signaling and regulatory molecules (1, 2). Gap junctions discriminate between molecules mainly on the basis of size (<1 kDa) and charge, and the channels are gated by transmembrane voltage (3) and chemically, involving cytoplasmic concentrations of hydrogen and calcium ions (4, 5). Accessory proteins (6, 7) and putative soluble factors (8) are also candidates in the regulation of the gating of gap junctions. Mutations detected in the cDNA encoding connexin (Cx) protein subunits of gap junctions have shown that Cx32 and Cx26 are implicated in Charcot-Marie-Tooth X-linked peripheral neuropathy and sensorineural deafness, respectively (9, 10).

A key event in the formation of gap junction channels is the oligomerization of connexins into hexameric hemichannels (connexons). These align and interact with connexons in the plasma membrane of adjacent cells to form an electrically sealed intercellular channel (11). Oligomerization of connexins was reported to occur after exit from the endoplasmic reticulum (ER) (12), and oligomeric assembly is promoted in the Golgi apparatus (13). These properties suggest that connexins are unusual membrane proteins since protein folding and oligomerization in the membrane are usually requirements for exit from the ER (14, 15).

Several studies have shown that connexins accumulate in intracellular stores that correspond to the ER/Golgi in cultured cells (16–18) and tissues (19), and growth factors appear to regulate connexin transport between these stores and the gap junction (20). Transport of membrane proteins in general from such intracellular stores to the plasma membrane is likely to involve tubulovesicular networks (21, 22). Gap junctions are dynamic structures, with their constituent connexin proteins having high turnover rates (16, 23). Since the extent of intercellular communication is a function of the number of gap junction channels participating in coupling (24) and because gap junctions are rapidly removed from the plasma membrane (16, 25, 26), the translocation of connexins from intracellular sites to the plasma membrane compensates for this high turnover to maintain and modulate coupling between cells.

We have used connexin-26, -32, and -43 fused to the calcium reporter aequorin to study the trafficking pathways that lead to the generation of functional gap junction channels (18, 27, 28). Gradations in the cytoplasmic calcium levels surrounding various stations as they trafficked along the secretory pathway and at the gap junction were determined (18). To overcome the nonfunctionality of the Cx26-Aeq chimera, the 16-amino acid carboxy-terminal tail of Cx26 was replaced with the 156-amino acid carboxy-terminal tail of Cx43, producing a construct (Cx26/43T-Aeq) whose behavior could not be distinguished from that of Cx26 (18). In this work, the inherent chemiluminescent activity of a series of Cx-Aeq chimeras was used to quantify levels of connexins and their oligomeric status in intracellular stores and at the plasma membrane, and a method was devised to measure the kinetics of translocation between these stores and the plasma membrane. Applications of drugs that disrupt protein trafficking and the cytoskeleton (brefeldin A, nocodazole, and monensin) pointed to differences

* This work was supported by the Medical Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Cardiology, Wales Heart Research Inst., Heath Park, Cardiff CF4 4XN, Wales, UK. Tel.: 44-1222-744518; Fax: 44-1222-743500; E-mail: georgech@cf.ac.uk.

§ Present address: Amersham Pharmacia Biotech, Forest Farm, Whitchurch, Cardiff CF4 7YT, Wales, UK.

The abbreviations used are: Cx, connexin; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum/Golgi intermediate compartment; Cx-Aeq, connexin-aequorin; KRH, Krebs-Ringer-Henseleit buffer.
in the properties of trafficking of the Cx26, Cx32, and Cx43 chimeras to plasma membranes and gap junctions.

**EXPERIMENTAL PROCEDURES**

**Materials**—All cell culture reagents and plasticware were from Life Technologies, Inc.; other reagents were obtained from Sigma unless specified.

**Measurement of Delivery of Connexins to the Plasma Membrane from Intracellular Stores**—COS-7 cells were transfected with plasmid cDNA encoding Cx32-Aeq, Cx43-Aeq, Cx26/43T-Aeq, and Cx32/43T-Aeq (18); trypsinized; seeded onto glass coverslips (8 × 10^5/100 ml); and processed for photon counting as described (27). Four h prior to commencement of experiments, cells were incubated in tissue culture medium containing 3 mM EGTA to give nominal free calcium and a 5 μM final concentration of coelenterazine (Molecular Probes, Inc.). Coverslips were fixed onto a perfusion chamber and brought into contact with a fiber-optic bundle attached to a photon-counting camera (Photek 216) (29). Cells were perfused with Krebs-Ringer-Henseleit solution containing nominal free Ca^{2+} (KRH—Ca^{2+}): 120 mM NaCl, 25 mM Hepes, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 1 mM EGTA, pH 7.4) for 10 min to remove excess coelenterazine. Plasma membrane-associated recombinant protein was selectively consumed by perfusing the cells with KRH—Ca^{2+} (120 mM NaCl, 25 mM Hepes, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 1 mM CaCl₂, pH 7.4). Recombinant protein that has been previously exposed to Ca^{2+} (causing the loss of bound coelenterazine) cannot contribute to further chemiluminescence measurements. This procedure is shown in Fig. 1.

**Translocation of Connexins to the Plasma Membrane**—Cell culture, transfection, and processing for photon counting were carried out as described above. Coelenterazine (5 μM final concentration) in Ca^{2+}-free Dulbecco’s modified Eagle’s medium was added 4 h prior to photon counting. The following inhibitors were added 1 h prior to start of experiments: brefeldin A (5 μg/ml), nocodazole (20 μg/ml; Calbiochem), and monensin (20 μM). Cells exposed to nocodazole were processed prior to experiments as described (31). During the experiments, all perfusion media (KRH—Ca^{2+} and KRH + Ca^{2+}) contained the appropriate drug at the specified concentrations. The effect of inhibitors on the recovery of chemiluminescence activity at the plasma membrane was measured at time intervals between 5 and 25 min using the above protocol.

**Oligomeric Status of Recombinant Connexins in Intracellular Stores**—COS-7 cells (1 × 10^5) transfected with cDNA encoding each recombinant connexin and propagated for 48 h were scraped into phosphate-buffered saline, pH 7.4; pelleted by centrifugation (1500 × g, 10 min); resuspended in 1 ml of homogenization buffer (10 mM Tris-HCl, pH 7.4; 50 mM NaCl, 50 mM potassium acetate, 1 mM dithiothreitol, and 250 mM sucrose, pH 7.4) supplemented with protease inhibitor mixture (0.5 μg/ml pepstatin A, 5 μg/ml leupeptin, 5 μg/ml chymostatin, 5 μg/ml antipain, 5 μg/ml aprotinin, and 10 μg/ml phenylmethylsulfonyl fluoride). Cells were dispersed by passage through a fine gauge needle (20 times) and sonicated (10 × 20 s; Decon Laboratories, Ltd.). The resultant suspension was centrifuged (500 × g, 15 min) to obtain a post-nuclear supernatant. The post-nuclear supernatant was separated on 15–40% (w/v) sucrose density gradients as described by Hammond and Helenius (32) into 16 (1 ml) fractions, and their densities were calculated from refractive index measurements. The subcellular compartments corresponding to the ER (17–22% (w/v) Nycodenz, fractions 1–5), ERGIC (13–16% (w/v) Nycodenz, fractions 6–11), and the Golgi (9–12% (w/v) Nycodenz, fractions 12–16) were verified by assay across the gradients of mannosyltransferase, Golgi 58-kDa protein, and galactosyltransferase, respectively (18). Fractions corresponding to each subcellular compartment were pooled. To determine the distribution of Cx-Aeq chimeras across the gradient, 500 μl from each pool was incubated with an equal volume of buffer (1 mM EDTA, 500 mM NaCl, 5 mM β-mercaptoethanol, and 10 mM Tris-HCl, pH 7.4) and 5 μM coelenterazine for 2 h in the dark at 4 °C. Chemiluminescence activity was then measured by luminometry (Berthold) after injection of 50 μM CaCl₂ into the sample. The remainder of the pooled ER, ERGIC, and Golgi samples from the Nycodenz gradients (see above) were dialyzed against distilled H₂O (removal of Nycodenz was monitored by refractometry). Cx-Aeq samples were then dialyzed under vacuum, lyophilized, and mixed with an equal volume of 2× solubilization buffer (20 mM triethanolamine, 20 mM EDTA, and 20 mM dithiothreitol, pH 9.2). Dodecyl maltoside (0.2%, w/v) was added to solubilize membranes, but to keep oligomeric forms of connexins intact (13), and each pooled sample was mixed by orbital rotation for 1 h at 4 °C. Pool fractions corresponding to the ER, ERGIC, and Golgi were added to separate preformed sucrose gradients (5 ml, 10–40% (w/v) sucrose) containing dodecyl maltoside (0.2%, w/v) and centrifuged for 22 h at 150,000 × g to determine the oligomeric status of connexins within the ER, ERGIC, and Golgi according to sedimentation rates (12). Fractions (10 × 0.5 ml) were collected from the bottom of the tube, and following the removal of 20 μl from each for refractive index measurements, the chemiluminescence activity of each fraction was measured. Under the above conditions (10–40% (w/v) sucrose, 22 h, 150,000 × g), the resultant sucrose gradient was categorized as follows: <15% (w/v) sucrose, monomer (connexin); 15–33% (w/v) sucrose, oligomeric intermediates; and >33% (w/v) sucrose, hexamer (connexon) (according to the migration of known molecular mass proteins) (13).

**Effect of Inhibitors on Plasma Membrane-associated Connexins**—COS-7 cells transiently expressing various Cx-Aeq chimeras were incubated in Ca^{2+}-free Dulbecco’s modified Eagle’s medium containing an inhibitor of protein trafficking (5 μg/ml brefeldin A, 20 μg/ml nocodazole, or 20 μM monensin) for 1–6 h prior to start of measurement of chemiluminescence. The amount of plasma membrane-associated recombinant connexin at each time interval was quantified for each protein as described above.

To investigate the effects of inhibitors on total recombinant protein in cells (at the plasma membrane and in intracellular stores), COS-7 cells (1 × 10^6) expressing Cx-Aeq chimeras were exposed to brefeldin A, nocodazole, or monensin for 2 or 6 h. Cells were subsequently harvested in hyposmotic buffer (1 mM EDTA, 5 mM β-mercaptoethanol, and 20 mM Tris, pH 7.4) and freeze-thawed (five cycles). Nuclei were removed from the suspension by centrifugation (500 × g, 15 min), and the protein concentration of the supernatant was determined (Pierce). Reconstitution of the extracted recombinant protein with coelenterazine and measurement of chemiluminescence activity were carried out as described above.

**Intercellular Transfer of Lucifer Yellow**—HeLa cells were microwaved with plasmid cDNA encoding wild-type or chimeric connexins (28). Transient expression of recombinant protein in >95% cells was observed 24 h later, and cells were then incubated in brefeldin A (5 μg/ml) or nocodazole (20 μg/ml) for 2 or 6 h. Lucifer yellow (5% (w/v) in 0.3 M LiCl) was injected into the cytoplasm, and 10 min later, cells were fixed in paraformaldehyde (4% (v/v) in phosphate-buffered saline). Dye transfer was quantified by viewing cells on a fluorescence microscope (Zeiss) fitted with appropriate filters.

**RESULTS**

**Delivery of Cx-Aeq Chimeras to the Plasma Membrane Occurs at Different Rates**—The addition of Ca^{2+} to cells previously maintained in Ca^{2+}-free media has been shown to selectively trigger plasma membrane-associated aequorin (18, 30). The remaining chemiluminescence in a population of cells expressing chimeric connexins (i.e. intracellular stores that are unaffected by the addition of KRH + Ca^{2+} to the cells formerly maintained in KRH — Ca^{2+}) was calculated at the end of each experiment by exposing cells to 5 mM Ca^{2+} in H₂O; this allowed the relative amounts of Cx-Aeq at the plasma membrane and sequestered in intracellular stores to be determined. Following consumption of all plasma membrane-associated recombinant protein, cells were perfused for variable times in KRH — Ca^{2+}. By measuring the recovery of chemiluminescence activity at the plasma membrane at different time points (Fig. 1), it became evident that the connexins were delivered to the plasma membrane at different rates (Fig. 2). After consumption of all Cx-Aeq chimeras initially present at the plasma membrane, the rate of appearance of chimeras at the plasma membrane was Cx26/43T-Aeq (5 min) > Cx32-Aeq > Cx32/43T-Aeq (15 min) (Fig. 2, C (panel i), B (panel ii), A (panel iii), and D (panel iii), respectively).

**Effects of Inhibitors on the Translocation of Connexins to the Plasma Membrane**—The amount of Cx32-Aeq (Fig. 3A), Cx43-Aeq (Fig. 3B), Cx26/43T-Aeq (Fig. 3C), and Cx32/43T-Aeq (Fig. 3D) detected at the plasma membrane increased with time. The translocation of various connexins to the plasma membrane was influenced by various inhibitors of protein trafficking and depended on the connexin isoform to which the aequorin reporter was attached. Delivery of Cx32-Aeq, Cx43-Aeq, and Cx26/43T-Aeq from intracellular stores to the plasma mem-
brane was inhibited by 83 ± 4, 87 ± 4, and 81 ± 4%, respectively, in cells treated with brefeldin A (Fig. 3, A, B, and D, respectively) compared with untreated cells. Treatment of cells expressing these chimeric proteins with nocodazole had a much lower relative effect on their movement to the plasma membrane; nocodazole reduced the amount of Cx32-Aeq, Cx43-Aeq, and Cx32/43T-Aeq at the plasma membrane by 29 ± 6, 4 ± 7, and 7 ± 7%, respectively (Fig. 3, A, B, and D, respectively). In contrast, delivery of Cx26/43T-Aeq to the plasma membrane was almost completely inhibited by nocodazole treatment (89 ± 5% inhibition of translocation to the plasma membrane). However, translocation of Cx26/43T-Aeq was affected to a lesser extent by exposure to brefeldin A (16 ± 11% inhibition of movement to the plasma membrane) (Fig. 3C). The movement of all connexins to the plasma membrane was inhibited by monensin treatment (Fig. 3, A–D). A combination of brefeldin A and nocodazole completely inhibited trafficking of all Cx-Aeq chimeras (data not shown).

**Intracellular Stores of Connexins: Location and Oligomeric Status**—The intracellular location of the Cx-Aeq chimeras was analyzed using a subcellular fractionation approach. Cx32-Aeq, Cx43-Aeq, and Cx32/43T-Aeq accumulated in the Golgi region of COS-7 cells (Table I) as determined by their comigration with galactosyltransferase (18). Cx26/43T-Aeq was found predominantly in the ERGIC (revealed by its comigration with Golgi 58-kDa protein), with only 21 ± 5% of the total chimera being transferred to the Golgi apparatus (Table I). Analysis of connexin assembly demonstrated that the oligomerization process commenced in the ER, and the formation of connexons, i.e. protein migrating at the 9 S position on sucrose gradients (13, 36), was promoted as the connexin trafficked along the secretory pathway (Fig. 4); the extent of oligomerization (determined on the basis of the amount of connexon formed) of each connexin was Cx43-Aeq > Cx26/43T-Aeq > Cx32-Aeq (Fig. 4, B, C, D, and A, respectively). However, the fusion of aequorin to the carboxyl-terminal tail of each connexin appeared to hinder the oligomerization of Cx-Aeq chimeras relative to that observed with wild-type connexins (28). The results show that Cx26/43T-Aeq was assembled into connexons at an earlier stage than observed with the Cx32-Aeq, Cx43-Aeq, or Cx32/43T-Aeq chimera, and its maximal oligomerization into connexons occurred, importantly, in the ERGIC environs of the cell (Fig. 4C). However, it was noted that the Cx-Aeq chimeras were not fully oligomerized in these

---

**FIG. 1.** Schematic diagram showing how the recovery of chemiluminescent activity of Cx-Aeq chimeras at the plasma membrane was determined. Reconstituted Cx-Aeq chimeras at the plasma membrane were maintained by incubation of cells in Ca²⁺-free Dulbecco’s modified Eagle’s medium/KRH–Ca²⁺ (A). Selective consumption of plasma membrane-associated Cx-Aeq chimeras by addition of 1.3 mM Ca²⁺ to the perfusion medium does not trigger intracellular stores of recombinant protein (B). Coelenterazine is unavailable to bind to the Cx-Aeq chimeras once it has been consumed at the plasma membrane. Subsequent incubation of cells in EGTA (KRH–Ca²⁺) permits trafficking of reconstituted Cx-Aeq (from pre-existing intracellular locations) to the plasma membrane (C). Newly delivered Cx-Aeq at the plasma membrane can be detected by re-addition of Ca²⁺ to the perfusion medium (KRH + Ca²⁺) as described above (D).

**FIG. 2.** Cx-Aeq chimeras are trafficked to the plasma membrane at different rates. Following initial consumption of aequorin (see Fig. 1B), the appearance of Cx32-Aeq (A), Cx43-Aeq (B), Cx26/43T-Aeq (C), and Cx32/43T-Aeq (D) at the plasma membrane was quantified after perfusion of cells in KRH–Ca²⁺ for 5 (panels i), 10 (panels ii), and 15 min (panels iii) and subsequent addition of KRH + Ca²⁺. The total amount of unconsumed Cx-Aeq (i.e. located intracellular stores) was determined following cell lysis in 5 mM Ca²⁺ as described under “Experimental Procedures.” Data are plotted as means (n = 4).
intracellular stores, and they existed as a mixture of hexameric connexons and non-hexameric oligomers (Fig. 4).

Removal of Connexins from the Plasma Membrane Occurs at Different Rates—Cx-Aeq chimeras specifically associated with the plasma membrane accounted for 2–5% of the total recombinant protein expressed by COS-7 cells, with the majority of Cx-Aeq chimeras being located in intracellular stores (Fig. 4). However, Cx32-Aeq (3 ± 0.2%), Cx43-Aeq (4 ± 0.5%), Cx26/43T-Aeq (5 ± 0.3%), and Cx32/43T-Aeq (4 ± 0.4%) (Fig. 5, A–D at t = 0, respectively) were detected at the plasma membrane in different amounts. Trafficking of Cx32-Aeq (Fig. 5A), Cx43-Aeq (Fig. 5B), or Cx32/43T-Aeq (Fig. 5D) to the plasma membrane was inhibited by incubating COS-7 cells expressing each of these chimeric connexins in brefeldin A. Under these conditions, the time required for the removal of 50% of Cx32-Aeq, Cx43-Aeq, and Cx32/43T-Aeq from the plasma membrane (t½) was 198 ± 24, 168 ± 19, and 165 ± 27 min, respectively. The majority of Cx32-Aeq (85 ± 5%), Cx43-Aeq (76 ± 4%), and Cx32/43T-Aeq (74 ± 6%) was removed from the plasma membrane following exposure to brefeldin A for 6 h. Treatment of COS-7 cells expressing Cx32-Aeq, Cx43-Aeq, or Cx32/43T-Aeq with nocodazole did not greatly affect the amount of recombinant protein associated with the plasma membrane even after 6 h of incubation (13 ± 7, 13 ± 6, and 21 ± 8% decreases, respectively) compared with untreated cells (Fig. 5, A, B, and D, respectively). In contrast, removal of Cx26/43T-Aeq from the plasma membrane took place more rapidly (t½ = 54 ± 10 min) when its trafficking to the plasma membrane was inhibited by nocodazole (Fig. 5C). Brefeldin A treatment of cells expressing Cx26/43T-Aeq resulted in a longer residence time of this chimeric connexin at the plasma membrane (13 ± 5% decrease) compared with other Cx-Aeq chimeras (Fig. 5C). Incubation of cells with monensin prevented trafficking of all Cx-Aeq chimeras to the

### TABLE I

| Cx-Aeq chimera    | Subcellular distribution of Cx-Aeq chimera |
|------------------|------------------------------------------|
|                  | ER    | ERGIC | Golgi   |
| Cx32-Aeq         | 14.7 ± 3.2 | 16.3 ± 4.1 | 69 ± 8.4 |
| Cx43-Aeq         | 13.2 ± 4.1 | 6.2 ± 1.2  | 80.6 ± 9.6 |
| Cx26/43T-Aeq     | 15.8 ± 3.7 | 62.2 ± 9.7  | 21.0 ± 4.5 |
| Cx32/43T-Aeq     | 24.6 ± 5.2 | 17.6 ± 4.2  | 57.8 ± 6.3 |

*FIG. 3. Effect of inhibitors of protein trafficking on the delivery of Cx-Aeq chimeras from internal stores to the plasma membrane. Trafficking of Cx32-Aeq (A), Cx43-Aeq (B), and Cx32/43T-Aeq (D) to the plasma membrane (PM) was inhibited by treatment of cells with brefeldin A (■), but was only slightly affected by nocodazole treatment (▲) compared with untreated cells (●). In contrast, movement of Cx26/43T-Aeq to the plasma membrane (C) was inhibited by incubation of cells in nocodazole and was relatively unaffected by brefeldin A treatment. Trafficking of all Cx-Aeq chimeras was blocked by monensin (■, A–D). All data are plotted as the mean ± S.E. (n = 4).*
plasma membrane (Fig. 3) and inhibited their subsequent removal from the cell periphery (Fig. 5, A–D). In complementary experiments using HeLa cells expressing various Cx-Aeq chimeras, both the amount of Cx-Aeq chimeras present at the plasma membrane and their loss from the cell surface induced by various inhibitors of protein trafficking were no different from what was observed in COS-7 cells (data not shown).

Incubation of cells with nocodazole or monensin for up to 6 h had little effect on the total levels of Cx-Aeq chimeras expressed (Table II). Prolonged exposure to brefeldin A (6 h) decreased the amount of all Cx-Aeq chimeras in cells (82 ± 9%). This probably represents removal from intracellular stores owing to degradation (16) and therefore did not affect the quantification of brefeldin A-induced loss of connexin from the plasma membrane.

**Intercellular Dye Transfer Depends on a Threshold Amount of Connexin at the Plasma Membrane**—Intercellular transfer of Lucifer yellow was determined following treatment of HeLa cells expressing Cx-Aeq chimeras and wild-type connexins with brefeldin A or nocodazole (Table III). After a 2-h incubation in brefeldin A (when 2–3% the total recombinant connexin was present at the plasma membrane), dye transfer between cells expressing Cx32 and Cx43 (as Cx-Aeq chimeras or wild-type connexins) was not affected (Table III). However, after a 6-h exposure to brefeldin A, conditions that lowered the amount of connexin at the plasma membrane to <1%, the transfer of Lucifer yellow between cells expressing these connexins was abolished (Table III). Despite its minor effect on the amount of Cx32-Aeq, Cx43-Aeq, and Cx32/43T-Aeq (13 ± 7, 13 ± 6, and 21 ± 8% decreases, respectively) at the plasma membrane, nocodazole treatment of HeLa cells expressing these chimeric connexins for 6 h resulted in a markedly lower efficiency of dye coupling.

Intercellular dye transfer between cells expressing Cx26/43T-Aeq or wild-type Cx26 was reduced by incubation in nocodazole for 2 h (~1.5% of total Cx26/43T-Aeq at the plasma membrane) or 6 h (~1% of total Cx26/43T-Aeq at the plasma membrane) (Fig. 5C). However, despite the loss of coupling observed following nocodazole treatment, a detectable level of dye transfer remained (Table III). Brefeldin A treatment of cells expressing Cx26/43T-Aeq or wild-type Cx26 did not significantly alter levels of cell-to-cell coupling (Table III).

**DISCUSSION**

The inherent chemiluminescent properties of connexins fused to the calcium-dependent photoprotein aequorin have allowed trafficking pathways to be studied. We showed that the time of transfer of various Cx-Aeq chimeras from intracellular stores to the plasma membrane depended on the connexin isoform. Stores of chimeric protein were detected in different intracellular locations. Cx43-Aeq resided in a different environment of the Golgi compared with Cx32-Aeq and Cx32/43T-Aeq, whereas Cx26/43T-Aeq was found predominantly in the ERGIC in COS-7 cells (18). The calcium environments surrounding these intracellular connexin stores were different (18). The present results do not allow us to distinguish between chimeras translocating at the same speed but from different intracellular locations to the plasma membrane or whether they trafficked at different rates from spatially separate stores (33). Since Cx32-Aeq and Cx32/43T-Aeq are in similar intracellular locations and take a similar time to reach the plasma membrane, both taking longer than Cx43-Aeq, it is concluded that the cytoplasmic carboxyl-terminal domain has little influence on the trafficking properties of these two chimeras. The results with Cx26/43T-Aeq as well as other constructs in which the carboxyl-terminal tail of Cx43 was removed or truncated (28, 34) support the conclusion that the trafficking of connexins to gap junctions is not dependent on the integrity of the carboxy-terminal tail.

Subcellular fractionation of transfected COS-7 cells demonstrated that oligomerization of connexins had commenced in the ER and increased as the proteins trafficked along the secretory pathway, broadly in agreement with other findings (12). However, the serial process of oligomerization of Cx-Aeq chimeras into connexons was incomplete, with up to 65% of chimeric protein in the hexameric form, and we propose that...
these intracellular stores exist as a mixture of partially and fully assembled chimeric connexons. These results raise questions regarding the oligomerization and assembly of connexons into gap junctions. First, proteins that oligomerize do so before exiting the ER (35). The subcellular fractionation and oligomerization approaches showed that the failure of recombinant connexins to oligomerize fully did not cause a major retention of chimeric connexins in the ER. The results suggest that both ERGIC and Golgi stations of the secretory pathway promote oligomerization and that the partial oligomerization initiated in the ER is sufficient to permit onward trafficking of connexins. Second, since the plasma membrane contains mainly hexameric connexons (13, 38), we propose that the Golgi possesses mechanisms that retain incomplete oligomers but permit the onward trafficking of fully oligomerized hexameric hemichannels to the plasma membrane. These and other results (12) reinforce the conclusion that oligomerization is completed in the Golgi and that oligomerization is a key determinant for the movement of connexons from the Golgi to the plasma membrane. Also, the results show that, despite being present in the Golgi in similar amounts compared with Cx43, Cx32 oligomerized to a lesser extent and was also present at the plasma membrane in lower amounts than Cx43, again highlighting the conclusion that the Golgi is a key organelle in the oligomerization of these two connexins.

Inhibitors were used to study the routes of gap junction assembly. The translocation of Cx32-Aeq, Cx43-Aeq, and Cx32/43T-Aeq chimeras to the plasma membrane was inhibited by brefeldin A, but nocodazole treatment (39–41) had only minor effects. These results argue strongly that trafficking of the Cx32 and Cx43 chimeras occurred via the Golgi apparatus since it was interrupted by disruption of this organelle. Delivery to the plasma membrane was compromised by nocodazole treatment, for Lucifer yellow transfer in cells expressing Cx43 and Cx32 chimeras was reduced by -30%, but this was probably caused by nocodazole-induced remodeling of the plasma membrane.

FIG. 5. Removal of Cx-Aeq chimeras from the plasma membrane occurs at different rates in COS-7 cells. The amount of Cx32-Aeq (A), Cx43-Aeq (B), Cx26/43T-Aeq (C), and Cx32/43T-Aeq (D) present at the plasma membrane (PM) was determined following incubation of cells in brefeldin A, nocodazole (A), or monensin (C) for 0–6 h. Quantification was by chemiluminescence measurements, and data are given as the mean ± S.E. (n = 4).

**TABLE II**

Effect of protein trafficking inhibitors on the amount of Cx-Aeq chimeras in COS-7 cells

The Cx43-Aeq content of cells was determined following treatment of cells with inhibitors of protein trafficking for 2 or 6 h. The results are expressed as the means ± S.E. (n = 3) and are representative of the effects of protein trafficking inhibitors on all Cx-Aeq chimeras.

| Inhibitor      | Chemiluminescent activity counts/µg cellular protein (×10⁻³) |
|---------------|------------------------------------------------------------|
|               | 2 h             | 6 h             |
| Untreated     | 6.55 ± 0.51     | 6.61 ± 0.52     |
| Brefeldin A   | 6.09 ± 0.61     | 5.42 ± 0.55     |
| Nocodazole    | 6.71 ± 0.51     | 6.34 ± 0.51     |
| Monensin      | 6.68 ± 0.49     | 6.45 ± 0.54     |
membrane (42, 43). As expected, disruption of the Golgi by brefeldin A treatment severely inhibited dye transfer by cells expressing Cx43 and Cx32 chimeras.

The effects of brefeldin A and nocodazole treatment on cells expressing Cx26/43T-Aeq were different, with implications for the trafficking route followed by this chimera. The delivery to the plasma membrane of the Cx26 chimera, in contrast to that of the Cx32 and Cx43 chimeras, was largely unaffected by brefeldin A treatment. Also, the cells, whether expressing Cx26 or its chimera, were functional as demonstrated by Lucifer yellow transfer. The brefeldin A insensitivity of the trafficking of the majority of the Cx26 chimera is highly suggestive that it may have reached the plasma membrane via a route that does not directly involve the Golgi apparatus. Furthermore, the sensitivity of the Cx26 chimera to nocodazole treatment suggests that its routing is crucially dependent on microtubules, in contrast to the minimal dependence under the same conditions of Cx32 and Cx43 trafficking. The drug effects on trafficking were mirrored by their influence on Lucifer yellow transfer, for cells expressing the Cx26 chimera with brefeldin A transferred dye almost as efficiently as those expressing wild-type Cx26. Also, in contrast to its effects on Cx32 and Cx43 chimeras as well as wild-type Cx32 and Cx43, nocodazole inhibited the assembly of gap junctions constructed of either wild-type Cx26 or the Cx26 chimera.

Although Cx32 and Cx43 followed the secretory pathway to the plasma membrane, it should be noted that the role of the Golgi apparatus in glycosylation, in contrast to oligomerization, is not a concern with connexins, for they are non-glycosylated proteins. With the Cx26 chimera, its targeting was not dependent on Golgi intactness as inferred from the lack of brefeldin A sensitivity, and this agreed with the oligomerization data, showing that it oligomerized in the ERGIC. The trafficking characteristics of the Cx26 chimeras led to the conclusion that an alternative pathway accounts for the bulk of the trafficking of this connxin to the plasma membrane. Intriguingly, the results suggest that ERGIC may serve as a sorting compartment in the trafficking of specific connexins. Since COS-7 cells are not polarized, it is unlikely that these two pathways followed by connexins correspond to those delivering proteins to the apical or basolateral plasma membrane domains (44). Results consistent with the existence of an alternative connxin trafficking route to the plasma membrane have been obtained by biochemical subcellular fractionation approaches, which show that the majority of Cx26 appears to bypass the Golgi apparatus in guinea pig liver (18).

Cx26/43T-Aeq was internalized from the plasma membrane of COS-7 cells more rapidly than other connexins. This result and the rapid appearance of Cx26 at the cell surface via a brefeldin A-insensitive route suggest that the amount of Cx26 at the plasma membrane can be modulated independently of other connexin isoforms. Kojima et al. (45) demonstrated that Cx26 alone was recruited into gap junctions in female rat liver expressing Cx26 and Cx32 with no associated increase in protein synthesis or mRNA levels and suggested that intracellular stores of Cx26 could be selectively mobilized to the gap junction in an estrogen-dependent manner. Indeed, other examples exist of proteins that may traffic to the cell surface independently of the Golgi apparatus (46–49), including major histocompatibility complex class I molecules (37).

The removal of connxin from the plasma membrane of COS-7 cells treated with brefeldin A or nocodazole, neither of which inhibits protein degradation (50, 51), showed that a threshold level of connxin (−1% of the total cell recombinant protein) was required at the plasma membrane to ensure functional cell-to-cell dye coupling. Only chimeras translocated from intracellular stores can be active at the plasma membrane; once exposed to Ca$^{++}$ at the plasma membrane, they become inactive due to the absence of the cofactor coelenterazine. Thus, even in the unlikely event of connexins being recycled back to the plasma membrane (26), these chimeras can play no further part in subsequent measurements. These results confirm that we have measured connxin movement from intracellular stores and not simply monitored recycling events as observed with other membrane proteins such as the transferrin receptor (51). The loss of connxin isoforms from the plasma membrane in cells incubated in brefeldin A or nocodazole was also abolished by lactacystin (20 μM) and leupeptin (100 μM) (data not shown), indicating that degradation of Cx-Aeq chimeras was mediated by proteasomal and lysosomal mechanisms, as also shown for wild-type connexins (25, 26).

No connxin-43 or connxin-32 was detected by immunofluorescence at the plasma membrane following treatment of cultured cells with brefeldin A for 6 h (16, 18). A disparity has thus arisen between the extent of intercellular coupling observed and the amount of protein that can be detected at gap junctions by immunofluorescence (26). The high sensitivity of our approach has allowed us to calculate that 0.5–1% of the total cellular chimeric protein remained at the plasma membrane even after 6 h incubation of the Cx26-Aeq, Cx32-Aeq, Cx26/43T-Aeq chimeras with brefeldin A or the Cx26/43T-Aeq chimera with nocodazole. This low amount of protein was insufficient to allow functional cell-to-cell coupling as assessed by Lucifer yellow transfer. It was estimated, based on chemiluminescent data (assuming that one photon equates to one molecule of recombinant connxin-aequorin (29)), that 1% (no com-

### Table III

| cDNA expressed | Intercellular transfer of Lucifer yellow | Nocodazole |
|----------------|----------------------------------------|------------|
|                | Untreated 2 h | Brefeldin A 2 h | 6 h | 2 h | 6 h |
| WT Cx26        | 44.3 ± 4.8   | 39.2 ± 5.0   | 36.1 ± 4.2 | 19.3 ± 4.2 | 13.4 ± 3.1 |
| WT Cx32        | 51.0 ± 4.9   | 41.1 ± 3.9   | 5.2 ± 2.4  | 39.4 ± 3.6 | 33.2 ± 2.9 |
| WT Cx43        | 48.6 ± 4.1   | 39.1 ± 4.7   | 4.4 ± 3.6  | 41.9 ± 4.8 | 28.5 ± 4.1 |
| Cx32-Aeq       | 24.6 ± 4.2   | 20.9 ± 3.9   | 3.1 ± 1.2  | 21.3 ± 3.9 | 18.3 ± 2.5 |
| Cx43-Aeq       | 39.3 ± 5.2   | 37.1 ± 4.5   | 5.2 ± 2.1  | 41.9 ± 5.0 | 27.4 ± 3.1 |
| Cx26/43T-Aeq   | 18.4 ± 3.6   | 16.4 ± 2.9   | 13.2 ± 3.2 | 7.2 ± 2.2  | 7.9 ± 1.4  |
| Cx32/43T-Aeq   | 17.8 ± 3.3   | 14.1 ± 3.1   | 4.9 ± 1.3  | 14.2 ± 2.1 | 11.6 ± 2.2 |
| EGFP           | 2.1 ± 0.6    | 1.9 ± 0.4    | 2.4 ± 0.4  | 2.4 ± 0.4  | 3.2 ± 0.6  |

a WT, wild-type; EGFP, enhanced green fluorescent protein.

The intercellular transfer of Lucifer yellow was assessed following treatment of HeLa cells expressing wild-type connexins or various Cx-Aeq chimeras with brefeldin A or nocodazole for 2 or 6 h (see "Experimental Procedures"). Enhanced green fluorescent protein was injected as a control. Data are given as the percentage of cells transferring Lucifer yellow to two or more neighbors (mean ± S.E.), and >20 colonies were injected in each experiment.
munication) or 2% (detectable cell-to-cell coupling) of total cellular connexin present at the plasma membrane approximated to 25 and 50 hemichannels, respectively. However, since unpaired hemichannels are impermeable to Lucifer yellow in the presence of external calcium (38) and intercellular dye transfer is conditional upon the successful docking of connexins, cell-to-cell coupling can probably be achieved by the assembly of relatively few gap junctional channels. Furthermore, since ~25 hemichannels calculated at the cell surface of HeLa cells do not permit intercellular transfer of Lucifer yellow, it is likely that most of these connexons are unpaired. Other approaches have suggested that intercellular communication can be mediated by as few as two gap junction channels (52, 53).

Although attachment of large reporter groups to proteins is unlikely to be functionally benign, the carboxyl-terminal tail switching studies with the Cx26 and Cx32 chimeras are strongly indicative that their trafficking behavior largely mirrors that of wild-type connexins, as also demonstrated with other reporters used to follow trafficking characteristics in living cells. For example, extensive studies with the green fluorescent protein attest to the validity of this general approach for kinetic analysis of their trafficking along the secretory pathway (54).

In conclusion, the present approaches have analyzed intercellular communication by studying connexon assembly and translocation from intracellular stores to the plasma membrane and the recruitment of these hemichannels into gap junctions. The results identify an alternative trafficking route to the gap junction that is followed by the bulk of Cx26. This routing contrasts with the conventional Golgi pathway used by Cx32 and Cx43. An alternative trafficking route can provide an important mechanism for rapidly generating homomer Cx26 channels independently of channels (homo- or heteromeric) constructed of other connexins. The identification of routes leading to the assembly of gap junctions with different permeability properties, constructed of homo- or heteromeric connexins, supports further the possibility that these intercellular communication channels exhibit regulatable selectivity to biochemical messengers.

REFERENCES

1. Kumar, N. M., and Gilula, N. B. (1996) Cell 84, 381–388
2. Sanderson, M. J., Charles, A. C., Boitano, S., and Dirksen, E. R. (1994) Mol. Cell. Endocrinol. 96, 173–187
3. Trellet, E. B., Bennett, M. V. L., Bargiello, T. A., and Verselis, V. K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5836–5841
4. Ek-Vitorin, J. F., Calero, G., Morley, G. E., Coombs, W., Taffet, S. M., and Klein, R. A., Hulser, D. F., and Willecke, K. (1995) J. Cell Biol. 129, 895–917
5. George, C. H., Kendall, J. M., Campbell, A. K., and Evans, W. H. (1998) J. Biol. Chem. 273, 29822–29829
6. Rahman, S., Carlile, G., and Evans, W. H. (1993) J. Biol. Chem. 268, 1260–1265
7. Nadaresk, B., Marenkova, H., Becker, D. L., Evans, W. H., and Parnavelas, J. G. (1998) J. Neurosci. 18, 7881–7890
8. Nakata, T., Terada, S., and Hirokawa, N. (1998) J. Cell Biol. 140, 659–674
9. Lippincott-Schwartz, J., Cole, N., and Presley, J. (1998) Trends Cell Biol. 8, 16–20
10. Fallon, R. F., and Goodenough, D. A. (1981) J. Cell Biol. 90, 521–526
11. Blumenhasset, M. G., and Garfield, R. E. (1991) Am. J. Physiol. 261C, C1001–C1009
12. Laing, J. G., and Beyer, E. C. (1995) J. Biol. Chem. 270, 26399–26403
13. Laing, J. G., Tadros, P. N., Westphale, E. M., and Beyer, E. C. (1997) Exp. Cell Res. 236, 482–492
14. Martin, P. R. M., George, C. H., Castro, C., Kendall, J. M., Capel, J., Campbell, A. K., Revilla, A., Barrio, L. C., and Evans, W. H. (1998) J. Biol. Chem. 273, 1719–1726
15. George, C. H., Martin, P. E. M., and Evans, W. H. (1998) Biochem. Biophys. Res. Commun. 247, 785–789
16. Campbell, A. K., Trewavas, A. J., and Knight, M. R. (1996) Cell Calcium 19, 211–218
17. Marsault, R., Murgia, M., Pozzan, T., and Rizzuto, R. (1997) EMBO J. 16, 1575–1581
18. Cole, N. B., Sciaf, N., Maretta, A., Song, J., and Lippincott-Schwartz, J. (1996) Mol. Biol. Cell 7, 631–650
19. Hammond, C., and Helenius, A. (1994) J. Cell Biol. 126, 231–238
20. Pous, C., Drehou, A., Rouiez, J. D., Guilboumbenche, J., Elmojjudah, A., and Durand, G. (1992) Eur. J. Biochem. 203, 277–283
21. Fishman, G. I., Moreno, A. P., Spray, D. C., and Leinwand, L. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3525–3529
22. Huntley, S. M., and Helenius, A. (1989) Annu. Rev. Cell Biol. 5, 277–307
23. Falk, M. M., Buchler, L. K., Kumar, N. M., and Gilula, N. B. (1997) EMBO J. 16, 2704–2716
24. Reimann, J., and Kaufmann, S. H. E. (1997) Curr. Opin. Immunol. 9, 462–469
25. Li, H. Y., Liu, T. F., Lazrak, A., Peracchia, C., Goldberg, G. S., Lamp, P. D., and Johnson, R. G. (1996) J. Cell Biol. 134, 1019–1030
26. Kidder, G. M., Rains, J., and McKeon, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3718–3722
27. Wacker, T., Kuehler, C., Kromer, A., Migula, A., Almers, W., and Gerdes, H. H. (1997) J. Cell. Sci. 110, 1453–1463
28. Grindstaff, K. K., Bacallao, R. L., and Nelson, W. J. (1998) Mol. Biol. Cell 9, 565–609
29. van Deurs, B., Van Bokel, V., Vilhardt, F., Holm, P. K., and Sandvig, K. (1996) J. Cell. Sci. 109, 1655–1665
30. Ribeiro, C. M. P., Reece, J., and Putney, J. W., Jr. (1997) J. Biol. Chem. 272, 26655–26656
31. Gut, A., Kappeler, F., Hyka, N., Balda, M. S., Hauri, H.-P., and Matter, K. (1998) EMBO J. 17, 1919–1929
32. Kajima, T., Sawada, M., Ito, K., Nakamura, M., Chiba, H., Isomura, H., and Mori, M. (1994) J. Cell Biol. 127, 3579–3590
33. Florkiewicz, R. Z., Majaka, R. A., Beuchler, D. R., and Florkiewicz, E. (1995) J. Cell. Physiol. 162, 388–399
34. Cid-Arregui, A., Parrot, R. G., Simons, K., and Dotti, C. G. (1995) J. Neurosci. 15, 4259–4269
35. Conrad, P. A., Smart, E. J., Ying, Y. S., Anderson, R. G. W., and Bloom, G. S. (1995) J. Cell Biol. 128, 1421–1433
36. Cleaves, A. (1997) Curr. Biol. 7, R318–R320
37. Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H.-P., Yuan, L. C., and Klausner, R. D. (1996) Cell 80, 821–836
38. Sakakibara, T., Yamashina, S., and Ohsaki, S. (1991) J. Biochem. (Tokyo) 109, 528–533
39. Dragun, A., Traub, R. D., Schmitz, D., and Jefferys, J. G. R. (1998) Nature 394, 189–192
40. Chen, L., and Meng, M. Q. (1995) J. Theor. Biol. 176, 39–45
41. Hirschberg, K., Miller, C. M., Ellenberg, J., Presley, J. P., Siggia, E. G., Phair, R. D., and Lippincott-Schwartz, J. (1998) J. Cell Biol. 143, 1485–1503