Cx43 has distinct mobility within plasma-membrane domains, indicative of progressive formation of gap-junction plaques

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Summary
Connexin 43 (Cx43) is a dynamic molecule, having a short half-life of only a few hours. In this study, we use fluorescent-protein-tagged Cx43 variants to examine Cx43 delivery to the cell surface, its residency status in various cell-surface membrane domains and its mobility characteristics. Rapid time-lapse imaging led to the identification of Cx43 being delivered to cell-surface domains that lacked a contacting cell, and also to its localization within membrane protrusions. Fluorescence recovery after photobleaching (FRAP) was used to investigate the mobility state of cell-surface-localized Cx43. Cx43 mobility within clustered cell-surface profiles of Cx43 could be categorized into those with generally a high degree of lateral mobility and those with generally a low degree of lateral mobility. Cx43 mobility was independent of cluster size, yet the C-terminal domain of Cx43 regulated the proportion of gap-junction-like clusters that acquired a low Cx43 mobility state. Collectively, these studies show that Cx43 establishes residency at all cell-surface membrane domains, and progressively acquires assembly states that probably reflect differences in either channel packing and/or its interactions with Cx43-binding proteins.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/122/4/554/DC1

Key words: Gap junctions, Connexins, Dynamics, Trafficking, Mobility, Delivery, Lateral diffusion

Introduction
Connexin 43 (Cx43) is one of 21 members of the human connexin family and assembles into gap junctions (GJs) in over 35 distinct cell types (Laird, 2006; Sohl and Willecke, 2003). GJs (often called gap-junction plaques) are dense clusters of porous intercellular channels consisting of paired hexameric arrangements of connexins; these unique channels are referred to as hemichannels or connexons (Goodenough et al., 1996) and allow for the intercellular passage of numerous small molecules, ranging from ions to larger metabolites (Goldberg et al., 1999). With over nine distinct human diseases associated with germline mutations in nearly half of the members of the connexin gene family, these communication networks are essential for normal development and tissue function (Laird, 2008). Connexins are dynamic molecules that turn over quickly, with half-lives typically ranging from 1 to 5 hours (Beardslee et al., 1998; Fallon and Goodenough, 1981). The unique features of connexons add to the fact that Cx43, and other family members, oligomerize into connexons after they exit the endoplasmic reticulum (Musil and Goodenough, 1993). Connexon delivery to the cell surface has been clearly shown to be facilitated by microtubules and involves carriers ranging from small vesicles to tubular extensions (Laird, 2006; Thomas et al., 2005). However, the targeting of connexin cargo to specific cell-surface microdomains has been far less clear. De novo assembly of GJs might also involve pathways that are distinct from steady-state conditions and there is no documented evidence of connexins being found at apical domains of polarized epithelial cells. A recent report using HeLa cells provides evidence for Cx43 targeting directly to adherens junctions that are juxtaposed to GJs, by using the microtubule plus-end-tracking protein EB1 and p150 (Shaw et al., 2007). Other studies employing tetracysteine tagging and photobleaching of fluorescent-protein-tagged Cx43 support a model in which Cx43 targets to non-GJ regions prior to coalescing into GJ plaques (Gaietta et al., 2002; Lauf et al., 2002). Consistent with the latter model, GJs were earlier proposed to undergo two stages of assembly, beginning with the loose packing of connexons (formation plaques) followed by full assembly into a crystalline structure (Johnson et al., 1974). Most evidence appears to support a role for cadherin-based cell adhesion in plaque formation, because the absence of adherent conditions disrupts or perturbs initial GJ formation (Herve et al., 2004). At least some of these processes appear to involve one or more of the over a dozen Cx43-binding proteins (Giepmans, 2004). For example, ZO-1 (encoded by Tjp1) binds to the edges of Cx43-based GJ plaques, and this interaction appears to regulate Cx43-channel accretion into GJs and overall plaque size (Hunter et al., 2005). However, interaction of Cx43 with ZO-1 is not essential for GJ formation (Giepmans, 2006). Fluorescently tagged connexins can be used to study connexin trafficking and channel formation (Jordan et al., 1999). Fluorescent-protein tagging to the C-terminal tail of Cx43 has a relatively mild effect on Cx43 by only modifying the fast voltage gating potential and by impairing the interaction of protein-binding partners that require an untagged C-terminal tail (e.g. ZO-1) (Bukauskas et al., 2000; Hunter et al., 2005; Jordan et al., 1999). When green fluorescent protein (GFP) is tagged to the N-terminal of Cx43 (GFP-Cx43), the trafficking and assembly of Cx43 into GJs is maintained and the C-terminal tail is available to interact with Cx43 binding partners. However, although the resulting GJ channels are not
functional, the N-terminal GFP tag serves to alter the kinetics of Cx43, resulting in Cx43 being enriched in non-GJ membranes (Contreras et al., 2003; Laird et al., 2001). In essence, the GFP-Cx43 variant might serve as an effective tool in studying Cx43 trafficking. In other studies, mutants have been widely used to dissect the mechanisms governing many molecular and trafficking pathways (Blanchetot et al., 2005; Itoh and Fukuda, 2006; Kontaridis et al., 2004). Importantly, successes in using fluorescent-protein tags as reporters of Cx43 trafficking and assembly allows for real-time-imaging kinetic studies to be performed. In the present study we employ rapid time-lapse imaging and fluorescent recovery after photobleaching (FRAP) to assess the delivery of Cx43 to the plasma membrane, the assembly steps leading to GJ formation and the dynamics of Cx43 within GJs. It is our hypothesis that the mobility properties of Cx43 will differ depending on the location of Cx43 within the plasma membrane.

Results

It has been widely accepted that bright fluorescence puncta observed between adjacent cells that express epitope- or GFP-tagged Cx43 correspond to GJs (structures frequently referred to as GJ plaques). The evidence for this understanding is notable given that light and electron microscopic studies have revealed that fluorescent-protein- or epitope-tagged Cx43 acquires the hallmark characteristics of dense channel packing and a narrow gap between juxtaposed membranes (Gaietta et al., 2002; Hand et al., 2001; Jordan et al., 1999; Sosinsky et al., 2003). In one case, this conclusion was reached by processing the very same cells as viewed by light microscopy for electron microscopy (Gaietta et al., 2002). Importantly, successes in using fluorescent-protein tags as reporters of Cx43 trafficking and assembly allows for real-time-imaging kinetic studies to be performed. In the present study we employ rapid time-lapse imaging and fluorescent recovery after photobleaching (FRAP) to assess the delivery of Cx43 to the plasma membrane, the assembly steps leading to GJ formation and the dynamics of Cx43 within GJs. It is our hypothesis that the mobility properties of Cx43 will differ depending on the location of Cx43 within the plasma membrane.

To study Cx43 dynamics, we first chose to express Cx43-GFP [GFP attached to the C-terminal tail of Cx43 via a seven-amino-acid linker sequence as described previously (Roscoe et al., 2005)] in the rat mammary tumor cell line, BICR-M1Rk, because these cells are known to express abundant levels of endogenous Cx43, and to actively assemble and turnover GJs (Laird et al., 1995). As expected, Cx43-GFP was localized to punctate structures at sites of cell-cell apposition (Fig. 1A). However, Cx43-GFP was more readily identifiable in multiple non-GJ and GJ membranes following a 6-hour BFA treatment (Fig. 1B) and a subsequent 1-hour recovery after BFA washout (Fig. 1C), as previously shown (Thomas et al., 2005). Similar to the distribution of Cx43-GFP after BFA washout, GFP-Cx43(Δ2) [previously described (Gong et al., 2007; Laird et al., 2001); lacks amino acids 381 and 382 of Cx43 and contains seven residual irrelevant amino acids remaining from the linker sequence of an earlier generation of Cx43-GFP from which it was derived] and GFP-Cx43 were generically localized to four distinct sites: GJs, areas next to GJs, free membrane surfaces and areas where cells juxtapose (Fig. 1D-F). To determine whether the distribution profile of GFP-Cx43(Δ2) was the same as Cx43-GFP when expressed in the same cell, GFP-Cx43(Δ2) and Cx43-RFP were co-expressed in BICR-M1Rk cells (Fig. 1G-H). Our results revealed that these two Cx43 variants typically colocalized (Fig. 1G-I). In essence, all of these Cx43 variants would be expected to intermix with endogenous Cx43, and indeed they appeared to at least transiently occupy similar intracellular compartments and cell-surface domains during their life cycle. Because GFP N-terminal-tagged Cx43 variants could readily be imaged at multiple cell-surface domains in the absence of drug treatments, these constructs were chosen for the analysis of steady-state GJ renewal.

Fig. 1. GFP-tagged Cx43 localizes to multiple cell-surface domains. (A,B) Cx43-GFP was localized to GJ-like clusters and non-GJ membranes in untreated cells (A) but, when BICR-M1Rk cells were treated with BFA for 6 hours, GJ-like clusters were not evident (B). (C) Following BFA-washout and recovery for 1 hour, GJ-like clusters were again evident, as was Cx43 at cell-surface areas where no apposing cell was apparent (arrows). (D-F) When expressed in BICR-M1Rk cells, GFP-Cx43(Δ2) (E) or GFP-Cx43(Δ2) (F) was localized to four distinct areas of the plasma membrane, as illustrated in the schematic model (D). The fluorescent images presented in E and F were overlaid on DIC images to denote contacting cells. (G-I) GFP-Cx43(Δ2) (G) and Cx43-RFP (H) colocalized (I, note yellow), demonstrating that these Cx43 variants have similar distribution profiles. Nuclei are stained with Hoechst (A-C). Scale bars: 10 μm.
Dynamic delivery of GFP-Cx43 to cell surfaces that lack a contacting cell

We next explored GFP-Cx43\textsuperscript{\textregistered} delivery to the cell surface of BICR-M1R\textsubscript{4} cells using time-lapse image acquisition. Vesicles carrying GFP-Cx43\textsuperscript{\textregistered} were observed to be moving, and they apparently merged with various microdomains of the plasma membrane (suggestive of vesicular fusion with the plasma membrane) (Fig. 2A-G; supplementary material Movie 1). Qualitative analysis suggested that delivery of vesicles to non-GJ plasma-membrane domains was spatially arbitrary. In cases in which a vesicle was observed to merge with the plasma membrane, a transient increase in fluorescent intensity was observed. This increase in fluorescence intensity disappeared quickly, suggesting that the newly delivered GFP-Cx43\textsuperscript{\textregistered} cargo laterally diffused within the plasma membrane (Fig. 2; supplementary material Movie 1).

Cx43 is localized to multiple non-GJ cell surfaces

Further live BICR-M1R\textsubscript{4} cell time-lapse imaging studies revealed that GFP-Cx43\textsuperscript{\textregistered} was present in dynamic membrane protrusions (Fig. 3A-C; see supplementary material Movie 2). As these membrane protrusions underwent characteristic ruffling, an enriched population of Cx43 was observed to follow the remodeling of the cellular extensions (Fig. 3D-H, arrows). Similarly, whereas Cx43-GFP could be visualized at the cell surface in non-GJ membranes as well as within membrane protrusions in BICR-M1R\textsubscript{4} cells recovering from BFA treatment (Fig. 3I-N), the intensity of the Cx43 signal was less than that observed with the GFP-Cx43\textsuperscript{\textregistered} variant. Collectively, these results suggest that all regions of the cell surface, including membrane protrusions, are enriched with connexons that may transiently communicate with the extracellular environment as hemichannels, or are possibly primed for GJ channel assembly upon contact with a neighboring cell.

FRAP revealed the presence of GJ-like populations with distinct Cx43 mobility characteristics

Although the GFP-Cx43\textsuperscript{\textregistered} and GFP-Cx43 variants could both be observed at multiple plasma-membrane domains, we focused our attention on the GFP-Cx43 variant, which has an intact and unblocked C-terminal tip and is thus suitable for binding to any...
Connexin-43 trafficking and dynamics

Truncated Cx43 exhibits two mobile GJ-like cluster populations independently of endogenous Cx43

In order to examine the role of the C-terminal domain in regulating the mobility of Cx43 within GJ-like clusters, we expressed a truncated Cx43\textsuperscript{244}-GFP variant in BICR-M1R\textsubscript{k} cells, which express endogenous Cx43 (Fig. 4C). This Cx43 variant lacks the C-terminal domain that is required for interaction with the vast majority of known C-terminal Cx43-binding proteins, with the possible exception of tubulin (Giepmans, 2006), yet it is still capable of forming functional GJs (Bates et al., 2007). To determine whether the endogenous Cx43 within BICR-M1R\textsubscript{k} cells played a part in governing the mobility properties of the truncated Cx43 variant, we used FRAP to investigate the mobility properties of Cx43 and truncated Cx43 in HeLa cells that lacked the expression of endogenous Cx43 (Hunter et al., 2005). To first determine whether GFP-Cx43 could form GJ-like clusters, HeLa cells were transfected with GFP-Cx43 followed by immunostaining for Cx43 (Fig. 5A-C). Cx43 immunostaining was found to colocalize in areas that expressed GFP-Cx43 (Fig. 5C, yellow) and GJ-like clusters were identified at sites of cell-cell apposition. Similar to BICR-M1R\textsubscript{k} cells, HeLa cells expressing GFP-Cx43 exhibited a similar array of high- and low-mobility GJ-like clusters as those found in BICR-M1R\textsubscript{k} cells that expressed endogenous Cx43 (Fig. 5D). Likewise, our FRAP analysis revealed both the high- and low-mobility GJ-like populations of Cx43\textsuperscript{244}-GFP when expressed in HeLa cells, consistent with what was found in BICR-M1R\textsubscript{k} cells. However, FRAP analysis of the highly mobile fraction of Cx43\textsuperscript{31-244}-GFP in HeLa cells did reveal that the fluorescence recovery did not reach a clear recovery plateau, but did recover to the same extent as that found in BICR-M1R\textsubscript{k} cells (Fig. 5E). Statistical analysis of the recovery curves for Cx43\textsuperscript{31-244}-GFP between BICR-M1R\textsubscript{k} and HeLa cells were significantly different (P<0.05), suggesting that there was a contribution from endogenous Cx43, probably due to intermixing of endogenous and truncated Cx43. In addition, the percentage of GJ-like clusters that exhibit a highly mobile fraction did not appear to change upon the truncation of Cx43 (Fig. 5F).

Truncation of Cx43, but not cluster size, affects the percentage of GJ-like clusters that exhibit low Cx43 mobility

We sought to determine whether the truncation of Cx43 would affect the proportion of GJ-like clusters that acquire a less-mobile state. Interestingly, approximately two-thirds of all

Fig. 4. Cx43 has a variety of mobilities at the cell surface and Cx43 GJ-like clusters can be categorized into two general populations in BICR-M1R\textsubscript{k} cells. (A) FRAP analysis of GFP-Cx43\textsuperscript{31-244}-expressing BICR-M1R\textsubscript{k} cells revealed that the mobility of Cx43 in GJ-like clusters was significantly less than Cx43 in all other plasma-membrane locations (*P<0.05). (B-E) Cells expressing Cx43-GFP (B,D,E), GFP-Cx43 (B) or Cx43\textsuperscript{244}-GFP (C) were subjected to FRAP, and fluorescence recovery into the photobleached area was monitored over a period of 300 seconds. Cx43\textsuperscript{31-244} represents GJ-like clusters that exhibited a highly mobile Cx43 fraction, whereas Cx43\textsuperscript{244} represents GJ-like clusters that exhibited a low-mobility Cx43 fraction. Note that the Cx43-GFP FRAP results presented in B were originally presented as one combined pool by Thomas et al. (Thomas et al., 2005). Scale bars: 2 μm.
Cx43-GFP GJ-like clusters reached a low-mobility state, whereas only one quarter of all GJ-like clusters reached a similar state when the C-terminus was removed (Fig. 6A). Thus, these studies support a role of the C-terminus in regulating the proportion of high- and low-mobility pools of Cx43. Assuming that low Cx43 mobility clusters represent a more fully assembled GJ configuration, the large C-terminal domain might facilitate this arrangement. Likewise, tagging GFP to the N-terminus of Cx43 also impaired the ability of Cx43 from reaching a low-mobility state within GJ-like clusters (Fig. 6A), a process that was independent of the C-terminal tail. Interestingly, there was no correlation between cluster size and the existence of high- or low-mobility Cx43 GJ-like populations for any of the Cx43 variants used in this study (Fig. 6B).

Reversible photobleaching is negligible and does not account for the fluorescence recovery after photobleaching. Given that EGFP has been reported to exhibit some level of recovery upon photobleaching as the fluorochrome reforms (Tsien, 1998), we systematically assessed whether reversible photobleaching could account for the rapid movement of Cx43 within GJ-like clusters. First, all imaging conditions were identical and thus reversible photobleaching would be expected to be common for all imaged clusters, which was not evident (compare Fig. 4A and B). Second, fluorescence recovery followed a pattern of moving inward from the edges of the photobleached areas towards the middle of the photobleached area, which is consistent with molecular movements and not recovery of previously bleached EGFP molecules (compare Fig. 4D and E). Finally, we employed another EGFP-tagged Cx43 mutant (Cx43T244-GFP, harboring two missense mutations; see Materials and Methods) that is retained in a combination of aggregated structures and within the endoplasmic reticulum when expressed in MDCK cells (Fig. 7A-D). Upon photobleaching of the aggregated structures and regions of the endoplasmic reticulum, fluorescence only returned to the photobleached endoplasmic reticulum and not the aggregates, further verifying that enhanced GFP (EGFP) did not recover from being photobleached simply by regaining its fluorochrome properties.

**Discussion**

Connexin trafficking is a highly regulated process, and a vital component of proper cellular function. Using time-lapse imaging and FRAP we examined the delivery events of Cx43 to the plasma membrane, dynamic activity of Cx43 at ruffling membrane edges and Cx43 cell-surface dynamics. In all cases, we employed GFP-tagged variants of Cx43 that collectively allow Cx43 to be trapped and visualized at multiple plasma-membrane compartments in cells that are actively building, remodeling and removing GJs. We show that Cx43 is found at non-GJ regions at the cell surface, including membrane protrusions, suggesting that cells are primed for GJ formation or that connexons can function in a hemichannel capacity within these non-GJ cell-surface domains. Cx43 was found to exhibit its greatest mobility within non-GJ regions, whereas mobility in GJ-like clusters was significantly reduced. Within GJ-like clusters, Cx43 mobility varied across a spectrum from low to high mobility and was independent of cluster size. Finally, the C-terminal tail of Cx43 regulated the percentage of GJ-like clusters that acquired a low Cx43 mobility status, possibly through the activity of Cx43-binding proteins or channel packing.

**Trafficking dynamics and delivery sites of Cx43**

In an earlier study, nascent GJ assembly, as assessed by recovery from BFA-induced loss of pre-existing GJs, revealed that Cx43 is first delivered to non-GJ plasma-membrane domains (Thomas et al., 2005). Although the evidence for this conclusion was compelling, this study did not preclude the possibility that when cells reach a steady-state of GJ formation and removal, an alternate pathway might exist in which Cx43 is more directly targeted to areas near pre-existing GJs, as suggested by Shaw and colleagues (Shaw et al., 2007). Our present study would support the notion that, even in the presence of pre-existing GJs, a pathway of Cx43 delivery to non-GJ membranes continues to exist and serves as a pool of...
Cx43 for GJ renewal. This conclusion is consistent with our findings using GFP-tagged Cx43 variants in steady-state conditions, where non-GJ populations of Cx43 could be observed at all cell-surface domains, ranging from areas with juxtaposed cells to free migrating cellular edges containing membrane protrusions. The reduction in Cx43 mobility within GJ-like clusters could further reflect differences in Cx43 interactions with scaffolding or cytoskeletal elements, or progressive increases in the state of channel or connexon interactions. These studies are consistent with the findings in which lateral diffusion and joining of connexon aggregates promotes GJ growth (Jordan et al., 1999; Ryerse et al., 1984; Windoffer et al., 2000).

In our study we used time-lapse imaging of GFP-tagged Cx43 variants to examine the delivery of Cx43 to the cell surface. Delivery of pleiomorphic vesicles to non-GJ regions on the cell surface appeared to be arbitrary and consistent with previous studies using functional variants of Cx43 (Lauf et al., 2002; Thomas et al., 2005). Given the difficulties in quantifying the delivery of Cx43 vesicles to the cell surface, we cannot rule out a quantitative preference of Cx43 delivery to specific plasma-membrane microdomains that might reflect pre-existing adherens junctions, as suggested by Shaw and colleagues (Shaw et al., 2007), or other areas of cell-cell interactions. The concept of untargeted delivery of Cx43 to non-GJ regions in the cell surface would necessitate the need for lateral diffusion of Cx43 within the plasma membrane as a pre-requisite to GJ formation. In support of this mechanistic step in GJ assembly, contents of exocytic GFP-Cx43-containing vesicles quickly dispersed following apparent fusion to the plasma membrane. Consistently, nascent Cx43 was abundantly found at the cell surface in non-GJ localization patterns (Thomas et al., 2005), further suggesting that Cx43 can be non-specifically targeted to the plasma membrane prior to GJ assembly. This mechanism of GJ assembly is further supported in vivo by the identification of non-GJ cell-surface populations of Cx43 in basal keratinocytes (Maher et al., 2005). In other studies, immunohistochemistry has revealed the presence of connexins within the filopodia of astrocytes at central nervous system lesions (Ochalski et al., 1995), and within cultured astrocytes in vitro (Wolff et al., 1998). Moreover, extracellular-loop-specific anti-Cx43 antibodies have been used to detect Cx43 in non-GJ membranes of astrocytes (Hofer and Dermietzel, 1998). In our studies, we have no direct evidence to distinguish whether Cx43 is localized at the cell surface in the form of a non-active connexon, a functionally active hemichannel, or both.

Interestingly, a second mechanism of direct microtubule-governed targeting of Cx43-YFP to adherens junctions in HeLa cells has been reported that would suggest that steady-state GJs are renewed systematically at sites adjacent to adherens junctions (Shaw et al., 2007). It is well established by these authors and others that cell adhesion and microtubules can facilitate, or are even necessary for in some cases, GJ assembly. But it is more uncertain as to whether Cx43 is exclusively targeted to adherens junctions via microtubules. Such a model does not account for the abundance of Cx43 at membrane protrusions and free cell surfaces that are devoid of adherens junctions, or for evidence of apparent Cx43 vesicle fusion events at cell surfaces that lack an adjacent cell. It is possible that the position of the GFP tag might act to limit the interaction of Cx43 with microtubules, resulting in a more random distribution of Cx43 variants to the cell surface, but this remains to be demonstrated. Collectively, these combined studies suggest that Cx43 can be delivered to the cell surface by two distinct mechanisms.

Clustered Cx43 has distinct mobility characteristics

On the basis of FRAP assessments of Cx43 mobility properties within GJ-like clusters, we identified a spectrum of Cx43 mobilities that could generally be grouped into two categories: those with...
relatively high mobility and those with relatively poor Cx43 mobility. It is important to note that Cx43 mobility exists within a continuum between these states (see supplementary material Fig. S1), suggesting that they represent differential states of GJ assembly. Because electron-microscopic evidence clearly demonstrates that Cx43 acquires a crystalline state once fully assembled (Unger et al., 1999), we attribute the lowest Cx43 mobility status to a greater state of Cx43 assembly. Our results are consistent with a model in which high-mobility Cx43 clusters transition into more densely packed low Cx43 mobility clusters as the GJ becomes more assembled.

Dynamic microdissection of GJ subdomains has previously been reported with respect to GJ turnover. For instance, it has been shown by using tetramethylrhodamine-tagging and pulse-chase experiments that GJ plaques can be segregated into newer and older subdomains (Gaietta et al., 2002). In general, new components of GJ plaques were found at the periphery, whereas older elements were found in the central domains (Gaietta et al., 2002). Although these results are intriguing, they do not address the differential mobility characteristics that might exist within populations of GJ-like clusters. To that end, we methodically determined the three-dimensional center of fluorescent GJ-like clusters and restricted our analyses to the putative ‘older’ elements of the clusters. This method of analysis avoids potential variations that might arise because of the dynamic aspects of GJ-plaque assembly at the outer edges.

The discovery of two broad categories of GJ-like clusters with distinctly different mobile populations of Cx43 is consistent with, and supports, a long-standing hypothesis that cells contain both ‘mature plaques’ and ‘formation plaques’, as originally suggested by Johnson and colleagues (Johnson et al., 1974). In these studies, freeze-fracture analysis revealed patch-like areas of loosely packed GJs (termed ‘formation plaques’) and GJs tightly clustered into crystalline arrays. On the basis of these studies, it is tempting to speculate that the GJs with lower Cx43 mobility represent GJs that have reached a more-advanced state of assembly or channel packing. The recruitment of additional GJ channels would mandate the exclusion of lipids and predict that channels within the crystalline structures would become less mobile. It is quite possible that the higher-mobility Cx43 fraction undergoes some combination of diffusion and/or transport processes to promote channel packing. These events could very well be mediated through transient interactions with any number of known Cx43-binding proteins (Giepmans, 2004). This concept is supported by Triller and Choquet (Triller and Choquet, 2005), who reviewed the evidence that transient binding of GABA, AMPA and glutamate receptors to scaffolds during extra-synaptic movement towards synapses creates changes in mobility (Triller and Choquet, 2005). Similar motility concepts have been found with the transferrin receptor (Sako and Kusumi, 1995), and with both mobile and immobile fractions of AMPA receptors within synapses (Tardin et al., 2003).

The C-terminal domain of Cx43 regulates the acquisition of a low-mobility status

In order to directly assess the role of the C-terminal tail in regulating the mobility fractions exhibited by Cx43, we employed the use of a Cx43 variant in which the C-terminal tail was deleted. These studies were designed to address whether the multitude of Cx43-binding proteins (Giepmans, 2004) or the protein kinases responsible for phosphorylating Cx43 (Lampe and Lau, 2004) were responsible in whole, or in part, for Cx43 acquiring both higher and lower categories of mobile fractions within GJs. Our findings suggest that eliminating the ~17-kD C-terminal domain greatly reduced the frequency of GJ-like clusters being assembled that exhibited a low Cx43 mobility status. The C-terminal of Cx43 has previously been shown to be highly phosphorylated and to interact with several scaffolding and cytoskeletal proteins (Giepmans, 2004; Laird, 2006). Despite the truncation of Cx43 at a site that eliminates almost all known binding sites for these proteins, Cx43T244-GFP displayed both high- and low-mobility pools of GJ-like clusters. Collectively, these studies suggest that Cx43-binding proteins are probably not essential for Cx43 to reach a low-mobility state within GJs, but they probably play a part in the efficiency of the process.

Location of the GFP tag affects Cx43 mobility

The majority of research regarding the N-terminus of connexins has revealed it to be a transjunctional voltage sensor (Harris, 2007). Additional studies have documented the importance of N-terminal residues in regulating the oligomerization compatibility of Cx43 (Falk et al., 1997; Lagree et al., 2003). Our studies extend these assignments and support the premise that this domain of Cx43 can play a role in either the packing of Cx43 into plaques or the tethering of GJs to Cx43-binding proteins. This conclusion is based on evidence showing that GFP-Cx43 exhibited a larger mobile fraction within GJ-like clusters than when GFP was tagged to the C-terminus of Cx43 (e.g. Cx43-GFP). Thus, both Cx43 variants can reach a similar level of low mobility within a GJ-like cluster, suggestive of a ‘mature’ GJ, but the efficiency of reaching such a dynamic state is inhibited when GFP is tagged to the N-terminal tail of Cx43.

In summary, our studies reveal that Cx43 is found in non-GJ and GJ-like clusters and exhibits differential dynamics within these when expressed in BICR-M1Rk cells. Cx43 GJ-like clusters exist in two general states, reflecting differences in Cx43 mobility that are mechanically consistent with GJs progressing to a greater state of assembly.

Materials and Methods

Cells and reagents

Rat mammary tumor (BICR-M1Rk; gift from Dieter Hulser, Stuttgart, Germany), and MDCK and human cervical tumor (HeLa) cells (obtained from the American Type Culture Collection, Manassas, VA) were grown in high glucose (4500 mg glucose/l) Dulbecco’s modified Eagle’s medium (Invitrogen Life Science Products, Burlington, ON) supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cell cultures were placed within a humidified environment that maintained 5% atmospheric CO2 and a temperature of 37°C. For the purpose of sub-culturing cells, 0.25% trypsin/1 mM EDTA solution (Invitrogen) was used to release the cells from the flasks.

Constructs, transfections and immunofluorescent labeling

The Cx43-GFP cDNA construct was generated to encode GFP attached to the C-terminus of Cx43 via a seven-amino-acid linker sequence as described previously (Roscoe et al., 2005). Cx43-GFP was engineered as described (Gong et al., 2007). The Cx43T244-GFP construct, which in which GFP was tagged to Cx43 truncated at residue 244, was previously reported by Bates et al., 2007; Langlois et al., 2008). A sequence variant of the Cx43T244-GFP mutant containing two additional point mutations resulting in a glutamine-to-arginine substitution at position 58 and a second serine-to-asparagine change at position 158 was used in FRAP control experiments. The GFP-Cx43D250 construct was previously described (Gong et al., 2007; Laird et al., 2001) and lacks amino acids 381 and 382 of Cx43, and contains seven residual irrelevant amino acids remaining from the linker sequence of an earlier generation of Cx43-GFP from which it was derived. Consequently, this construct, as well as Cx43-GFP, is incapable of binding to ZO-1 (Hunter et al., 2005). Finally, a construct encoding GFP attached to the N-terminus of full-length Cx43 (GFP-Cx43) was generated using the pEGFP-C2 vector and the KpnI and BamHI restriction enzymes. All constructs were verified by sequencing.

For transient transfections, BICR-M1Rk and HeLa cells were grown to ~70% confluence overnight in 35-mm MatTek glass-bottom dishes or in 60-mm culture dishes containing glass coverslips. To prepare the transfection mixtures, 250 μl of OptiMEM-I medium (Life Technologies, Burlington, ON) was aliquoted into two glass tubes. 1-2 μg of cDNA was added to one tube whereas 2 μl Lipofectamine
2000 (Invitrogen) was added to a second tube. Both tubes were combined within 5 minutes, gently mixed and allowed to sit at 24°C for 20 minutes. Transfection mixtures were then added to OptiMEM-I-medium for 24 hours, followed by washout.

For immunolabeling studies, a mixture of 80% methanol/20% acetone was added to the cells for 5 minutes at 4°C, then replaced with PBS. Cells were treated with 2% bovine serum albumin in PBS for 1 hour to block non-specific binding sites. Rabbit anti-Cx43 polyclonal antibody (Sigma Aldrich, Oakville, ON; diluted 1:500) was applied to each coverslip for 1 hour. Coverslips were then washed three times for 5 minutes each using PBS. A secondary anti-rabbit antibody conjugated to Texas red was applied to each coverslip for 1 hour. Finally, cells were washed four times with PBS and water prior to mounting in preparation for imaging.

Imaging

In the case of fixed cells, fluorescently labeled cells were imaged on a Zeiss LSM 510 META confocal microscope. All GFP-tagged Cx43 variants were excited using a 488 nm argon laser, and emissions were captured using a 505-550 nm band pass filter. Texas red was excited with a 543 nm helium-neon laser line, with emissions captured using a 580-620 nm long pass filter. Confocal images were compiled into movie format using Adobe Premiere Pro Software, version 7.0.

For time-lapse imaging, cells were excited using a 488 nm argon laser, and emitted fluorescence was collected after passage through a 500-550 nm band pass filter. Optical sections were fixed at a z-plane and at constant brightness and contrast settings were continuously line-scanned at a rate of ~2 seconds/frame over a time period of ~30 minutes. Individual time-lapse frames were compiled into movie format using Adobe Premiere Pro Software, version 7.0.

In cases in which photobleaching of GFP fluorescence was performed, rectangular regions of interest (ROI) of approximately 0.2 μm² were selected within GJ-like clusters, along free membranes, at juxtaposed membranes, or at regions next to GJ-like clusters. Lengths and widths of ROIs were adjusted to best fit the area of plasma membrane being analyzed; however, an average ROI area of 0.2 μm² was always maintained. To ensure that photobleaching occurred throughout the entire z-depth of the ROI, and not just in the focus plane alone, control experiments were performed in which microscope parameters (laser strength, number of bleach iterations) were tested within ROIs having an average area of 0.2 μm². The integrity of photobleaching was observed in two ways: with repeated visualization of the bleached area through microscope binocular lenses, which would allow the entire thickness of the cell to be visualized; and through a repeated manual confocal laser scanning up and down through the cell thickness following the photobleaching. Time-lapse imaging using the LSM software incorporated an imaging protocol (Time Series) together with a photo-bleaching procedure (‘Edit Bleach’) to allow for uninterrupted tracking of fluorescence before and after bleaching. Photobleaching was performed using eight to ten successive iterations (combined time ≤ a few milliseconds) of the 488 nm laser line at 100% emission intensity. Pre- and post-bleach images were scanned at ~1 second/frame using a 488 nm laser line at 0.05% laser intensity. All emitted fluorescence was captured through a 500-550 nm band pass filter, and the confocal pinhole was set to acquire a 1.2-μm-thick optical slice. Pinhole size and detector gain remained constant throughout all experiments. Imaging of plaques before and after photobleaching was performed at a fixed z-plane, which corresponded to the central location of the plaques. Fine manual focus adjustments, while performing confocal laser scanning, were used to establish the central location of the plaques. The three-dimensional central regions were chosen for analysis.

In a control series of experiments to assess recovery from photobleaching that might be attributed to fluorochrome auto-recovery from laser bleaching, and not to molecular reorganization within the plaques, photobleaching experiments were performed using 60 μg/ml propidium iodide (unlabeled) scan iterations at 488 nm on a Zeiss 10× oil-immersion (numerical aperture 1.4) objective. Recovery of photobleached fluorescence was then compared to non-rebleached controls having an average ROI area of 0.2 μm².

Quantification and statistical analysis

Fluorescence recovery was quantified as follows: mean fluorescence in ROIs was determined using the ‘Mean ROI’ function in the Zeiss 510 META software. Background intensities of ROIs were recorded at the following time periods: prior to photobleaching (pre-bleach), immediately after photobleaching (t=0 seconds), and at 25, 50, 100, 150, 200 and 300 seconds post-bleach. Prior to the plotting of FRAP curves, post-bleach intensities for all samples were corrected for any residual, non-bleached fluorescence using the following equation (Lippincott-Schwartz et al., 2001; Nichols et al., 2001; F(t) = F(t=0) – F(t=0)/[F(t=0) – F(t=0)]) where F(t=0)-normalized fluorescence at time point t, F(t) is the overall fluorescence at time points 25, 50, 100, 150, 200 or 300 seconds post-bleach, F(t=0) is fluorescence immediately upon completion of the photobleaching procedure; and F(t=0) is fluorescence intensity prior to photobleach procedure. For each Cx43 variant, F(t=0) values from all replicates were combined, and non-linear regression (GraphPad Prism software) was used to create a best-fit FRAP curve. The mobile fraction for each connexin variant was determined as being the extent of fluorescence recovery following complete removal of the plateau of the curve. To compare the mobile fractions across Cx43 variants, an ANOVA was used. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (San Diego, CA).

The authors would like to thank Karen Jordan for her contributions to the data presented in Fig. 7. This study was supported by the Canadian Institutes of Health Research to D.W.L.

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