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Adhesive Properties of Connexin Hemichannels

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Abstract

Gap junctions are intercellular channels formed by hemichannels (or connexons) from two neighboring cells. Hemichannels, which are composed of proteins called connexins, can function as conduits of ATP and glutamate, and interact with adhesion molecules and other signaling elements. As a result, their functional repertoire is expanding into other roles, such as control of cell growth or cell migration. Here we further elucidate the involvement of hemichannels in cell-cell adhesion by analyzing how connexins regulate cell adhesion without the need of gap junction formation. Using a short-term aggregation assay with C6-glioma and HeLa cells stably transfected with connexin (Cx) 43 or Cx32, we found that the connexin type dictates the ability of these cells to aggregate, even though these two cell types do not usually adhere to each other. We have also found that high expression of Cx43, but not Cx32 hemichannels, can drive adhesion of cells expressing low levels of Cx43. Aggregation was not dependent on high levels of extracellular Ca^{2+} , as Ca^{2+} removal did not change the aggregation of Cx43-expressing cells. Our data confirm that connexin hemichannels can establish adhesive interactions without the need for functional gap junctions, and support the concept that connexins act as adhesion molecules independently of channel formation.

Keywords

gap junctions; adhesion; glioma cells; calcium

INTRODUCTION

Connexins are the constituents of the intercellular channels known as gap junctions. Gap junctions allow the exchange of ions and small molecules up to 1 kDa in size and are therefore primarily thought of as communicating channels. There are about 21 different connexin genes, which are expressed in almost every tissue, making gap junctions an important signaling element in many tissues (Söhl et al. 2005).

As gap junctions require that cells come into close contact for functional channels to form, junction formation relies on proper adhesive interactions between cells. So, the relation between gap and adherens junctions has been amply documented (Jongen et al., 1991, Frenzel and Johnson, 1996). Antibodies against N-cadherin and E-cadherin can alter the formation of gap junctions (Meyer et al., 1992; Jongen et al., 1991). Surprisingly, antibodies against connexin (Cx) 43 can also inhibit the assembly of adherens junctions (Meyer et al., 1992). In addition, Cx43 can associate with N-Cadherin in a multi-protein complex in

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NIH3T3 cells, and blocking the expression of either one affects the membrane localization of the other (Wei et al., 2005). Studies in mice lacking specific connexins have shown their impact on the expression of other proteins. Complementary DNA microarray comparison between wild-type and Cx43-null astrocytes from neonatal mouse brains has shown that 9% of the most significantly altered genes relate to cell junctions, adhesion and extracellular matrix proteins (Jacobas et al., 2004), although no gross morphological changes are apparent in mice with targeted inactivation of Cx43 (Theis et al., 2003). Transfection of NIH3T3 cells with siRNA that targets Cx43 promotes a decrease in the membrane localization of N-Cadherin and a distribution of N-cadherin-associated proteins (Wei et al., 2005). So, it is not clear to what extent connexins on their own can mediate adhesive interactions.

We have previously shown that cellular adhesivity of a glioma cell line increases upon transfection with Cx43 or Cx32 (Lin et al., 2002). These cells acquired an epithelial morphology and changed their migration and invasiveness properties. Interestingly, adhesion *per se* only required the docking of two opposing hemichannels, but not a functional gap-junction channel. The need for connexin-mediated adhesion during migration has been reported in neuron-radial glia interactions during cortical development (Elias et al., 2007). Furthermore, mice conditionally deficient for Cx43 showed disorganization in cortex, hippocampus and cerebellum. This effect was attributed to alterations in the migration of neuronal precursors (Wiencken-Barger et al., 2007). These observations indicate that connexin hemichannels have adhesive properties.

Given the emerging evidence that hemichannels might be involved in a range of functions that directly affect neural physiology, such as the release of glutamate and ATP (Ye et al., 2003; Spray et al., 2006), we investigated the adhesive properties of connexin hemichannels in a setting that does not involve migration or channel formation. Using a short-term aggregation assay, we tested whether Cx43 is sufficient to establish adhesion between heterotypic cell lines that normally do not interact. We then asked whether connexins other than Cx43 also show adhesive properties. Last, we showed that connexin-mediated adhesion does not require high levels of Ca^{2+} .

These results further the notion that connexins not only contribute to the formation of intercellular channels, but also behave as non-traditional adhesion molecules, independent of channel function.

MATERIALS AND METHODS

Cell culture and transfection

Cell cultures were grown in DMEM-F12 (GIBCO, Invitrogen) supplemented with 1.6% glucose, antibiotic-antimycotic and 10% fetal bovine serum (Atlanta Biologicals) and kept in 5% CO_2 at 37°C. Cells were passaged every 3 days. C6 glioma cells, INS1, HEK293, and N2A teratocarcinoma cells were transfected using lipofectin (Invitrogen, Carlsbad, CA) as previously described (Cotrina et al., 1998). Three independent clones transfected to stably express Cx43, Cx32 or the plasmid vector without connexin were used for all assays. Cx43-GFP construct was obtained from J. Weiss, David Geffen School of Medicine, UCLA (John et al., 1999). Stable transfectants were generated in C6 cells similar to the Cx43 and Cx32 clones. HeLa cells expressing Cx43 and Cx32 were supplied by K. Willecke, Bonn University, Germany (Elfgang et al., 1995). Expression of each connexin was assayed by immunolabeling with polyclonal antibodies (courtesy of B. Nicholson, State University of New York, Buffalo and D. Paul, Harvard Medical School) and by functional dye transfer on a bi-weekly schedule.

Immunocytochemistry and functional coupling assay

Cells were plated on 12-mm uncoated coverslips ($0.5\text{--}1 \times 10^5$ cells/mL) and fixed after two days with 4% paraformaldehyde. After permeabilization with 0.1% Triton X-100 cells were blocked with 10% normal goat serum. Anti Cx43 or anti Cx32 staining was performed as described (Cotrina et al., 1998).

To evaluate functional coupling the dye transfer technique was used as described (Cotrina et al., 1998). Briefly, cells were loaded with 5 (and 6)-carboxy-2',7'-dichlorofluorescein diacetate (CDCF diacetate) for 5 min, washed and trypsinized. After resuspension, the cells were labeled with 10 μM DiIC18 (Molecular Probes) for 10 min. and mixed with unlabeled cells at a 1:250 ratio. One hour after plating on polylysine-coated dishes, dye transfer from the CDCF/DiIC18 labeled cells (donor) to unlabeled cells (recipient) was evaluated using confocal scanning microscopy. Counts of the labeled donor and recipient cells were performed manually. The coupling index was defined as the fraction of donor cells that transferred dye to surrounding cells, multiplied by the mean number of receiving cells.

Short-term aggregation assay

To perform the cell aggregation assay (adapted from Götz et al., 1996), we first obtained a homogeneous population of single cells. To this end, cell monolayers were first disrupted in a solution of HBSS $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free containing 1mM EDTA for 15–30 min. at 37 °C. The culture was then dissociated mechanically. After two washes to eliminate EDTA residues, each cell population was labeled with the fluorescence cell trackers CMTMR (2 μM , red) or 5-chloromethylfluorescein diacetate (CMFDA, 2 μM , green) from Molecular Probes in DMEM-F12 minus serum for 30 min, according to the manufacturer instructions. After washing, the cells were mixed, and complete cell dissociation was verified. Mixtures containing more than 10 cell clumps of the same color were discarded. A total of 4×10^5 cells were added to a 24-well plate containing 400 μL DMEM/F12 (serum-free). Cells were then allowed to reaggregate on a rotary shaker (89 rpm) for 30–60 min and fixed by addition of 4% paraformaldehyde. Some mixtures were allowed to aggregate for 2h. Cellular aggregates were counted without mounting. For quantification, the number of cells in aggregates, the number of aggregates per field and the number of single cells per field were counted in a minimum of 5 different fields. Clusters of more than 10 cells were considered an aggregate. The percentage of aggregation was defined as the average percentage of cells in aggregates per field.

Selective removal of cell adhesion molecules (CAMs)

To remove distinct classes of CAMs, dissociated C6/Cx43 cells were incubated for 15 minutes in the following solutions prior to the aggregation assay: condition A, Ca^{2+} -free Hank's balanced salt solution (HBSS) containing 1 mM EDTA (positive control, all CAMs are left intact); condition B, 0.0001% trypsin in Ca^{2+} -free HBSS containing 1 mM EDTA (only Ca^{2+} -independent molecules, CIDs, remain); condition C, 0.01% trypsin in HBSS containing 10 mM Ca^{2+} (only Ca^{2+} -dependent molecules, CADs, remain); condition D, 0.01% trypsin in Ca^{2+} -free HBSS containing 1mM EDTA (negative control, no CAMs remain). Cells were then washed twice in DMEM-F12 containing 10% fetal calf serum to stop all enzymatic reactions. Aggregation assays were then performed as described above.

RESULTS

Connexins allow adhesion between heterotypic cell lines

We first asked whether Cx43 is sufficient to mediate adhesion between two different cell types that do not naturally adhere to each other. We transfected C6 glioma and HeLa cells with Cx43, labeled them with a different intracellular dyes (CMFDA (green) for C6 cells

and CMTMR (red) for HeLa cells) and allowed them to aggregate in a short-term aggregation assay. In this assay, only adhesion entirely mediated by cell-surface molecules is tested, as the assay is performed in solution, not allowing the cells to establish interactions with the substrate.

C6 and HeLa cells do not adhere to each other naturally and form aggregates that exclude each other. However, transfection with Cx43 enabled the two cell types to form abundant mixed aggregates of both colors (Figure 1A). Numerous punctate immunosignals were observed at the interface between the two cell types. These represented functional gap-junction channels, as they allowed dye transfer from labeled HeLa/Cx43 cells to unlabeled C6/Cx43 cells. In contrast, when C6/Cx43 cells were mixed with HeLa mock cells, only green-labeled cells (C6/Cx43 cells) associated and excluded red HeLa cells, which remained in a dissociated state (Figure 1B). Furthermore, no dye transfer or immunoreactive plaques were observed in mixed cultures of HeLa mock and Cx43 transfected cells.

We obtained similar results when we repeated the experiment with another connexin—Cx32. As before, C6 and HeLa cells formed mixed aggregates only when Cx32 was expressed in both cell types (Figure 1C, 1D). And, again, gap-junction plaques and dye transfer were detected in mixtures of C6-Cx32 and HeLa-Cx32 cells (Figure 1C). We did not observe any mixed aggregation between C6-Cx32 and HeLa mock cells. In these mixtures, only green, C6-Cx32 cells formed aggregates, whereas most of the red, HeLa mock cells remained as single cells (Figure 1D, 1E). These results indicate that expression of a Cx protein is sufficient to allow adhesion between two cell types that otherwise would not stick together.

Transfection of Cx43 in other cell types confirmed that connexins can determine the adhesive capability of the cell. The insulin-producing cell line INS-1 and HEK293 cells also increased their aggregation capability after forced expression of Cx43 ($213 \pm 40\%$ and $187 \pm 13\%$, respectively). In contrast, N2A neuroblastoma cells did not show increased aggregation after Cx43 transfection ($97 \pm 12\%$ of mock-transfected control, $n=4$). Importantly, whereas most INS-1 and HEK293 cells (about 70%) displayed Cx43-immunoreactive plaques, less than 20% of N2A cells did so. This finding suggests that connexins can function as CAMs only if expressed by most of the cells.

High Cx expression can drive adhesion with cells expressing low Cx levels

To complement the above data and establish the strength of connexin as adhesion moiety, we tested adhesive interactions between cells expressing high and low levels of connexins. We took advantage of the fact that wild-type C6 cells express low levels of Cx43 but no Cx32. As Cx32 and Cx43 do not form heterotypic channels, we predicted that mock-transfected or wild-type cells would only interact with Cx43-C6 cells and not with Cx32-C6 cells in the aggregation assay. When we tested this prediction, we surprisingly found that most mock-transfected or wild-type cells were engaged in mixed aggregates with Cx43-C6 cells, resembling aggregates formed only by C6-Cx43 cells (Figure 2A,B and Table 1).

We next tested if wild-type or mock-C6 glioma cells that express low levels of Cx43 (Figure 2G) could also adhere to C6-Cx32 cells expressing high levels of Cx32, but low levels of Cx43. In this case, we detected mostly pure aggregates of C6-Cx32 cells, and very few cells in these aggregates were C6-mock transfected cells (Figure 2D,F and Table 1).

These data show that the cell's ability to adhere is dependent on the compatibility between the connexins expressed because wild-type C6 glioma cells with low levels of Cx43 bind to Cx43, but not to Cx32-expressing cells. The low level of endogenous Cx43 in wild-type C6 cells was possibly sufficient to permit homotypic binding to the C6 cells expressing high

levels of Cx43. Indeed, the inhibition of aggregation of wild-type Cx43 transfectants (Lin et al., 2002) by anti-Cx43 antibodies argues in favor of this possibility. Although we observed aggregation between C6-Cx43 and C6-Cx32 cells (Table 1), we believe this aggregation is due to the contribution of the endogenous, low levels of Cx43 expression of the mock-transfected cells rather than a true docking event between Cx43 and Cx32 hemichannels. This conclusion is supported by the facts that Cx43 and Cx32 do not form heterotypic channels (White et al., 1995) and that we didn't observe differences in the level of aggregation between these two mixtures (61 ± 7 in Cx43/Cx32 vs. 74 ± 7 or 68 ± 6 in Cx43/mock-transfected cells; Table 1).

Importantly, the ability of the cells to adhere when expressing different connexins did not correlate with their ability to form stable gap-junction channels, as indicated by the coupling index from dye-transfer assays (Table 1). The same phenomenon occurred with cells expressing high and low levels of connexins: despite the fact that aggregation is very high, coupling index is almost undetectable.

Cx-mediated adhesion is independent of extracellular Ca^{2+}

Extracellular Ca^{2+} is an important regulator of adhesion. So, CAMs can be classified as CADs or CIDs on the basis of the requirement of Ca^{2+} to form adhesive interactions (Brackenbury et al., 1981). Likewise, extracellular Ca^{2+} ions are critical modulators of the opening and closing of gap-junction channels (Gomez-Hernandez et al., 2003). So, we next sought to establish whether the adhesion ability of connexins was dependent on the concentration of extracellular Ca^{2+} .

As a means of specifically assessing the role of Ca^{2+} in Cx-dependent adhesion, we used the same short-term aggregation assay, but this time we selectively removed CADs and/or CIDs (Takeichi, 1977; Gotz, 1996) prior to assessing their relative contributions to adhesion. The protocol for preferential digestion is based on the fact that CADs are highly sensitive to trypsin in the absence of Ca^{2+} ions. In the presence of Ca^{2+} , though, they are very resistant to trypsin, allowing the preferential digestion of CIDs. We also took advantage of the fact that C6 cells aggregate poorly regardless of the presence or absence of Ca^{2+} (Asano et al., 2004). The cells aggregate only after transfection with N-cadherin, even after trypsinization, as long as there is Ca^{2+} in the assay conditions. This indicates that there are no significant CADs or CIDs in C6 cells that can significantly contribute to aggregation under our assay conditions.

Dissociated C6/Cx43 cells were incubated for 15 min. in the following solutions prior to the aggregation assay: condition A, Ca^{2+} -free HBSS containing 1 mM EDTA (positive control, all CAMs are left intact); condition B, 0.0001% trypsin in Ca^{2+} -free HBSS containing 1 mM EDTA (only CIDs remain); condition C, 0.01% trypsin in HBSS containing 10 mM Ca^{2+} (only CADs remain); condition D, 0.01% trypsin in Ca^{2+} -free HBSS containing 1 mM EDTA (no CAMs remain). For this experiment to work, it is critical to incubate the cells for only 15 min. in each condition, as removal of Ca^{2+} for longer periods of time results in irreversible dissociation of the cells due to effects on other Ca^{2+} -dependent processes.

Removing CADs (condition B) did not reduce aggregation (59 ± 13 vs. 55 ± 16 in condition A, $n=4$). In contrast, when CIDs were removed (condition C), adhesion was considerably impaired, similar to what was observed when no CAMs remained (condition D; 5 ± 1 and 15 ± 8 , respectively). These results suggest that adhesion of Cx-expressing C6 cells does not require extracellular Ca^{2+} (Figure 3).

To confirm these results, we selectively removed CADs and allowed the cells to aggregate for a short period of time in the presence or absence of Ca^{2+} . Under these conditions, big

aggregates formed, regardless of the absence of Ca^{2+} (aggregation index 58 vs. 52, in HBSS plus and minus Ca^{2+} , respectively) indicating again that Cx43 did not require Ca^{2+} for adhesion to occur.

Connexins are highly mobile membrane proteins

We next performed time-lapse imaging studies in C6 cells expressing a Cx43-GFP construct in an attempt to analyze the dynamics of transport of Cx43 hemichannels towards the cell membrane. C6 cells expressing the Cx43-GFP construct were dissociated as before, allowed to adhere at low density, and their GFP fluorescence followed by confocal microscopy. Initially after plating, intense fluorescence was observed surrounding the Golgi apparatus, a phenomenon previously described for Cx proteins (Lauf et al., 2002). Two hours after plating, Cx43-GFP fluorescence was observed as irregular ruffles along the edge of the cell body, but also at the tips of the cellular protrusions (Figure 4A–C). Ruffles were observed even in cells that were not physically in contact with other cells. In this case, they constant protruded and retracted, as if sensing the environment. As soon as the cells encountered other cells, gap-junction plaques started to form, as judged by the concentration of punctate immunosignals within larger dots (white arrows in Figure 4B,C) and by the disappearance of elongated fluorescent structures.

These observations confirm the notion that connexins are highly mobile proteins in the membrane without the need of cell-cell contact. So, hemichannels can aggregate or disaggregate rapidly, according to the cellular needs. The existence of a large reservoir of highly diffusible hemichannels, potentially ready for adhesive interactions, is consistent with reports about other CAMs, such as IgCAMs (Thoumine et al., 2005).

DISCUSSION

This study shows that Cx43 and Cx32 can mediate cell-cell interactions and therefore have adhesive properties. We found that cells expressing high levels of Cx43 aggregated better among themselves and with mock-transfected, low Cx43-expressing cells. In contrast, Cx32⁺ cells did not adhere to low Cx43-expressing cells and excluded these cells from their aggregates. Thus, adherence between Cx43⁺ cells probably involves the same docking rules as those required to form a gap-junction channel, as Cx43 and Cx32 do not naturally form gap junctions. Together with the observation that Cx43 enabled adhesion between cells from different tissues and even species, which otherwise would segregate from each other, our data point to the connexins themselves as the proteins directly responsible for aggregation.

Several lines of evidence have suggested that Cx expression increases the adhesive properties of transfected cells and promotes cell migration (Lin et al., 2002; Elias et al., 2007). This study explored the adhesion capability of connexins in the context of pure adhesion, excluding other cell functions such as migration and channel formation. Our data provide clear evidence for a direct role of Cx43 and Cx32 in establishing stable cellular contacts.

The extracellular domains of connexons dock with connexons in neighboring cells and thereby link adjacent cells through a mechanism similar to CAMs (Johnson et al., 1974), but gap junctions have traditionally not been considered as such. The current concept about gap junction formation is that cadherins establish initial cell-cell contacts, and gap junctions only form after cell adhesion has been established. While our study does not question this sequence of events, it suggests the novel concept that docking of connexin hemichannels both directs and stabilizes the contact from the beginning. We observed that cells expressing high levels of Cx drive adhesion with cells expressing low levels of the same Cx. This adhesion capability did not correlate with the functionality of the gap-junction channel

because the same cell types do not show significant dye transfer. These observations could be explained if we assume that 1) the probability of interaction with the cell expressing low Cx levels is increased when one of the binding parties expresses high Cx level in the membrane and/or 2) the probability of channel opening does not change with the presence of more connexons in the neighboring cell.

Likely, additional steps are needed to transition to the state that requires higher amounts of connexons recruited in one single area for gap junctions to be formed. Alternatively, it is also possible that the sensitivity of our assay conditions to detect dye transfer is lower than the sensitivity of our adhesion assay to detect cell-cell interactions. In any event, the formation of stable cell-cell contacts was driven by connexin subtypes, rather than by cell-specific differences. The fact that chaotropic treatment with agents like urea is required to split existing gap junctions suggests that the assembly of a gap junction is extraordinarily strong (Manjunath et al., 1984).

We must not forget that the occurrence of gap junction channels in the membrane without subsequent opening seems to be very high, at least in cultured cells. Bukauskas et al. (2000) examined the relationship between clustering of gap-junction channels and electrical coupling, and estimated that only when there are gap junction plaques formed by more than 400 channels electrical coupling is detected. More strikingly, fewer than 2% of these channels are actually open. Thus, adhesion mediated by gap junctions independent of channel opening can be a much more relevant phenomenon than initially considered.

Cx37 can regulate monocyte adhesion in atherosclerotic lesions (Wong et al., 2006). In this case, adhesion is modulated by the release of ATP via hemichannels. However, we did not see any differential adhesive effect in the presence of apyrase, an enzyme that rapidly degrades ATP (data not shown) supporting the idea that the adhesion effect we report is exclusively mediated by the extracellular domains of connexins. However, we cannot entirely rule out the Cx-mediated regulation of other CAMs at the level of the plasma membrane to help establish stronger adhesive interactions between cells once the initial contact has been formed.

Ca²⁺ dependence of connexin-mediated adhesion

We have found that Cx-mediated adhesion does not depend on extracellular Ca²⁺, as removing CADs in low levels of trypsin and Ca²⁺-free medium did not alter the connexins' ability to mediate adhesion. To promote adherence, connexins must have a docking capability similar to what is required to form intercellular channels, as mutations that affect the docking residues interfere with adhesion (Lin et al., 2002). Opening hemichannels in nonjunctional membrane stimulates gap junction formation in oocyte pairs (Beahm et al., 2004), and the opening probability of hemichannels increases as Ca²⁺ concentration decreases (Gomez-Hernandez et al., 2003; Thimm et al., 2005). Indeed, the optimal concentration of Ca²⁺ to detect gap junction channel conductance is low—about 0.1 mM (Dahl et al., 1992).

Most of these studies have manipulated Ca²⁺ to detect functional gap-junction channels. However, there are no studies establishing the optimal Ca²⁺ conditions to start contacts between two apposing hemichannels, as the available studies have also measured channel conductance and not just physical contact. Here we provide the first evidence that the initial contact between apposing connexins does not require Ca²⁺, as removing Ca²⁺ did not alter connexin-mediated adhesion. However, we think that hemichannel docking to form a channel and to mediate adhesion are likely to be similar processes, as they occur in a setting of physiological extracellular Ca²⁺. Later on, it is possible that the areas where junctions

form may contain a low Ca^{2+} microenvironment that favors the optimal conditions for opening the channel.

The requirement of Ca^{2+} for gap-junction channel formation in epidermal cell lines is primarily due to the Ca^{2+} dependence of CADs (Jongen et al., 1991). In that study, a direct correlation between connexins and E-cadherin expression was found. However, Xu et al. (2001) have shown that N-cadherin-deficient neural crest cells still contain numerous Cx-positive membrane contacts despite the fact that dye coupling is virtually eliminated. These results are consistent with our observation that Cx-mediated adhesion is independent of the presence of extracellular Ca^{2+} . Indeed, the fact that it is possible to dissociate adhesion from coupling (Oliveira et al., 2005) suggests that additional domains may have to be engaged in order to establish a functional gap-junction channel.

What is the relevance of connexins as CAMs?

The presence of connexins has been observed in a broad array of states, from development to disease. Connexins are detected early in the embryo, and their capability to function as CAMs fits well with a role during early stages of development.

In the developing nervous system, proliferating cells in the rostral migratory stream (RMS) may provide an example of connexins' dual function in adhesion and communication. In the RMS, neuroblasts from the subventricular zone migrate towards the olfactory bulb while they are still proliferating. The existence of neurogenic areas of the RMS that are highly coupled has been reported (Menezes et al., 2000). These cells are apposed to cells of the astrocytic lineage and, in this interphase, there might be a dynamic interplay of adhesion-separation in which connexins might simultaneously subserve adhesion and communication functions.

A similar scenario takes place in the developing heart, where cardiac neural crest cells migrate for tissue remodeling in the conotruncal cardiac region (Huang et al., 1998). These cells migrate as groups of cells organized in sheets and streams. Both Cx43 expression and gap-junction communication are abundant in this migratory route. When N-cadherin is downregulated, there are still abundant Cx43-mediated contacts at the cell surface despite the fact that gap junction communication is markedly reduced. Only when the levels of Cx43 decrease significantly, as in mice lacking Cx43, the rate of migration is highly reduced, resulting in fewer cells in the outflow tract towards the heart (Xu et al., 2001). These studies highlight the importance of Cx43 contacts during group migration, evidencing again the dissociation between adhesion and channel function in another cell population. They also evidence the separation between the roles of N-cadherin and Cx43 in neural crest cells, even though both molecules have the ability to regulate migration.

A recent study on the mechanisms of radial neuronal migration in the developing neocortex has confirmed these observations. Radial glial cells provide a scaffold for the movement of neuronal precursors to their final cortical destinations. The downregulation of Cx26 or Cx43 promoted a reduction in the number of neurons reaching their final targets (Elias et al., 2007). In accordance with our previous data (Lin et al., 2002), only the residues involved in cell-cell contact via connexins, but not those required for the formation of a gap-junction channel, were important for migration to occur. Interestingly, Elias et al. did not find a change in the expression pattern of other cell-cell and cell-matrix adhesion molecules like N-cadherin, zona occludens-1 or β 1-integrin, supporting a role for connexins as the sole mediators of the adhesion effect.

High coupling and expression of connexins is prevalent in adult astrocytes. Although other connexins have been reported, Cx43 is the major astrocytic Cx. So, this Cx can be a critical

CAM for the maintenance of the astrocytic syncytium. In addition, glioma cells expressing high levels of Cx43 can interact with host astrocytes and invade the brain parenchyma (Lin et al., 2002), contributing to the dissemination of astrocytomas. Oliveira et al. (2005) have confirmed that Cx43 expression enables glioma cells to migrate from the tumor core and invade the adjacent parenchyma. In this case, the migrating ability depends on the capacity of Cx43 to form functional gap-junction channels and not just cell-cell contacts. In contrast, the same study shows that chemical inhibition of gap junction coupling prevented migration but enhanced the adhesive interactions between homotypic cells. This observation points once more to the dissociation of these two cellular functions—channel formation (in this case, allowing migration) and adhesion (restricting movement). It also confirms previous data on the involvement of different domains to mediate each particular function.

Unfortunately, it has not been possible to accurately measure the number of gap-junction channels that are open *in vivo* because most of the studies refer of functional coupling in terms of dye-transfer capability or measuring electrical conductance without stating the actual percentage of opened gap junctions involved. It is therefore likely that, in physiological conditions, ‘adhesive’ gap junctions are more relevant than initially thought. At this point, however, it is not possible to firmly establish the biological importance of Cx-mediated adhesion.

In summary, our results provide evidence that Cx expression is sufficient to permit adhesion between glial cell lines. We have shown that, even in the absence of other CAMs, Cx expression may be sufficient to establish adhesive interactions without intervention of a functional gap-junction channel.

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Figure 1. Transfected C6 and HeLa cells aggregate when expressing the same Cx
A, Aggregates formed by Cx43-C6 cells (red) and Cx43-HeLa cells (green). *Insert top* Cx43 punctate immunosignals (green, white arrows) in the interface between a Cx43 HeLa cells (red) and a Cx43-C6 cell (not visible). *Insert bottom* Dye transfer between a HeLa-Cx43 (yellow nuclei, pre-labeled with a red membrane dye-DiIC₁₈ and a green intracellular dye, CDCF) and Cx43-C6 cells (green). **B**, Only aggregates of Cx43-C6 cells (green) form when mixed with mock-transfected HeLa cells that do not express any connexin in their membranes (red). **C**, Aggregates composed of Cx32-C6 cells (green) and Cx32-HeLa cells (red). *Insert top* Punctate immunosignals are observed (white arrows) between Cx32-C6 and Cx43-C6 (not visible). Scale bar, 3 μ m. *Insert bottom* Functional dye coupling is also observed from a pre-labeled Cx32-HeLa cell to unlabeled Cx32-C6 cells. Scale bar, 16 μ m. **D**, Aggregates primarily form among Cx32-C6 cells (green) if mixed with mock-transfected HeLa cells (red). Scale bar, 35 μ m. **E**, Quantitative analysis of the aggregates. Histograms show the percentage of cells in aggregates. The relative ratio of red and green in the aggregates is indicated by the color. Data represent mean $\pm$ SEM of 4 independent assays.

Figure 2. C6-Cx43 cells aggregate with mock-transfected and wild-type C6 cells

A, Aggregates formed by Cx43-C6 cells pre-labeled with either CMTMR (red) or CMFDA (green). **B**, Similar aggregates are observed when Cx43-C6 cells (green) are mixed with mock-transfected C6 cells (red). **C**, Aggregates formed by red and green Cx32-C6 cells. **D**, An aggregate composed primarily of Cx32-C6 cells prelabeled with CMFDA (green). The mock-transfected C6-cells (red) fail to intermingle with the Cx32-C6 cells. Scale bar, 30 μ m. **E,F**, Quantitative analysis of the aggregates formed by transfected C6 cells: C6-Cx43/C6-Cx43, C6-Cx43/C6-mock, C6-Cx32/C6-Cx32 and C6-Cx32/C6-mock.

Histograms show the percentage of all cells incorporated within aggregates. Relative ratios of red:green cells in the aggregates are indicated by the respective colors. Data represent mean $\pm$ SEM of 4–16 assays. **G**, Expression of Cx43 or Cx32 in transfected and wild-type C6 cells. A low level of endogenously expressed Cx43, but not Cx32, is observed in control, mock-transfected C6 cells.

Figure 3. Connexin-mediated aggregation is independent of extracellular Ca^{2+}
 Histogram of the effect of selective removal of CAMs on aggregation of C6-Cx43 cells. Treatment **A**, Ca^{2+} -free HBSS containing 1 mM EDTA; **B**, 0.0001% trypsin in Ca^{2+} -free HBSS containing 1 mM EDTA; **C**, 0.01% trypsin in HBSS containing 10 mM Ca^{2+} ; **D**, 0.01% trypsin in Ca^{2+} -free HBSS containing 1mM EDTA. *Statistically different from A and B (Student t-test). $P < 0.05$. Percent aggregation was calculated as the average number of cells that formed aggregates of more than 10 cells per field.

Figure 4.

Temporal dynamics of Cx43-GFP fluorescence following plating. **A** At 20 min after plating intense staining is evident over the Golgi with little fluorescence in the remaining parts of the cell body. **B** At 2 hr, Cx43-GFP forms irregular ruffles along the edge of the cell body but is also concentrated in the tips of the cellular protrusion. A few highly fluorescence punctate immunosignals have formed (arrows). **C** The same pattern is observed at 4 hr, but more of the Cx43-GFP fluorescent signals are detected in contact areas between cells. Scale bar, 10 μm .

Table 1

Comparison between dye transfer and adhesion in mixtures of C6-mock and Cx43- or Cx32-transfected cells.

Cell Mixture	Adhesion (% aggregation)	Coupling Index
Cx43/Cx43	68 ± 2 (7)	7 ± 0.9 (9)
Cx43/Cx32	61 ± 7 (7)	0.09 ± 0.05 (5) **
Cx43/mock1	74 ± 7 (4)	n.d.
Cx43/mock2	68 ± 6 (6)	0.01 ± 0.01 (7) **
Cx32/Cx32	54 ± 3 (10)	18 ± 0.1 (6)
Cx32/Cx43	61 ± 7 (7)	0 (4) **
Cx32/mock1	28 ± 10 (4) *	n.d.
Cx32/mock2	n.d.	0.01 ± 0.007 (6) **
Mock1/mock1	15 ± 4 (9) *	0.14 ± 0.04 (3) **
Mock2/mock2	29 ± 3 (5) *	0.004 ± 0.003 (9) **

Number of experiments is in parenthesis, n.d. Not determined.

* Significantly different from mixtures with C6-Cx43 cells. $p < 0.05$

** Significantly different from Cx43 or Cx32 pure cells. ANOVA for group comparison with *post hoc* t-test to establish significant difference between the groups. $p < 0.01$.