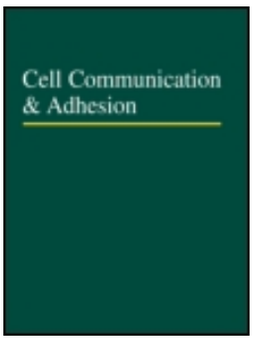

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Differential Oligomerization of Endoplasmic Reticulum-Retained Connexin43/Connexin32 Chimeras

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To examine early events in connexin oligomerization, we made connexin constructs containing a C-terminal di-lysine based endoplasmic reticulum (ER) retention/retrieval signal (HKKSL). Previously, we found that both Cx32-HKKSL and Cx43-HKKSL were retained in the ER. However, Cx32-HKKSL oligomerized into hexameric hemichannels, but Cx43-HKKSL was retained as an apparent monomer. To define elements that prevent Cx43-HKKSL oligomerization in the ER, we made a series of HKKSL-tagged Cx43/Cx32 chimeras. When expressed by HeLa cells, some chimeras were retained in the ER as apparent monomers, whereas others oligomerized in the ER. To date, the second and third transmembrane domains and the cytoplasmic loop domain provide the minimal sufficient Cx43 element to inhibit ER oligomerization.

Keywords. Assembly, connexin, gap junction, hexamer, transmembrane protein

INTRODUCTION

Connexins oligomerize into hexameric hemichannels prior to transport to the plasma membrane. We previously used a di-lysine endoplasmic reticulum (ER) retention/retrieval motif, HKKSL, to study early events in connexin oligomerization. A di-lysine-tagged connexin43 construct (Cx43-HKKSL), expressed by HeLa cells, was preferentially localized to the ER as an apparent monomer (4), consistent with the notion that Cx43 oligomerizes in a post-ER compartment (3, 7, 11, 16). In contrast to our results with Cx43-HKKSL, an ER retained Cx32 construct (Cx32-HKKSL) oligomerized into hexamers (4). This, and other studies (7, 12, 14), suggested the possibility the connexin oligomerization might also occur in the ER. Given the difference in oligomerization state between Cx43-HKKSL and Cx32-HKKSL, we decided to make a series of HKKSL-tagged Cx43/Cx32 chimeras to define elements required for post-ER oligomerization by HeLa cells.

MATERIALS AND METHODS

Polyclonal rabbit anti-Cx43 antisera were generated using 6his-tagged C-terminal tail constructs as previously described (2). Monoclonal anti-Cx32 was from Zymed (South San Francisco, CA). Fluorescent secondary antibodies and horseradish peroxidase conjugated secondary antibodies were from Jackson ImmunoResearch (Malvern, PA).

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Construction and characterization of rat Cx43-HKKSL and rat Cx32-HKKSL were previously described (4). Cx43/32 chimeras were produced by two step PCR amplification using established protocols (9) and inserted into the pcDNA3 expression vector. HeLa cells were transiently transfected with Cx43/Cx32-HKKSL chimeras using Lipofectamine (Invitrogen, Carlsbad, CA). For some biochemical studies, stable HeLa transfectants were generated by selection using medium containing 2 mg/ml G418 (Invitrogen). HeLa cells stably transfected or transiently transfected with Cx43/Cx32-HKKSL chimeras gave comparable results.

Sucrose gradient fractionation, immunoblot and immunofluorescence were done as described (3, 4, 11). Monomeric chimeras were identified as proteins collected in fractions that formed a single peak centered at ~7–9% sucrose. Chimeras resolved by sucrose gradient fractionation where the fractions peaked at 11% sucrose or greater were considered oligomerized. The hexamer fraction showed a peak at ~15% sucrose.

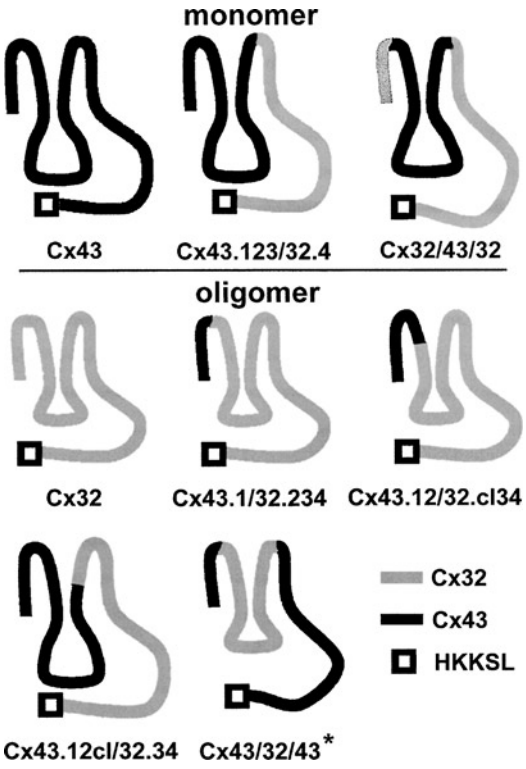

Figure 1. Oligomerization of ER-retained Cx43/Cx32 chimeras. Shown are diagrams depicting chimeras where black represents protein elements derived from Cx43 and light gray represents protein elements derived from Cx32. The box represents the C-terminal HKKSL ER retention/retrieval motif. Chimeras were categorized as being monomers or oligomers based on sucrose gradient fractionation analysis of transfected HeLa cells. The chimeras classified as oligomers showed predominantly hexamers, except for Cx43/32/43-HKKSL (*) which was primarily sub-hexameric oligomers.

RESULTS AND DISCUSSION

We produced a series of Cx43/Cx32-HKKSL chimeras summarized in Figure 1. One advantage to using ER-retained constructs was that they were rarely transported to the cell surface, which eliminated complications due to hemichannel docking. Also, this meant that we could take advantage of extracellular protein domains that were highly homologous between Cx43 and Cx32 as splice sites for chimera construction and leave membrane interface regions intact.

By immunofluorescence microscopy, all of the constructs were retained in the ER (unpublished data). Two of the constructs, Cx43.123/Cx32.4-HKKSL and Cx32/43/32-HKKSL, were retained in the ER as apparent monomers. The remaining constructs were able to oligomerize in the ER. For Cx43.123/Cx32.4-HKKSL, we partly confirmed that this was not due to incompatibility, since untagged Cx43.123/Cx32.4 formed structures resembling gap junction plaques at membrane interfaces (unpublished data).

Since Cx43.123/Cx32.4-HKKSL was monomeric and Cx43.12cl/Cx32.34-HKKSL oligomerized, this suggests that an aspect of the third transmembrane/extracellular domain is critical for regulating post-ER oligomerization of Cx43. It is not clear whether this domain is sufficient for post-ER oligomerization. The current minimal element for post-ER oligomerization in these studies was defined by the Cx32/43/32-HKKSL construct, which also contains the second transmembrane domain and cytoplasmic loop of Cx43. Future chimeras consisting

of the third transmembrane domain of Cx43 on a Cx32-HKKSL backbone are anticipated to help determine whether the cytoplasmic loop domain is required to prevent ER oligomerization.

It has been found that the third transmembrane domain of Cx32 is required for oligomerization (1), which makes the third transmembrane domain an appealing site for regulating Cx43 oligomerization. Two distinct motifs, a di-tryptophan motif for Cx32 and a leucine-arginine motif for Cx43, are close to the interface between the cytoplasmic leaflet and third transmembrane domain and may help regulate the specificity of oligomerization (13). However, the Cx43.12cl/Cx32.34-HKKSL chimera has the Cx43-like leucine-arginine motif, suggesting that these residues are not sufficient to prevent ER oligomerization of this chimera.

Also, it is likely that the third transmembrane domain is the major helix that lines the gap junction channel aqueous pore (17, 18). Cysteine-scanning mutagenesis suggests that most of the charged amino acids in the third transmembrane domain that are conserved between Cx32 and Cx43 are likely to be buried rather than exposed to the aqueous pore (17). Thus, extensive charge shielding from the hydrophobic portion of the lipid bilayer is probably not required to maintain Cx43 as a monomer. However, Cx43 has a polar residue (glutamine 173) that might require some shielding by a putative chaperone to stabilize monomeric Cx43 in the bilayer. Helix shielding would best be accomplished by an interaction with a putative transmembrane chaperone, although dimer formation might also accomplish this as well.

Aside from being exposed to the aqueous environment, the third transmembrane domain is normally not likely to be exposed to the bulk membrane lipid environment when connexins are oligomerized. This is underscored by the difficulty that TMPred (10) and comparable algorithms have in assigning the membrane interface regions of the third connexin transmembrane domain. Conceivably, binding of a putative chaperone to the second extracellular loop could help stabilize the position of the third transmembrane domain relative to the membrane bilayer for monomeric Cx43. This putative chaperone would not necessarily have to be a transmembrane protein in this case and could be a peripheral lumenal protein. Also, the second extracellular loop plays a critical, although not necessarily exclusive, role in determining heterotypic connexon compatibility (5, 6, 8, 19, 20). This suggests that the binding of a putative chaperone to this region of the protein might not only prevent Cx43 oligomerization, but might also help prevent aberrant docking interactions involving extracellular loops.

Unlike the other constructs that oligomerized, Cx43/32/43-HKKSL predominantly formed subhexameric oligomers. Cx43.1/Cx32.234-HKKSL completely oligomerizes, arguing against a role for the first transmembrane domain in preventing oligomerization. However, the Cx43/32/43-HKKSL chimera also contains the fourth transmembrane domain and carboxyl terminus of Cx43. This raises the possibility that Cx43/32/43-HKKSL oligomerized because it lacks the Cx43 third transmembrane domain, however, the other elements of Cx43 in this chimera might limit this to dimerization. Consistent with this possibility, we have found that Cx43 can form stable dimers with a Cx43-β–galactosidase fusion protein (3). Also, elements of the Cx32 carboxyl terminus may be required for full oligomerization to occur (15). Further, we have not yet ruled out the possibility that this chimera cannot oligomerize due to incompatibility. Experiments examining the assembly state of untagged Cx43/32/43 are pending.

In summary, we have used a series of Cx43/Cx32-HKKSL chimeras to define Cx43 motifs likely to be required for post-ER oligomerization. The third transmembrane domain appears to be critical, although other Cx43 elements may also help regulate post-ER oligomerization. Also, given that we can isolate different ER-retained chimeras that are either monomeric or oligomerized, these results further support the notion that Cx43 and Cx32 can oligomerize in different intracellular compartments.

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