Gap Junction Protein Cx37 Interacts With Endothelial Nitric Oxide Synthase in Endothelial Cells

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Objective—The gap junction protein connexin37 (Cx37) plays an important role in cell-cell communication in the vasculature. A C1019T Cx37 gene polymorphism, encoding a P319S substitution in the regulatory C terminus of Cx37 (Cx37CT), correlates with arterial stenosis and myocardial infarction in humans. This study was designed to identify potential binding partners for Cx37CT and to determine whether the polymorphism modified this interaction.

Methods and Results—Using a high-throughput phage display, we retrieved 2 binding motifs for Cx37CT: WHK...[K,R]XP... and FHK...[K,R]XXP..., the first being more common for Cx37CT-319P and the second more common for Cx37CT-319S. One of the peptides (WHRTPRLPPPVP) showed 77.7% homology with residues 843 to 854 of endothelial nitric oxide synthase (eNOS). In vitro binding of this peptide or of the homologous eNOS sequence to both Cx37CT isoforms was confirmed by cross-linking and surface plasmon resonance. Electrophysiological analysis of Cx37 single channel activity in transfected N2a cells showed that eNOS-like and eNOS(843–854) increased the frequency of events with conductances higher than 300 pS. We demonstrated that eNOS communoprecipitated with Cx37 in a mouse endothelial cell (EC) line (bEnd.3), human primary ECs, and a human EC line transfected with Cx37-319P or Cx37-319S. Cx37 and eNOS colocalized at EC membranes. Moreover, a dose-dependent increase in nitric oxide production was observed in ECs treated with Cx37 antisense.

Conclusion—Overall, our data show for the first time a functional and specific interaction between eNOS and Cx37. This interaction may be relevant for the control of vascular physiology both in health and in disease. (Arterioscler Thromb Vasc Biol. 2010;30:827-834.)

Key Words: connexin37  ■  GJA4  ■  endothelial nitric oxide synthase  ■  gene polymorphism

Connexins are integral membrane proteins that oligomerize to form intercellular channels called gap junctions. Gating, permeability, and regulatory properties of a gap junction channel vary depending on the connexin isof orm. The present study focuses on connexin37 (Cx37), an isof orm expressed mostly in endothelial cells (ECs), monocytes, and macrophages but also found in vascular smooth muscle cells.1 Studies on genetically modified mouse models suggest an involvement of Cx37 in atherogenesis.2 Additional studies have shown atherosclerosis-related changes in Cx37 expression; in particular, Cx37 can no longer be detected in the endothelium overlaying advanced atheromas, whereas there is high expression in macrophage foam cells and in previously Cx37-deprived medial smooth muscle cells.3 Studies in humans indicate that a polymorphism at position 1019 of the GJA4 gene, encoding human Cx37, may be a prognostic marker for atherosclerosis.1 This C1019T polymorphism codes for a proline-to-serine substitution (P319S) in the C-terminal domain of the protein. Two separate studies showed a correlation between the Cx37-319S isoform and an increased incidence of myocardial infarction in the sampled population.4,5 The Cx37-319S genotype was also shown to predict survival after an acute coronary syndrome.6 In 3 additional studies, the alternative isoform (Cx37-319P) was found to segregate with an increased incidence of stenosis in the coronary arteries7,8 or the carotid arteries.9 Studies at the cellular level recently showed that Cx37 expression inhibited monocyte adhesion to the endothelium; this effect was more pronounced in monocytes expressing Cx37-319P than in those expressing Cx37-319S.2 Overall, the data support the notion that Cx37 could play a protective role against the development of atherosclerosis, and they leave open the possibility that variations in the sequence of the C terminus of the gene could influence this protective effect.
Cx37 (Cx37CT) could have functional effects relevant to pathophysiology.

Little is known about the functional role of Cx37CT. Primary sequence analysis of this region identifies several potential consensus sites for phosphorylation and for the binding of Cx37 to other molecules. It is thus reasonable to speculate that, as in the case of other connexin isoforms, the C-terminal domain is the major regulatory domain of Cx37, and, perhaps, the P319S polymorphism alters Cx37 regulation. However, an unbiased search for potential molecular partners of Cx37 remains to be conducted.

In the present study, we have carried out a high-throughput phage display screening in search for peptide sequences that bind to Cx37CT. Our results show that Cx37-319P preferentially binds to peptides containing the motif WHK...[K,R]XP... , whereas Cx37-319S displays a preference for FHK...[K,R]XXP... motifs. Moreover, we identified a particular peptide sequence homologous to a region of endothelial nitric oxide synthase (eNOS), an enzyme of fundamental importance to vascular biology and disease. Additional studies showed that this peptide (called eNOS-like) or the homologous sequence of eNOS (called eNOS[843–854]) could affect the function of Cx37 channels. Moreover, these results led us to demonstrate that Cx37 associates with eNOS in the native environment of ECs, thereby functionally affecting the enzyme. Overall, our data indicate a possible cross-talk between these 2 molecules and suggest a functional role for this interaction in the production of nitric oxide (NO) in the vascular endothelium.

**Methods**

An expanded Methods section is available in the Data Supplement, available online at http://atvb.ahajournals.org. In brief, production of recombinant Cx37CT, phage display, surface plasmon resonance (SPR), and cross-linking experiments on Cx37CT-319P or Cx37CT-319S were performed following methods previously described. Cell culture of murine neuroblastosoma cells (N2a; American Type Culture Collection, Manassas, VA), a mouse EC line (bEnd.3), a human umbilical vein ECs followed standard protocols. Transfection of EA.hy926 and N2a cells with Cx37-319P or Cx37-319S was performed following methods previously described.

**In Vitro Binding Detected by SPR**

Phage display allows screening for potential binding sequences, but it does not give information on the characteristics of the binding reaction. We therefore used SPR to quantitatively characterize binding of selected peptides to Cx37CT. Our studies focused on the peptide showing the highest homology to eNOS (eNOS-like) and a 12-mer peptide corresponding to sequence 843 to 854 of eNOS (Table), with the eNOS[843–854] sequence containing both [K,R]XP and [K,R]XXP motifs. Given the biological importance of eNOS in cells where Cx37 is naturally present, we pursued further characterization of these peptides and the C-terminal domain of Cx37.

**Table. Alignment of eNOS-Like Peptide With eNOS(843–854)**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS-like</td>
<td>N/WHRTPLPPPVPP-C</td>
</tr>
<tr>
<td>eNOS(843–854)</td>
<td>N/WVRDPRLLPPCTL-C</td>
</tr>
</tbody>
</table>

We calculated the probability of occurrence of these motifs by chance alone, taking into account the experimental abundance of individual amino acids. The results indicate that the actual occurrence of the specific sequences was significantly higher than their expected probability (Supplementary Tables III and IV). Overall, the data support the notion that Cx37CT-319P bound with enhanced selectivity to peptides containing the motif WHK...[K,R]XP... , whereas the sequence FHK...[K,R]XXP... was selected by Cx37CT-319S. Moreover, sequence alignments against the National Center for Biotechnology Information protein database (BLAST) indicated homology between one of the selected peptides and amino acids 843 to 854 of eNOS (Table), with the eNOS[843–854] sequence containing both [K,R]XP and [K,R]XXP motifs. Given the biological importance of eNOS in cells where Cx37 is naturally present, we pursued further characterization of these peptides and the C-terminal domain of Cx37.
with Cx37CT-319S was too weak to allow for proper quantitative analysis. These K_D values were not statistically different (N=3). As a control for specificity, both peptides were presented to mouse Cx40CT or rat Cx43CT, which did not result in any significant resonance shift (Supplementary Figure II). Overall, our data show that these peptides are able to interact specifically with a recombinant Cx37CT.

### Cross-Linking of Cx37CT to Peptides

Whereas SPR analysis requires the physical constraint of the bait protein to the sensor chip matrix, the use of a cross-linker reagent allows for the study of intermolecular interactions when the purported molecular partners are free in solution. Figure 2 shows the results obtained from the cross-linking of Cx37CT to either eNOS-like (Figure 2A) or eNOS(843–854) (Figure 2B). After separation by SDS-PAGE, samples were stained with Coomassie Blue. A band of ≈11 kDa representing Cx37CT could be seen in all samples. In the samples incubated with the cross-linking reagent (Figure 2A and 2B, first, second, fourth, and fifth lanes from the left in each panel), some supplementary bands were observed in the range between 22 and 36 kDa, likely resulting from polymerization of Cx37CT. The second, third, fifth, and sixth lanes in Figure 2A and 2B show results obtained in the presence of peptides. Bands of low molecular mass, corresponding to monomers or multimers of the peptides, can be observed. Interestingly, in the second and fifth lanes (Figure 2A and 2B), where samples contain one isoform of Cx37CT, eNOS-like or eNOS(843–854) peptides and the cross-linker, a supplementary band can be seen above the Cx37CT band. The mobility of this band corresponds to the estimated molecular mass of a Cx37CT-peptide complex (≈12 kDa). The density of this band was actually higher than that of the multimers of Cx37CT, suggesting a higher affinity for the peptide-protein interaction than for oligomerization of the free Cx37CT. A control experiment performed in the presence of 37,40Gap26, a Cx37 channel-blocking peptide, did not reveal any cross-linking of this peptide and Cx37CT (data not shown). Our results are consistent with those obtained by SPR and suggest that eNOS-like and eNOS(843–854) bind to both Cx37CT isoforms.

### Effect of eNOS-Like and eNOS(843–854) on the Function of Cx37 Channels

The ability of eNOS-like and eNOS(843–854) peptides to bind Cx37CT led us to hypothesize that the peptides may also...
change the behavior of Cx37 channels. Gap junction currents were recorded from N2a cells transfected with Cx37-319P or Cx37-319S. To reduce macroscopic currents, cell pairs were superfused with 2 to 4 mmol/L octanol.

Frequency histograms of unitary conductances obtained from Cx37-319P or Cx37-319S cell pairs in the presence of a scrambled peptide (Figure 3A and 3D, respectively) show that, as previously described, Cx37-319S channels transition between various conductive states up to 400 pS,11 with a trend for smaller unitary conductance in cells transfected with Cx37-319P or Cx37-319S. To reduce macroscopic currents, cell pairs were superfused with 2 to 4 mmol/L octanol.

To study the possible interaction between eNOS and Cx37 in a cellular environment, we performed coimmunoprecipitation studies using the mouse EC line bEnd.3 which constitutively expresses all vascular connexins. Cx37 antibody immunoprecipitated a protein recognized by Cx37 antibodies (Figure 4A, second lane of right panel). No signal for eNOS or Cx37 was observed in control experiments, where the immunoprecipitating antibody was omitted from the procedure (Figure 4A, right lane of each panel). The subcellular localization of eNOS and Cx37 was next examined by immunofluorescence. As expected,19,22 a strong Cx37 signal was observed at cell-cell contacts in bEnd.3 cells. After incubation with eNOS antibodies, bEnd.3 cells showed a staining in the perinuclear region and at cell membranes. Cx37 and eNOS colocalization was observed mostly in regions of cell-cell contacts (Figure 4B). In contrast, Caveolin-1 (Cav1), a protein known to interact with eNOS, was mostly localized intracellularly in bEnd3. When examined by confocal microscopy, this protein did not colocalize with Cx37 in bEnd3 cells (Supplementary Figure IV).

Previous studies indicated that eNOS enzyme activity might be affected by interacting proteins.23,24 The colocalization and coimmunoprecipitation of eNOS and Cx37 in the EC line led us to hypothesize that the interaction may also affect the activity of eNOS. In the next series of experiments, we exposed bEnd.3 cells to Cx37 antisense. As illustrated in Figure 4C, Cx37 expression was considerably decreased in response to 50 μmol/L Cx37 antisense (right panel), whereas the same concentration of sense oligonucleotides did not affect the expression of the protein (left panel). Constitutive NO production by bEnd.3 cells, as evaluated by the measure of the end product nitrite, was 0.403 ± 0.030 nmol/h per 10^5 cells (N=8). Interestingly, this nitrite production dose-dependently increased with increasing concentration of Cx37 antisense (Figure 4D). This effect was not due to an upregulation of eNOS expression nor to a modification in expression levels of Cx40, Cx43 or Cav1 (Figure 4E). Inducible nitric oxide synthase (iNOS), an alternative source of NO, could not be detected under all conditions compared with a 5-fold smaller amount of stimulated macrophage lysate. Moreover, Cx37 antisense did not affect eNOS or Cav1 subcellular localization (Supplementary Figure IV). On the other side,
downregulation of Cx40 or Cx43 with their respective antisense oligonucleotides did not consistently modify nitrite production in bEnd.3 (Supplementary Figure V). Overall, the data suggest that the interaction with Cx37 decreases the activity of eNOS in a specific way.

As bEnd.3 cells have been transformed with polyomavirus middle T antigen, they might harbor functional differences from native ECs. We therefore carried out experiments using human umbilical vein endothelial cells (HUVECs; passages 1 to 2). Similar to the mouse cell line, Cx37 immunoprecipitation of HUVECs showed a ≈140-kDa protein consistent with eNOS, which was absent in the control condition (Figure 5A). To assess the functional consequence of this interaction on eNOS, HUVECs were exposed to Cx37 antisense. Incubation with 50 μmol/L Cx37 antisense effectively repressed Cx37 expression (Figure 5B, bottom panel) compared with the same concentration of Cx37 sense (middle panel) or the absence of oligonucleotides (top panel). Similar to bEnd.3 cells, production of nitrite by HUVECs increased in response to Cx37 antisense exposure (Figure 5C). Constitutive nitrite production from HUVECs was 0.062±0.008 nmol/h per 10^5 cells (N=6). These data confirm that the interaction of Cx37 and eNOS is also present and functionally relevant in primary human ECs.

To assess the effect of the Cx37 P319S polymorphism on this interaction, we transfected the human EC line EA.hy926 with Cx37-319P or Cx37-319S. Cx37 antibody immunoprecipitates obtained from the lysates of ECs transfected with both isoforms of Cx37 contained a ≈140-kDa protein that was immunoreactive to eNOS antibodies (Figure 5D, second and fifth lanes). Control experiments using EA.hy926/empty vector transfectants, HeLa/Cx37-319P or HeLa/Cx37-319S transfectants showed the absence of an eNOS-immunoreactive protein in the precipitate (Figure 5D, first, third, and fourth lanes), further illustrating the specificity of this reaction.

Discussion

We have used phage display to identify peptides capable of binding to Cx37CT-319P and Cx37CT-319S. Our results identified 2 main consensus motifs, [K,R]XP and [K,R]XXP, in the peptidic sequences binding to Cx37CT. By comparing the sequences we obtained with the protein database, we found one particular peptide with homology to a region of the enzyme eNOS. Binding of this peptide, as well as of the corresponding sequence of eNOS to both isoforms of Cx37CT, was then assessed by various in vitro methods and in a cellular environment. In addition, functional consequences of the interaction were evaluated. Before discussing these results and their implications, we will address a few technical aspects of our study.

Technical Considerations

Phage display is a method that allows for high-throughput screening of binding sequences. However, it has limited sensitivity, and as such, it is possible to miss sequences of interest. Although the peptides found are capable of interacting with Cx37CT fragments, we likely failed to detect peptides of biological relevance that correspond to sequences of native Cx37 partners. It is also likely that some of the identified peptides lack biological relevance. In this study, we decided to focus our attention on one peptide (eNOS-like), as
it opened the possibility that these 2 proteins, Cx37 and eNOS, may interact in intact cells.

Surface plasmon resonance is a powerful technique to measure binding kinetics of a peptide to a protein. To detect a resonance shift, the target (in our case each isoform of Cx37CT) was covalently bound to a matrix. The consequence of this is that the configuration of Cx37CT might be modified and might not reflect the proper structural order of the protein when in solution (or in vivo). Moreover, SPR is limited in its ability to detect low-affinity interactions, particularly when these involve molecules of low mass. However, those limitations notwithstanding, SPR allowed us to characterize the interaction of eNOS-like to Cx37 and determine, comparatively, the interactions to the 2 Cx37 polymorphs. Cross-linking experiments are an alternative method to assess binding in vitro. They allow for studying the interaction of peptidic molecules in a soluble conformation. In our study, this method confirmed the results obtained by SPR, which indicate that both synthetic peptides, eNOS-like and the corresponding sequence of eNOS, interact with both isoforms of Cx37CT with comparable affinities. However, those experiments were performed with small peptides and therefore do not prove an interaction between Cx37 and the complete eNOS protein.

Binding Motifs

Based on the occurrences of the 2 motifs identified, we constructed a consensus motif for sequences binding to each isoform of Cx37CT. For Cx37CT-319P, the consensus motif would be WHK...[K,R]XP... and for Cx37CT-319S, FHK...[K,R]XXP.... Our results showed that the Cx37CT consensus binding motifs are rather similar to those previously identified for Cx43. This indicates that, though different in primary sequence, the two C-terminal domains may share higher order structures. The identification of the RXP motif led to the discovery of a peptide (RXP-E) that is able to interfere with the regulation of Cx43. Whether peptides known to affect the function of Cx43 could also affect the function of Cx37 (or vice versa) remains to be determined. However, eNOS-like and eNOS(843–854) did not bind to Cx43CT in vitro, suggesting a specific interaction with Cx37CT.

eNOS and Cx37CT

We have shown by 3 different methods that a peptide similar to a part of eNOS binds to Cx37CT. The homologous sequence extracted from amino acids 843 to 854 of eNOS bound to Cx37CT as well, even though with a lower affinity. These in vitro studies suggest a possible relationship between those 2 proteins in vivo. The synthesis of NO by eNOS in ECs is well known to play a vasoprotective role by different mechanisms. Thus, endothelium-derived NO controls vascular tone, inhibits leukocyte adhesion to the endothelium, inhibits platelet aggregation, and decreases endothelial permeability. Endothelium-derived NO also inhibits vascular smooth muscle cell migration and proliferation. Adequate levels of NO are thus important to preserve normal vascular physiology; diminished NO bioavailability can lead to endothelial dysfunction and increased susceptibility to atherosclerosis. Interestingly, Cx37 can also be considered a vasoprotective and antiatherosclerotic protein. Although the normal endothelium displays sizable Cx37 expression throughout the vascular tree, Cx37 expression is decreased in response to factors inducing endothelial dysfunction. Moreover, Cx37-deficient mice show enhanced susceptibility to atherosclerosis. The close association between Cx37 expression levels and eNOS activity led us to hypothesize that the 2 proteins might interact in ECs and affect each other’s function.

In agreement with previous studies, we have shown here that human Cx37 channels in N2a cells transit between multiple conductive states up to ~400 pS. As shown in Figure 3A and 3D, the frequency of high-conductance events (>300 pS) in the presence of a scrambled peptide was higher for Cx37-319P channels (33%) compared with Cx37-319S channels (14%). In analogy to Cx43, this may reflect differences in posttranslational modification, such as phosphorylation, between the 2 polymorphic proteins. Cx37CT contains indeed multiple consensus sequences for phosphorylation by various protein kinases, including the serine at position 319. We have also shown that the frequency of high-conductance events was increased in the presence of eNOS-like (Figure 3B and 3E) or eNOS(843–854) (Figure 3C and 3F), suggesting that eNOS-Cx37 interaction could...
modify Cx37 channel properties. In addition, it has recently been shown that exposure to an NO donor may reduce the permeability of Cx37 gap junctions to small molecules. The regulation of Cx37 gap junctions by eNOS could therefore implicate several pathways, either via a direct protein-protein interaction or via its product NO.

Consistent with our in vitro binding experiments (SPR and cross-linking), the two peptides induced similar modifications of channel properties for either polymorphic protein. This suggests that the interaction of Cx37CT and eNOS does not involve the region modified by the P319S polymorphism.

The mouse EC line bEnd.3, known to constitutively express Cx37, allowed for studying the interaction of eNOS with Cx37 in a cellular environment. Our studies revealed that eNOS coimmunoprecipitated with Cx37 in these cells. Because the use of polyomavirus middle T antigen transformation to immortalize the bEnd.3 cell line might result in aberrant protein regulation and intracellular localization, and hence in artificial coimmunoprecipitation results, another, more physiological model was needed to confirm these results. Primary ECs, such as HUVECs, are usually avoided for this kind of study because the expression of Cx37 progressively decreases on repeated passage with virtually no remaining protein at passage 3 to 4. To overcome this limitation, we restricted the use of primary cells to HUVECs at very early passages (passage 1 or 2) for our study. As in the mouse cell line, eNOS coimmunoprecipitated with Cx37 in these primary ECs, which strengthens the relevance of this newly identified interaction. The next logical step is to clarify whether the Cx37 polymorphism affects this interaction in human cells. As it is ethically problematic to genotype cells isolated from umbilical cords, we transfected the human EC line EA.hy926 with both Cx37 polymorphic proteins. The coimmunoprecipitation of eNOS with both isoforms of Cx37 confirmed the absence of functional effect of this polymorphism on the interaction of Cx37 with eNOS.

Our functional studies demonstrated that decreasing Cx37 expression enhanced production of nitrite, the end-product of NO, in bEnd.3 cells as well as HUVECs, and that this increase was not caused by modifications in eNOS or Cav1 expression and localization. Moreover, an indirect effect of Cx37 on eNOS via other endothelial connexins could be excluded, suggesting that the eNOS-Cx37 interaction could directly modify enzyme activity. Structural studies have revealed that eNOS is a multidomain enzyme consisting of an N-terminal oxygenase domain (amino acids 1 to 492), and a reductase domain (amino acids 493 to 1205). During NO synthesis, NADPH-derived electrons pass into the reductase domain flavins (flavin mononucleotide and flavin-adenine dinucleotide) and are then transferred to the heme located in the oxygenase domain that catalyzes stepwise NO synthesis from L-arginine. Calmodulin binding is known to activate NO synthesis by enabling the reductase domain to transfer electrons. The 843 to 854 binding motif resides in the eNOS reductase domain, a part that also contains a Cav1 binding domain. Although the possible modes of Cx37-based inhibition remain to be explored, one could imagine that, similar to Cav1, Cx37 binding to eNOS reductase compromises its ability to bind calmodulin, thereby inhibiting NO synthesis.

Even though other connexins are known to interact with caveolins and Cx37 was demonstrated to be expressed in caveolae-like vesicles in aortic ECs, Cx37 did not colocalize with Cav1 in the bEnd.3 cells used for our studies.

As mentioned above, both Cx37 and eNOS play a vasoprotective role, and thus, the observation that downregulation of Cx37 leads to an increased NO production was unexpected. Several factors may contribute to this apparent discrepancy. For instance, the present study has been performed in vitro and thus under static conditions. A disturbed or absent flow is known to promote endothelial dysfunction and to affect NO synthesis. Therefore, the situation may be quite different in vivo, where an absolutely static condition is almost never present. It is also known that proatherogenic factors, such as hypercholesterolemia and hypertension, can cause eNOS uncoupling, a deleterious condition leading to increased reactive oxygen species production. It is conceivable that in vivo, the inhibitory property of Cx37 on eNOS may mostly affect reactive oxygen species production under pathological conditions rather than NO synthesis in more physiological states.

In summary, the data presented here demonstrate for the first time an interaction between Cx37 and eNOS in ECs. However, this study could not detect a difference between the interactions of each isoform of Cx37CT with the peptides corresponding to eNOS. This suggests that the interaction of Cx37CT and eNOS does not involve the region modified by the P319S polymorphism. It also indicates that the epidemiological correlation between the Cx37 polymorphism and atherosclerosis likely does not result from a modification in eNOS function. Nevertheless, this newly identified interaction may lead to new insights in vascular physiology and pathologies, such as hypertension.

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Disclosures
None.

References


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Expanded Methods

Production and purification of recombinant Cx37CT

Recombinant Cx37CT was produced following methods previously described\(^1\). Briefly, cDNA derived from human Cx37-319P was inserted into pGEX-6P-2 plasmids (Amersham) and expressed in BL-21 competent cells. The resultant GST-fusion protein was cleaved from the GST by PreScission Protease® (Amersham Biosciences). The recombinant product after cleavage from GST contained the sequence 233-333 of human Cx37. Protein concentration was measured using the Bio-Rad DC Protein Assay. Protein purity was assessed by SDS-PAGE and Coomassie staining.

The Cx37CT-319S clone was produced using the QuikChange Site-Directed Mutagenesis Kit® (Stratagene) on the Cx37CT-319P pGEX-6P-2 plasmid. The following primers were used forward, GAA TGG CCA AAA ATC CCC AAG TCG TC and reverse, GAC GAC TTG GGG ATT TTT GGC CAT TC.

Mouse Cx40CT and rat Cx43CT were produced as previously described\(^2\).

Phage display

Phage display is a high-throughput method for identification of molecules binding to a ligand of interest. Here, we applied it to the detection of potential ligates of the CT domain of Cx37. The protocol was similar to that previously described\(^2\). Briefly, recombinant Cx37CT-319P, or recombinant Cx37CT-319S were used as baits. A well of a 24-well plate was coated with 15µg of the pertinent recombinant protein. The well was treated with blocking buffer (0.1mol/ L NaHCO\(_3\) (pH 8.6), 5mg/ml BSA, 0.02% NaN\(_3\)) for 1 hour. A phage library consisting of
2.7x10⁹ different M13KE bacteriophages displaying a random 12-mer peptide in their minor coat protein (Ph.D.-12™ Phage display peptide library kit; New England BioLabs Inc.) was first pre-cleared in an uncoated well, and then presented to the bait protein. Low-affinity binders were first eluted using a 100µg/ml solution of free Cx37CT in TBS. The well was overlaid with a culture of *E. coli* ER2738, which was then amplified for 4.5 hours. The amplified phages were precipitated with PEG-NaCl. The extracted phages were subsequently used for a new round of panning. After four rounds, the phages were grown on a lawn of *E. coli* ER2738 for plaque purification. A total of 120 plaques, each one representing a single clone, were amplified for 4.5 hours. Phages were precipitated with PEG/NaCl and their ssDNAs were extracted and sequenced. The sequences were analyzed using the ExPasy translate tool. Homology to human proteins was determined using the NCBI protein-protein BLAST (Basic Local Alignment Search Tool). Proteins to be further investigated as potential binding partners for Cx37 were selected according to expression pattern and pathophysiological relevance.

**Motif analysis**

The significance of particular motifs in the retrieved phage display sequences was assessed by comparing their actual frequency to their theoretical occurrence based on the experimental ratio of each amino acid. If for example the frequency of K in the display sequences is 10.2%, the frequency of R is 8.9% and the frequency of P is 16.1%, then the probability of finding a [K,R]XP motif in a 12-mer would be

\[
p([K,R]XP) = 10 \times (p(K) + p(R)) \times p(P) = 30.8\%
\]

even without a selection for this motif. These probabilities were then compared to the obtained data.
**Surface plasmon resonance**

Surface plasmon resonance (SPR) is a spectroscopic method to determine the amplitude and kinetics of binding between two molecules in real time\(^3,4\). Recombinant Cx37CT-319P, Cx37CT-319S, Cx40CT or Cx43CT were covalently bound to a carboxymethyl dextran matrix (Sensor Chip CM5, Biacore), and synthetic peptides were presented to assess binding. All experiments were performed at pH 7.4. Dissociation constants (K_D) were calculated from the recorded association and dissociation phases of the peptide-protein interaction, using a 1:1 Langmuir association and dissociation kinetic model (Biacore software package). In both phases (association and dissociation), the first 8 seconds of recording were not included in the fit to avoid artifacts resulting from peptide distribution within the flow cells\(^4\).

**Cross-linking experiments**

Cross-linking reagents enable the formation of covalent bonds between molecules in close proximity. A total of 0.25mmol/L of recombinant Cx37CT and 0.5mmol/L of a peptide were incubated for 1 hour at room temperature with 1mM of the cross-linker reagent BS\(^3\) at pH 7.4\(^5\). The reaction was subsequently blocked with 100mmol/L ethanolamine for 10 min. The samples were separated by SDS-PAGE (4-20%) and stained with Coomassie-Blue.

**Cell culture**

Three different cell lines were used: a) a communication-incompetent line of murine neuroblastoma cells (N2a; American Type Culture Collection; Manassas, VA), b) a mouse endothelial cell line (bEnd.3)\(^6\) which endogenously expresses all three endothelial connexins\(^7\), and c) a human endothelial cell line (EA.hy926; ATCC) which has no detectable endogenous expression of Cx37. Cells were grown in DMEM (Gibco, Invitrogen, Grand Island, NY) and
supplemented with 10% fetal bovine serum, 5mg/L L-glutamine, 5000U/L penicillin and 5mg/ml streptomycin (Mediatech, Herndon, VA).

For the isolation and culture of primary endothelial cells, human umbilical cords were obtained after approval by the institutional ethics committee of the University Hospital of Geneva, in accordance with the Helsinki declaration. Written consent was obtained from a parent of each cord donor. Endothelial cells (HUVEC) were isolated from umbilical cord veins as described previously. Briefly, the veins were rinsed with RPMI 1640 (Gibco BRL-Life Technologies) supplemented with 5000U/L penicillin and 5mg/ml streptomycin, and perfused for 10 min at 37°C with 1mg/ml collagenase (Sigma Chemical or CLS type 1 Worthington Biochemical). Cells were collected by flushing the vein with 50 ml of RPMI 1640. After centrifugation (5 min, 1000 rpm), cells were cultured in EGM-2 medium (Lonza, Walkersville, MD) at 37°C in a humidified atmosphere containing 5% CO2. EGM-2 medium includes 2% fetal calf serum (FCS), VEGF, IGF, FGF-B, EGF, ascorbic acid, gentamycin and heparin. Cells were passaged by trypsin–EDTA (Gibco BRL-Life Technologies or Seromed Biochrom KG)) treatment and used at passages 1–2.

cDNA preparation and transfection into cells

GJA4-1019T and GJA4-1019C cDNAs were subcloned into the pIRES2-eGFP mammalian expression vector (Clontech) using EcoRI and BamHI restriction sites. These vectors were separately transformed into DH5α competent cells (Invitrogen) and selected as positive kanamycin-resistant clones, according to the manufacturer's protocol. For transfection of EA.hy926 cells, 5-10µg of plasmid DNA were electroporated into 5×10⁵ cells using a Gene Pulser II device (Bio-Rad) at 220V and 975µF capacitance. After 5 min on ice, cells were transferred to a six-well tissue-culture plate. Selection was initiated in 350µg/ml G418 (Invitrogen) after 24 hours of recovery time. After 14-21 days, cells were selected by
expression of eGFP using flow cytometry (FACSVantage; Becton Dickinson); Cx37 expression was verified by immunostaining. Transiently transfected cells were used within one month after starting G418 selection. In the case of N2a cell transfection, cells were grown to 35-40% confluence and transiently transfected with the above-mentioned plasmids. Transfections were carried out using 0.5µg of plasmid DNA and Effectene (Qiagen, CA) according to the manufacturer’s instructions. Transfection efficiency ranged between 70 and 80%.

**Patch clamp recording and analysis**

Electrophysiological recordings were performed 24-48 hours post-transfection. eGFP positive (green) pairs of cells were identified by illumination under monochromatic light (495nm) and emitted fluorescence filtered at 520nm. Electrophysiological recordings were then made using the dual whole-cell patch-clamp technique. Patch electrodes had resistances of 5MΩ and were filled with (in mmol/L) 130 CsCl, 0.5 CaCl₂, 10 HEPES, 10 EGTA, pH 7.2. In some recordings eNOS-like peptide (WHRTPLPPPVP), eNOS(843-854) peptide (WVRDPRLPPPCTL) or scrambled peptide (PDPWPVLTCRLR) was added to the pipette solution at a concentration of 0.1mmol/L. The external solution was (in mmol/L) 160 NaCl, 10 CsCl, 10 CaCl₂, 0.6 MgCl₂, 10 HEPES, pH 7.4. All experiments were carried out at room temperature.

Single channel recordings were obtained from transfected N2A cell pairs. For initial average Gj values larger than 1.2 nS, cell pairs were exposed to brief periods of octanol superfusion (2-4mmol/L) to resolve single channel events. Junctional current traces were acquired during repetitive pulses of 20s to Vj=30mV using pClamp software (version 10.0, Axon instruments, CA). The holding potential was -40mV. Signals were filtered at 200 Hz and sampled at 2 kHz. Single channels events lasting more than 50ms were considered for
further analysis. Gaussian distribution best fits were constructed for all-events histograms using Origin software. (Version 7.0, Microcal, MA).

Co-immunoprecipitation studies and Western blots
Confluent cultures of bEnd.3 cells, transfected EA.hy926 cells or HUVECs were rinsed in phosphate-buffered saline (PBS), pH 7.4, and lysed in 500µl RIPA buffer (50mmol/L Tris-HCl, pH 7.4, 0.25mmol/L sodium-deoxycholate, 150mmol/L NaCl, 2mmol/L EGTA, 0.1mmol/L Na$_3$VO$_4$, 10mmol/L NaF, 1mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, Complete protease inhibitor (Roche Applied Science). Lysates were gently mixed at 4°C for 20 min, and then spun at 12,000rpm for 20 min.

For Western blots, equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane. After 2-hour blocking with 5% milk, the membrane was exposed to various primary antibodies (anti-Cx40 (Santa Cruz); anti-Cx43, anti-eNOS and anti-Cav1 (BD Sciences); anti-iNOS (Upstate Biotechnology) anti-GAPDH (Chemicon)). The appropriate secondary horseradish peroxidase-conjugated antibodies were added, followed by ECL detection (Millipore). For iNOS detection in bEnd.3 cells, a five-fold smaller quantity of proteins extracted from mouse macrophages stimulated with IFNγ/LPS (BD Sciences) was used as a positive control.

For co-immunoprecipitation studies, 50µl of Dynabeads Protein G (Invitrogen) was rinsed twice with 200µl of RIPA buffer and then separated on a magnet for 1 min (DynMag-Spin, Invitrogen). A 250µl volume of bEnd3 or transfected EA.hy926 cell lysate or a 1ml volume of HUVEC cell lysate was pre-cleared 2 hours at 4°C with the rinsed beads in gentle rotation separated on a magnet. Following the preclearing step, 7.5µg of Cx37 antibody (Cx37A11-A, ADI) or eNOS antibody (BD Sciences) were added to half of the protein lysate, and incubated using gentle rotation for 4 hours at 4°C. Thereafter, 50µl of Dynabeads Protein G were added,
and the samples were incubated overnight at 4°C. Negative controls included omission of antibodies from the procedure. The following day, the samples were separated on a magnet and the supernatants were discarded. The beads were rinsed 6 times in RIPA buffer, incubated in 40µl of loading buffer at 95°C for 10 min, and then placed immediately on ice for 2 min. The samples were then separated on a magnet and the loading buffer removed from the beads for running on Western blots using 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane, and eNOS or Cx37 were detected with the appropriate antibodies.

**Immunofluorescence**

bEnd.3 cells or HUVECs were grown to confluence onto gelatine–coated coverslips, fixed in ice-cold methanol for 10 min at -20°C, and immunolabeled with Cx37 alone (ADI) or with Cx37 and eNOS (BD Sciences) antibodies, as described previously. Alternatively, cells were fixed in ice-cold acetone for 5 min at -20°C, and immunolabeled with Cav1 alone (BD) or with Cav1 and Cx37 antibodies. Slides were mounted with Vectashield mounting medium (Vector laboratories, Burlingame, CA) and examined with a TMD300 microscope (Nikon AG, Küsnacht, Switzerland) equipped with a high-sensitivity CCD Visicam camera (Visitron systems GmbH, Puchheim, Germany) connected to a personal computer. Images were captured using the software Metaflour 4.01 (Universal Imaging Corp., Downington, PA). Alternatively, slides were examined with a LSM 510 Meta confocal microscope (Zeiss) connected to a personal computer, and images were captured using the software provided by the manufacturer. All images were processed using Adobe Photoshop.

**NO release**

NO release assays were performed using 96-well plates (Falcon). Ten thousand bEnd.3 cells or 7000 HUVECs were plated per well and grown to confluence for 48 hours in the presence
of 10, 25 or 50 µmol/L Cx37 anti-sense oligonucleotides (mCx37AS: 5’-GTCCCTTCGTGCCCTCTATCTC-3’; hCx37AS: 5’-GAAGCCCCAGTCACCCATGGC-3’), Cx40 anti-sense oligonucleotide (mCx40AS: 5’-CTCCCCCAGGAAGCTCCAGTCACCCAT-3’) or Cx43 anti-sense oligonucleotide (mCx43AS: 5’-GTAATTGCGGGCAGGAGGAATTGTTTCTGTC-3’). Cx37 sense oligonucleotides (mCx37S: 5’-TGCTAGACCAGGTCCAGGAAC-3’; hCx37S: 5’-GCCATGGGTGACTGGGGCTTC-3’), Cx40 scrambled oligonucleotide (mCx40S: 5’-ATACTGTATCATGCCATCCCAGCTTC-3’) and Cx43 sense oligonucleotide (mCx43S: 5’-GACAGAAGAACATTCTCCTGGCAGTAC-3’) were used as controls. Total nitrite and nitrate release to culture supernatant was measured during the final 16 hours using the Total Nitric Oxide Assay Kit (Assay Designs) according to the manufacturers’ instructions. NO release was assayed in duplicate.

Statistics

Results are presented as mean ± SEM. Unpaired t-test was used to compare differences between two groups and ANOVA for comparison of multiple groups. Data were considered statistically significant at P<0.05.

References


Supplementary Table I. Phage display peptidic sequences

<table>
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<th>Peptidic sequence</th>
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* eNOS-like
Red: [K,R]XP motif
Blue: [K,R]XXP motif
Purple: overlapping motifs
Numbers represent occurrence of peptidic sequence in each display.
## Supplementary Table II. Frequency of acidic and basic residues in phage display sequences

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Supplementary Table III. Frequency of [K,R]XP motif in Cx37CT-319P and Cx37CT-319S displays versus theoretical occurrence

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Supplementary Table IV. Frequency of [K,R]XXP motif in Cx37CT-319P and Cx37CT-319S displays versus theoretical occurrence

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**Figure legends**

**Supplementary Figure I:** SPR on Cx37CT and scrambled peptide

Cx37CT-319P (panel A) or Cx37CT-319S (panel B) were fixed on a carboxymethyl dextran matrix, which was superfused with a solution containing 500$\mu$mol/L of scrambled peptide and binding was assessed in real time. Upwards arrow: beginning of superfusion of the peptide. Downwards arrow: washout.

**Supplementary Figure II:** SPR on Cx40CT and Cx43CT

Cx40CT (A and B) and Cx43CT (C and D) were fixed on a carboxymethyl dextran matrix, which were superfused with a solution containing eNOS-like (A and C) or eNOS(843-854) peptides (B and D). Peptides were superfused at concentrations of 1mmol/L (black), 500$\mu$mol/L (red), 250$\mu$mol/L (green), 125$\mu$mol/L (blue) and 62.5$\mu$mol/L (light blue).

**Supplementary Figure III:** Single channel traces from N2a cells transfected with Cx37-319P and Cx37-319S

Single channel traces with the corresponding all points histograms obtained from cells transfected with Cx37-319P (top) or Cx37-319S (bottom) and exposed to scrambled (left), eNOS-like (middle) or eNOS(843-854) peptides (middle). All-events histograms for the same conditions are presented in Figure 3. Panel A (Cx37-319P channels exposed to scrambled peptide) is representative of 4 cell pairs, and a total of 211 events. Average junctional conductance (Gj) values for each pair at the onset of recording was 1.01nS, 0.93nS, 1.15nS and 0.30nS. Panel B (Cx37-319P channels; eNOS-like peptide) was obtained from 5 cell pairs (447 events in total). Macroscopic Gj values were 24.86nS, 10.47nS, 0.81nS, 0.28nS and
For panel C (Cx37-319P channels exposed to eNOS(843-854) peptide), 462 events were recorded from 4 cell pairs with initial average Gj values of 19.39nS, 13.74nS, 0.47nS and 0.65nS. Panel D (Cx37-319S recorded in the presence of scrambled peptide) shows an example among 286 events acquired from 5 different cell pairs with initial Gj values of 1.37nS, 0.97nS, 0.76nS, 0.34nS and 0.34nS. Panel E (Cx37-319S channels exposed to eNOS-like) depicts a single channel trace among 205 events recorded from 4 cell pairs. Initial average Gj values were 17.3nS, 0.47nS, 0.14nS and 0.29nS. Finally, data in panel F (Cx37-319S; eNOS(843-854) peptide) were obtained from 3 cell pairs (276 events) with initial Gj values of 8.3nS, 22.4nS and 0.51nS. Channels were acquired for a total of 200 seconds for each cell pair for all conditions tested. For the measurements reported in this Figure, Gj was calculated from the transjunctional current measured during a voltage step. Given the time-dependent changes in the amplitude of the current, all points acquired during the last 15 seconds after the onset of the voltage pulse were averaged and the average value was divided by the transjunctional voltage. In some cells, only one or two channels were active during the voltage pulse. Consequently, average Gj values reflect the combination of unitary conductance (when the channel was open) and baseline (when the channel was closed). Minimum dwell time for a transitory current state to be counted as an event was 50 msec. To avoid duplication, transitions were counted only in the direction of current amplitude increase. That is, from either closed or “residual” to a higher conductance state. Accordingly, for the traces in panel A, four transitions were counted as events: one from closed to open, one from residual to open and two from closed to residual. For panel B: One from closed to open, and two from residual to open. For panel C: one from closed to open, and two from residual to open. For panel D, one from closed to open, two from residual to
open and one from closed to residual. For panel E, one from closed to open, and three from residual to open and for panel F, one from closed to open and four from residual to open.

**Supplementary Figure IV: eNOS and Cav1 localization**

A: Cx37 (green) and Cav1 (red) immunostaining in bEnd3. Cav1 was found almost exclusively perinuclearly, whereas Cx37 was mainly detected at sites of cell-cell contact. Merged images were unable to detect any colocalization (yellow). B: eNOS (top panels) and Cav1 (bottom panels) immunostainings in bEnd3 in control conditions (left) or incubated with 50uM Cx37 sense (middle) or antisense (right). Bars represent 20µm.

**Supplementary Figure V: Effect of Cx40 and Cx43 downregulation on eNOS function**

A: NO production (measured by total nitrite production) by bEnd.3 cells incubated with Cx40 sense or 10, 20 or 50µmol/L Cx40 anti-sense for 48 hours. Overnight nitrite production was not affected by exposure to anti-sense. N=3-5. B: NO production by bEnd.3 cells incubated with Cx43 sense or 10, 25, 50 or 100µmol/L Cx43 anti-sense for 48 hours. Overnight nitrate production was significantly reduced after exposure to 25µmol/L Cx43 anti-sense, but not after exposure to lower or higher concentrations. N=6-9. Error bars show SEM and * P<0.05.
Supplementary Figure I

A. Cx37CT-319P

B. Cx37CT-319S
Supplementary Figure II
Supplementary Figure III

A: Cx37-319P (scrambled)
- O: 330 pS
- R: 101 pS
- C: 330 pS

B: eNOS-like
- O: 285 pS
- R: 83 pS
- C: 98 pS

C: eNOS(843-854)
- O: 300 pS
- R: 98 pS
- C: 72 pS

D: Cx37-319S
- O: 248 pS
- R: 93 pS
- C: 306 pS

E: Cx37-319S (scrambled)
- O: 333 pS
- R: 80 pS
- C: 72 pS

F: Cx37-319S (eNOS-like)
- O: 306 pS
- R: 72 pS
- C: 306 pS
A

![Supplementary Figure IV](image)

B

![Supplementary Figure IV](image)
Supplementary Figure

A  Cx40 antisense

B  Cx43 antisense