The N Terminus of Connexin37 Contains an α-Helix That Is Required for Channel Function*

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The cytoplasmic N-terminal domain of connexins has been implicated in multiple aspects of gap junction function, including connexin trafficking/assembly and channel gating. A synthetic peptide corresponding to the first 23 amino acids of human connexin37 was prepared, and circular dichroism and nuclear magnetic resonance studies showed that this N-terminal peptide was predominantly α-helical between glycine 5 and glutamate 16. The importance of this structure for localization of the protein at appositional membranes and channel function was tested by expression of site-directed mutants of connexin37 in which amino acids leucine 10 and glutamine 15 were replaced with prolines or alanines. Wild type connexin37 and both substitution mutants localized to appositional membranes between transfected HeLa cells. The proline mutant did not allow intercellular transfer of microinjected neurobiotin; the alanine mutant allowed transfer, but less extensively than wild type connexin37. When expressed alone in Xenopus oocytes, wild type connexin37 produced hemichannel currents, but neither of the double substitution mutants produced detectable currents. The proline mutant (but not the alanine mutant) inhibited co-expressed wild type connexin37. Taken together, our data suggest that the α-helical structure of the connexin37 N terminus may be dispensable for protein localization, but it is required for channel and hemichannel function.

Gap junction channels allow intercellular passage of ions and small molecules up to 1000 Da. They are oligomeric assemblies of members of a family of related proteins called connexins (CX) (reviewed in Ref. 1). Six connexin monomers assemble to form a hemichannel or connexon (Fig. 1, top panel), which, in turn, forms a complete gap junction channel by docking with a hemichannel from an adjacent cell. Based on sequence similarities, connexins have been separated into subfamilies designated by Greek characters (2, 3). The majority of connexins are members of the α- and β-subfamilies. Connexin polypeptides span the plasma membrane four times and have three cytoplasmic regions: the N terminus (NT), a cytoplasmic loop between the second and third transmembrane domains, and the C terminus (Fig. 1, middle panel). Structural studies of gap junctions have revealed that each hemichannel contains a ring of 24 transmembrane spanning helices (4, 5). Most topological models suggest that the NT of α-subfamily connexins contains 23 amino acids (illustrated for connexin37, CX37, in Fig. 1, bottom panel) and that of β-subfamily connexins contains 22 amino acids.

The importance of the connexin NT has been emphasized by the identification of a number of connexin mutants that cause amino acid substitutions within this region and are linked to diseases including sensorineural deafness (CX26, CX30, and CX31), Charcot-Marie-Tooth disease (CX32), oculodentodigital dysplasia (CX43), and congenital cataracts (CX46 and CX50). Among the disease-linked mutants that have been studied, some show impaired protein trafficking to the cell surface, whereas others traffic properly, but show loss or alterations of channel function (6–16). Heterologous expression of site-directed mutants and chimeric connexins has demonstrated the influence of NT amino acids upon channel properties, including transjunctional voltage (VT)-dependent gating, unitary conductance, permeability, and sensitivity to regulation by polyamines (17–22). Lagree et al. (23) have provided evidence that the NT influences the compatibility of connexin hetero-oligomerization.

The structure of the NT domain of a β-group connexin, CX26, has been investigated through circular dichroism (CD) and nuclear magnetic resonance (NMR) of a synthetic peptide corresponding to part of the predicted CX26NT (24, 25). Based on their data, Purnick et al. (24) proposed a model for the NT of CX26 with an α-helix extending from position 1 to 10 and a critical bend at positions 11 and 12 that was suggested to act as a "hinge" allowing the first 10 amino acids to swing into the pore and block the channel. Oshima et al. (5) have published structural studies of a “permeability” mutant (M34A) of CX26 (26) showing a density within the pore of the channel that they suggested might represent a bundle of N termini acting as a “plug” to close the channel.

We have been studying CX37, an α-group connexin that is expressed in endothelial cells (27), which may be important for development of atherosclerotic disease (28) and that can form large conductance channels and hemichannels (27, 29). We
have shown that as much as half the length of the CX37NT can be deleted without affecting formation of gap junction plaques, but a full-length N terminus is required for hemichannel gating and intercellular communication (30). These observations suggested that the CX37NT may have a structure that is required for function. Therefore, the present experiments were designed to determine the structure of the NT of CX37 and the importance of that structure for protein localization and formation of functional channels and hemichannels. Differences between our data and those previously reported in studies of CX26 suggest that the structure of the NT in α-group connexins may differ from that in β-group connexins.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were obtained from Sigma unless otherwise specified.

**Synthetic Peptides**—A peptide containing the first 23 amino acids of human CX37 (Fig. 1) was synthesized and purified by reverse phase HPLC in the peptide synthesis and protein chemistry cores of the University of Chicago Biological Sciences Division. Additional wild type peptide and peptides containing substitutions of alanines (AA) or prolines (PP) for leucine 10 and glutamine 15 (MGDWGFLKALDQVAEHSTVVGK and MGDGWFLEKPLDQVEHSTVVGK) were synthesized by GeneScript Corp. (Piscataway, NJ); the purities of these peptides were 95.6% (wild type), 97.1% (AA), and 99.6% (PP) as assessed by HPLC. Secondary structures of synthetic peptides were predicted using the GOR IV (31) and SOPMA (32) methods.

**Circular Dichroism (CD)**—Circular dichroism measurements were made using an Aviv model 202 circular dichroism spectrometer. The 23-amino acid wild type and mutant CX37 peptides were prepared at a concentration of 1 mM in 50 mM potassium phosphate buffer (pH 6.5) containing 100 mM KCl. This concentration of peptide was used to match the concentration necessary for NMR experiments. Initial CD spectra of the wild type peptide were acquired using a quartz sandwich cell with a path length of 0.1 mm at 25 °C between 190 and 280 nm in 1-nm increments, using a 1.00-nm bandwidth, a 5.0-s averaging time, 1.0-s settling time, and a 0.2-s delay between each of the scans used for signal averaging. Ellipticity measurements at 222 nm were taken from 1 through 75 °C in 2 °C increments to assess cooperativity in melting behavior. Additional CD spectra of all three peptides were obtained similarly, except for the use of a cell with a path length of 1.0 mm and a settling time of 3 s. The percentage composition of different secondary structures was calculated using the CDSSTR algorithm in the Olis GlobalWorks software package (Olis, Inc., Bogart, GA) (33) and reference data set SP43 (which includes data for 43 soluble proteins).

**NMR Spectroscopy**—NMR spectra were assigned using a combination of total correlation spectroscopy (TOCSY, 15- and 60-ms mixing times) (34–36), nuclear Overhauser enhancement spectroscopy (NOESY, 150 and 300 ms mixing times) (36, 37), constant time-correlation spectroscopy (CT-COSY) (38), and heteronuclear single quantum coherence spectroscopy (1H-15N HSQC, natural abundance) (36, 39) spectra. Data were acquired on a Varian Inova 600 MHz system with a standard H/C/N probe with z-gradients. Per standard analysis, spin systems were identified with TOCSY, CT-COSY, and HSQC spectra, and sequential assignments were accomplished using NOESY spectra. Chemical shift index values (CSI), which indicate secondary structure based on comparing chemical shifts to those expected for random coil peptides (38), were calculated for 1H α signals and were used to guide molecular modeling (45).

**Molecular Modeling**—To obtain atomic coordinates for models of CX37NT, we performed simulated annealing with NMR-derived restraints using the program CNS (40). We performed 50 rounds of heating, molecular dynamics, slow cooling, and minimization using default parameters. As is common for many peptides, we did not have sufficient data to determine a traditional high resolution NMR structure. The lack of a large globular structure obviates the possibility of detecting large numbers of NOE contacts for every residue, so models were loosely restrained using the data available. The CD data, CSI patterns, and inter-residue NOEs strongly suggested α-helical structure, so loose helical dihedral angle restraints were imposed during modeling. For residues 5 through 22, φ angles were allowed to range freely between −62 to +30°, and were subject to energy penalties outside this range. Force constants were set to 1.0 kcal/mol rad2 for residues 5 through 15, and 0.05 kcal/mol rad2 for residues 16 through 22 to reflect the more limited data associated with the structure of the C-terminal portion of the peptide. Likewise, ψ angles were allowed to range freely between −41° to +30°, and subject to energy penalties outside this range; force constants were set to 1.0 kcal/mol rad2 for residues 5 through 15, and 0.05 kcal/mol rad2 for residues 16 through 22. In all, 36 φ and ψ restraints were used. Distance restraints based on NOEs were imposed on intraresidue and sequential interresidue amide and α hydrogen atoms from residues 2 through 9, 12 through 16, and 18 through 23; atoms were allowed to range from 1.8 to 6.0 Å without penalty. Thirty five inter-residue and 20 intra-residue NOE restraints were used. Hydrogen bond distance restraints were imposed between residue pairs 4/8, through 12/16, in accordance with the NOEs, CSI data, and CD data suggesting α-helical structure. Nine O…N and 9 O…H restraints were used for hydrogen bonds. Final structures were visualized with the program PyMol (Delano Scientific LLC, Palo Alto, CA).

**Generation of GFP-tagged CX37 Substitution Mutants**—The DNA sequence encoding a fusion protein containing the coding region of human CX37 with a C-terminal tag corresponding to the Emerald variant (Invitrogen) of green fluorescent protein (GFP) was previously produced and subcloned into pBSSK-XG (where it is bracketed by the 5′- and 3′-untranslated regions of the Xenopus globin gene) for oocyte expression and into pcDNA3.1/Hygro(+) (Invitrogen) for expression in mammalian cells (30).

Substitution mutants were obtained by PCR using the wild type CX37-GFP construct in both plasmids as templates and the Phusion High Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA). Primers facing opposite directions and incorporating the desired changes at amino acid positions 10 or 15 were designed to amplify the sequence of the full constructs (including the vector sequences); circular plasmids were gener-
ated by ligation of the PCR products (30, 41). The coding region of all constructs was fully sequenced at the Cancer Research Center DNA Sequencing Facility of the University of Chicago to ensure that PCR amplification did not introduce additional mutations.

**Cell Culture and Transfections**—All cell culture media and supplements were obtained from Invitrogen unless otherwise noted. HeLa cells were grown in minimal essential medium supplemented with 0.1 mM non-essential amino acids, 10% fetal bovine serum (U. S. Bio-Technologies Inc., Pottstown, PA), 2 mM glutamine, 10 units/ml penicillin G, and 10 μg/ml streptomycin sulfate. Transient transfections were carried out as described previously (30) using Lipofectin Transfection Reagent and PLUS Reagent following the manufacturer's instructions; transfection efficiencies were ~50% for all constructs.

**Fluorescence Microscopy**—Transfected cells cultured on 4-well chamber slides (LAB TEK, Nalge Nunc International, Naperville, IL) were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 30 min at room temperature. After rinsing cells with phosphate-buffered saline, coverslips were mounted using 2% n-propyl gallate in phosphate-buffered saline/glycerol (1:1).

Specimens were observed using a Zeiss Plan Apochromat ×40 objective in an Axioscop 2 microscope (Carl Zeiss, München, Germany). Images were captured with an AxioCam digital camera (Carl Zeiss) using ZSS AxioVision software. Composite figures were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA).

**Detergent Solubility**—Solubility of expressed connexins in 1% Triton X-100 was examined essentially according to the technique described by Musil and Goodenough (42), which we have previously used to study CX37 (43).

**Microinjection of Gap Junction Tracers**—Cells were cultured on glass coverslips. When they reached 80–90% confluence, the cells were transferred to F-12 medium buffered with 15 mM HEPES, pH 7.6, and 4% neurobiotin (Vector Laboratories, Burlingame, CA) in water for 3 min using a picospritzer (model PLI-188, Nikon Instruments Inc., Melville, NY). After microinjection, cells were fixed in 4% paraformaldehyde for 30 min and then permeabilized with methanol/acetone (1:1) for 2 min at room temperature. The neurobiotin tracer was detected by incubating the cells with Cy3-streptavidin conjugate (Sigma) for 30 min at room temperature. CX37 channels do not allow transfer of Lucifer yellow, which has molecular mass of 457 Da and a charge of –2 (30, 44); however, co-injection of this tracer facilitated identification of microinjected cells to score for the presence or absence of neurobiotin transfer. The extent of intercellular transfer of neurobiotin was determined by counting the number of adjacent cells containing the tracer. Results are reported in the text as mean ± S.E. Statistical analysis was performed using one-way analysis of variance with Tukey's test and Bonferroni's method at the 99% confidence level. Both Tukey and Bonferroni methods gave similar significance results.

**Xenopus Oocyte Expression**—Preparation of transcripts, Xenopus oocytes, and electrophysiology methods routinely used in our laboratory have been described previously (29, 30). Briefly, pBSK-XG plasmids containing DNA encoding wild type and mutant CX37 were linearized with XbaI and transcribed using the T7 mMessage mMachine Kit (Applied Biosystems/Ambron, Austin, TX).

Stage V and VI oocytes were harvested from adult female Xenopus laevis under tricaine anesthesia, treated with collagenase (2 mg/ml for about 90 min) in 0 Ca2+/OR2 (90 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, and 5 mM HEPES, pH 7.6), and manually defolliculated in OR2 + (90 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 0.27 g/liter sodium pyruvate, 2 mg/liter gentamicin, and 5 mM HEPES, pH 7.6). Oocytes were injected with 10–50 ng of cRNA in a total volume of 50 nl. These concentrations of wild type CX37 cRNA produced large currents that increased with increasing cRNA injection. Antisense oligonucleotides directed against the endogenous Xenopus CX38 protein (1 μg/μl) were included to inhibit endogenous connexin expression. For the co-expression experiments, the amount of CX37WT was held constant and varying amounts of mutant cRNAs were co-injected to give ratios of 1:1 or 0.2:1 (mutant to CX37 cRNA). Injected oocytes were incubated in zero divalent OR2 (90 mM NaCl, 2.5 mM KCl, and 5 mM HEPES, pH 7.6, without Ca2+ or Mg2+) before recording to relieve divalent block of CX37 hemichannels. Data derived from multiple experiments were combined by normalizing the currents to the mean current measured for wild type CX37 at −80 mV for each day of recording.

**Electrophysiology**—Electrophysiological recordings on oocytes were performed using two-microelectrode voltage clamp in zero divalent OR2 unless otherwise indicated (29, 30). Pipettes were pulled to a resistance of 0.3 to 2.0 MΩ and filled with 3 M KCl. Recordings were performed using a Dagan CA1 oocyte clamp (Dagan Corporation, Minneapolis, MN). Signals were digitized with a Digidata 1600 analogue/digital converter (Axon Instruments, Inc., Sunnyvale, CA) at 1 kHz using pCLAMP 8 (Axon Instruments, Inc.). All experiments were performed at room temperature (20–24 °C).

Data analysis was performed using MATLAB version 6.5 (The Math Works, Inc., Natick, MA) and Microcal Origin version 7.5 (OriginLab Corporation, Northampton, MA). Group statistics are reported as mean ± S.E. Data from inhibition experiments that were performed on multiple days were pooled after normalizing the data to the mean current measured for wild type CX37 at −80 mV on that day. Statistical significance was determined using analysis of variance with Tukey’s method of multiple comparisons and Bonferroni's method at p = 0.05 and 0.01 (Origin). Both Tukey and Bonferroni methods gave similar results.

**RESULTS**

**Circular Dichroism Suggested a High α-Helical Content in CX37NT**—To elucidate the structure of the CX37NT domain, we synthesized a peptide corresponding to the first 23 amino acids of CX37 (Fig. 1, lower panel). We initially used circular dichroism to test the CX37NT peptide for structure. The CD spectrum of CX37NT at 25 °C exhibited the double well char-
characteristic of α-helices (Fig. 2). Spectral fitting suggested that the peptide was 60% helical (31% α-helix and 29% distorted α-helix). A melting curve was obtained to assess the thermal stability of the peptide (Fig. 2, inset). The spectrum showed that the CX37NT peptide was stable between −5°C and 25°C, but melted over a broad temperature range with no clear cooperative transition at higher temperatures. This lack of cooperativity is consistent with absence of tertiary structure.

NMR Also Suggested That CX37NT Was α-Helical—We then analyzed the CX37NT peptide by NMR spectroscopy. The structural statistics are presented in Table 1. The NOESY spectrum was analyzed for inter-residue contacts indicative of secondary structure. The critical contacts are summarized in Fig. 3A. NOE cross-peaks were observed for Hα(i) to HN(i+3) contacts for residues Asp-3 through Gly-22, except for those that were indeterminate due to spectral overlap (Fig. 3B). Like the CD spectra, these data showed that the peptide was significantly α-helical. Other NOE patterns indicating helical character, such as Hα(i) to Hβ(i+3) and HN(i) to HN(i+2), were observed primarily between residues Asp-3 and His-17. The chemical shifts of all the 1Hα and 1Hβ signals were compared with their residues’ random coil chemical shifts, and their corresponding CSIs were calculated to provide more indicators of secondary structure (Fig. 3B). No residue exhibited the CSI for β-sheet. The CSIs suggesting α-helical were located between residues Gly-5 and Gly-16, inclusive (Fig. 3B). Thus, the CSI and NOE data suggested that CX37NT was primarily helical between residues Gly-5 and Gly-16 with partial helical character from His-17 through Gly-22.

Molecular Modeling Provided Descriptions of the Structure of the Peptide—Using NMR data to guide a set of restrained simulated annealing routines, we generated a family of models representing the CX37NT structure. The peptide data were not abundant enough for highly redundant, restrained modeling as would be performed for a globular protein. Thirteen of the resulting energy-minimized structures were aligned so the α-carbons of residues 5 through 15 had a minimal root mean squared deviation (Fig. 4). This portion of the molecule was helical in all models. The part containing the first few N-terminal residues was unstructured. Residues 16 through 22 were helical in some, but not all, members of this structure family, indicating partial helical character for this segment.

Substitution Mutants Were Designed to Test the Importance of the α-Helix—To test the importance of the α-helical NT structure, we replaced two well spaced amino acids (Leu-10 and Gln-15) within the helical region of CX37NT with amino acids that were predicted to disrupt or maintain the α-helix. Secondary structure predictions suggested that replacing amino acids Leu-10 and Gln-15 with prolines (PP) would substantially reduce or abolish the α-helix, whereas replacing them with alanines (AA) would have little effect on the levels of α-helix (Fig. 5A). Fig. 5B depicts a model showing the locations of these residues within the α-helix. CD spectra were obtained from synthetic peptides corresponding to AA and PP (supplemental Fig. S1). Fitting of these spectra showed that the helical characters of both peptides were reduced as compared with that of CX37NT; it was 38% for AA (18% α-helix and 20% distorted α-helix) and 21% for PP (6% α-helix and 15% distorted α-helix).

Wild Type CX37 and Double Substitution Mutants Showed Similar
To test the importance of the α-helical NT structure for connexin trafficking and function, we generated CX37 mutants with the AA or PP substitutions. GFP was added to the C terminus of all constructs to facilitate identification of cells expressing these proteins and visualization of their subcellular distributions by fluorescence microscopy. Connexin localization was studied in HeLa cells transiently transfected with CX37 or the substitution mutants. In HeLa cells transfected with wild type CX37, the distribution of GFP fluorescence was very prominent as puncta and short lines at appositional membranes as expected for a gap junction protein (Fig. 5C, WT). Both double substitution mutants (AA and PP) showed similar distributions of fluorescence with fluorescent spots or lines at appositional membranes (Fig. 5C). Acquisition of insolubility in 1% Triton X-100 has previously been associated with incorporation of connexins into gap junction plaques (42). As expected based on previous studies (43), immunoblots showed that HeLa cells transfected with wild type CX37 contained a substantial amount of immunoreactive material in the 1% Triton X-100-insoluble fraction (supplemental Fig. S2, lanes labeled P). In HeLa cells expressing the AA or PP mutants, the total abundance of immunoreactive CX37 and the relative abundance of Triton X-100-insoluble CX37 material were comparable with those detected in cells expressing wild type CX37 (supplemental Fig. S2).

The Double Substitution Mutants Differed in Their Ability to Support Gap Junction-mediated Intercellular Communication—We also tested the importance of the α-helical NT structure for formation of conducting gap junction channels by assaying the intercellular transfer of neurobiotin (molecular mass, 323 Da; charge, +1) in HeLa cells expressing CX37 or the double substitution mutants. Cells expressing wild type CX37 consistently exhibited transfer of neurobiotin (100% of injections) to a large number of neighboring cells (14.6 ± 1.1) (Fig. 5D). Occasional neurobiotin transfer was observed in untransfected HeLa cells (30% of injections; 1.8 ± 0.8 neighbors) likely due to low level expression of endogenous connexins (Fig. 5D). Neurobiotin transfer was essentially absent in PP-transfected HeLa cells (14% of injections; 0.2 ± 0.1 neighbors), suggesting that this construct did not support intercellular communication. However, neurobiotin transfer was frequently observed in AA-expressing HeLa cells (91% of injections); this construct allowed neurobiotin transfer to a significantly smaller number of neighboring cells (7.0 ± 0.9) than wild type CX37, but significantly more than PP-expressing or

**TABLE 1**

| Constraints      | Total | Intraresidue | Sequential | Long range | Dihedral | H-bond |
|------------------|-------|--------------|------------|------------|----------|--------|
|                  | 100   | 20           | 35         | 0          | 36       | 9      |

Root mean square deviations:\n
- Backbone (5–15): 0.55 ± 0.14 Å
- Heavy atom (5–15): 2.02 ± 0.46 Å
- Backbone (all residues): 3.61 ± 0.94 Å
- Heavy atom (all residues): 4.77 ± 1.11 Å

a Mean ± S.D.

**FIGURE 3.** NMR analysis of CX37NT peptide reveals an α-helical segment. A, portion of the 300-ms mixing time NOESY spectrum showing amide to α cross-peaks. B, summary of critical NOE contacts and CSI indicating secondary structure. d indicates the distance between either α or amide (H) hydrogens. Residues with hydrogens within 5 Å of one another are indicated by lines or bars drawn between the corresponding residues. NOESY cross-peak intensities are indicated by the thicknesses of the lines. Dashed lines indicate contacts that could not be verified due to spectral overlap. CSI values were calculated for each residue; positive bars indicate helical structure, and null bars indicate resonance frequencies expected for random coil.
untransfected cells (Fig. 5D). Thus, the extent to which wild type CX37 and the substitution mutants supported intercellular transfer of neurobiotin followed the order, CX37 > AA > PP.

**The Double Substitution Mutants Did Not Form Conducting Hemichannels**—The ability of CX37 and the double substitution mutants to induce hemichannel currents was examined in cRNA-injected Xenopus oocytes using two-electrode voltage clamp. GFP fluorescence confirmed production of protein from each CX37 construct. Control oocytes injected only with the CX38 antisense oligonucleotide showed small currents (Fig. 6A). Large currents were detected in oocytes injected with wild type CX37 cRNA (Fig. 6B); they had similar magnitudes to those previously observed (29, 30). CX37 hemichannels passed current linearly with respect to voltage over the range of −100 to +10 mV (Fig. 7, A and B). Oocytes injected with PP or AA cRNA did not show connexin hemichannel currents above the levels recorded from control oocytes (Figs. 6, C and E, and 7, A and B).

**The Double Substitution Mutants Inhibited CX37WT Hemichannel Currents**—To test whether the double substitution mutants could co-assemble with wild type CX37 and alter...
its function, these constructs were co-expressed in *Xenopus* oocytes. In the mixing experiments, the amount of wild type CX37 cRNA was held constant and varying amounts of mutant cRNAs were added to produce ratios of mutant to wild type CX37 of 1:1 and 0.2:1. If the mutant constructs did not co-assemble with wild type CX37, the expected current magnitudes in co-injected oocytes should equal those in oocytes injected with wild type CX37 alone, because homomeric mutant channels would not contribute to hemichannel currents. In contrast, the currents measured for mixtures of AA with wild type CX37 at the 1:1 ratio were not statistically different from those of wild type CX37 alone (Fig. 7, B and C). Co-expression of AA with wild type CX37 at a 0.2:1 ratio produced hemichannel currents that were significantly greater than those produced by wild type CX37 alone (on average, 1.3-fold) (Figs. 6F and 7, B and D).

**DISCUSSION**

Our spectroscopic analysis of a synthetic peptide corresponding to the predicted N terminus of CX37 provides evidence that the CX37NT is α-helical between amino acid residues 5 and 16. In fact, the α-helix might even extend beyond amino acid 16 in the full-length protein, because our data indicate some α-helical character in this region, and anchorage of the NT to the first transmembrane domain would prevent fraying that may have occurred in the peptide.

The importance of the α-helix structure for gap junction and hemichannel function was demonstrated by the results obtained with the substitution mutants. CD spectra obtained from synthetic peptides corresponding to these substitution mutants showed that AA contained reduced helical content as compared with the wild type CX37NT and that PP contained very little helical content (supplemental Fig. S1).

Both of the substitution mutants exhibited functional deficits. The PP mutant did not permit gap junction mediated transfer of neurobiotin when expressed in transfected HeLa cells nor did it induce hemichannel currents when expressed by itself in *Xenopus* oocytes. Co-expression with wild type CX37 showed that PP acted as a potent dominant negative inhibitor of wild type CX37 hemichannel currents. This dominant negative behavior implies that PP can co-assemble with wild type CX37, but the resulting heteromeric hemichannels are not conducting. The AA mutant allowed intercellular neurobiotin transfer, but less extensively than wild type CX37. AA expression in *Xenopus* oocytes did not induce hemichannel currents. Although this functional discordance has not been explained, two other connexin mutants, Cx50H161N and CX46N63Q, have been identified that also form conducting intercellular channels, but not hemichannels (46, 47). When AA was co-expressed with wild type CX37 in equal amounts, the hemichannel current magnitude was not different from that produced by wild type CX37 alone. However, when AA was co-expressed with wild type CX37 at a low ratio (0.2:1, AA:WT), the hemichannel currents were 1.3-fold greater than in wild type-injected oocytes. An explanation for these results is that AA and wild type CX37 can co-assemble and can form functional hemichannels at optimal ratios of mutant to wild type. Thus, a small number of AA subunits may be tolerated when coassembled with wild type CX37 (~1 per hexamer) leading to an increase in the total number of functional hemichannels at the 0.2:1 coexpression ratio; however, higher proportions of mutant subunits may not be tolerated. Thus, the AA mutant exhibited better channel function and less deleterious effects on wild type hemichannel function than PP, but neither PP nor AA had functional properties comparable with

**FIGURE 6.** The CX37L10P,Q15P double substitution mutant does not induce hemichannel currents and inhibits co-expressed wild type CX37. A–F, hemichannel current traces from oocytes injected with no cRNA (A, control), or CX37 (B, WT), CX37L10P/Q15P (C, PP), a 0.2:1 mixture of CX37L10P/Q15P and wild type CX37 (D), CX37L10A/Q15A (E, AA), or a 0.2:1 mixture of CX37L10A/Q15A and wild type CX37 (F) cRNAs. Current traces recorded in zero divalent OR2 were elicited by a series of voltage pulses held for 1 s from a holding potential of −40 mV to a potential of +30 mV in 10-mV increments. The short line on the left of each panel indicates the current at 0 mV.
Structure of the CX37 N Terminus

NT structure is not absolutely required for CX37 localization to gap junction plaque-like structures. The results obtained from our structural studies of the NT of CX37, an α-connexin, differ in several aspects from studies of the NT of CX26, a β-connexin. First, based on NMR studies of a 15-amino acid peptide, Purnick et al. (24) concluded that the NT of CX26 contains an α-helix from residues 1 through 10, whereas we found that the α-helical domain extends beyond amino acid 10 in the CX37NT. Second, the α-helix domain in the CX26NT is followed by a flexible turn at positions 11 and 12; the corresponding CX37 residues (12 and 13) lie within the middle of the α-helical domain. It is possible that the difference between our results and those obtained by Purnick et al. (24) originates from studying a longer peptide (23 amino acids) that may have allowed detection of a structure that included amino acids 11–15 and beyond. However, it is also possible that the structure of the NT may differ among members of different connexin subfamilies, because the amino acid sequences of the NT domain are more closely conserved among members of each subfamily than among members of different subfamilies. Recently, Oshima et al. (5) reported the structure of a Cx26 mutant that contained a density within the pore that they interpreted as representing a plug made of NT domains, and Maeda et al. (50) reported the structure of wild type CX26 in which the NT domains line the pore entrance and form a funnel. Members of the α-subfamily (e.g. CX37) have more charged amino acid residues within the NT than do β-connexins (e.g. CX26). The amino acid residues corresponding to the “hinge” in CX26 (Gly-11, Gly-12) are very different in the α-connexins than among members of each subfamily (e.g. CX26). Because our data indicate that Asp-12 and Gln-13 within the α-helical structure, α-connexins may not have an NT hinge at the corresponding position. This difference suggests that the structure of some of the components responsible for channel gating of α-connexins may differ from those determined for β-connexins.

The distribution of amino acid residues within the α-helical region of the CX37NT reveals several interesting features (Fig. 8). One face of the α-helix contains three aligned, negatively charged amino acid residues (Glu-8, Asp-12, and Glu-16) and a positively charged residue (Lys-9), whereas the opposite face is...
Structure of the CX37 N Terminus

FIGURE 8. The CX37NT α-helical segment is amphipathic. A–C, different views of a model of the α-helical portion of the CX37NT. Negatively charged residues (Glu-8, Asp-12, and Glu-16) are indicated in red and the positively charged residue (Lys-9) is indicated in blue. Uncharged and hydrophobic amino acid residues within the α-helix are indicated in gray. A, the model is oriented to show the face of the α-helix that contains the aligned negatively charged residues at positions Glu-8, Asp-12, and Glu-16. B, the model has been rotated 180° along the long axis to show the opposite face of the α-helix that contains predominantly hydrophobic amino acid residues. C, the model has been tilted and rotated so the beginning of the NT is now on top to illustrate the charged face.

predominantly composed of hydrophobic amino acid residues giving the α-helix an amphipathic character. This distribution suggests that the different faces of the NT may be involved in different interactions. The hydrophilic face likely is exposed to the aqueous environment and may form part of the vestibule of the channel pore. A concentration of negatively charged residues in the vestibule of the channel pore may play a role in the limited permeation of negatively charged molecules through CX37 gap junction channels (44). Oh et al. (51) have found that charges within the NT contribute to determining the charge selectivity of a chimeric CX32 hemichannel. Our preliminary studies suggest that substitution of at least some of these charged residues results in altered gating of CX37 hemichannels (52). The charged residues might also form intramolecular or intermolecular salt bridges with charged residues from other connexin subunits or participate in hydrogen bonds with other non-charged residues. In Cx26, the funnel formed by the NT domains is stabilized by hydrogen bonds between Asp-2 and Thr-5 and between Trp-3 and Met-34 (50). The CX37NT hydrophobic face may interact with other hydrophobic regions in the connexin polypeptide or possibly with membrane lipids. Such NT interactions may be important for keeping the channel in an open conformation, because we have previously observed that mutants containing partial deletions of the CX37NT do not form opening hemichannels or support intercellular communication (30).

Several studies have suggested the importance of the charged residues in the NT of other α-connexins for gap junction function. Tong et al. (21, 53) found that Arg-9 is a critical determinant of V<sub>1</sub>-dependent gating in CX46 and its chicken ortholog, CX56. Musa et al. (20) showed that reversal of the charges at Glu-9 and Glu-13 in CX40 reduces V<sub>1</sub> sensitivity and the extent of block by spermine. Lin et al. (54) showed that Lys-16 of CX40 is required for spermine block.

The features of the CX37NT structure may help elucidate the abnormalities produced by NT amino acid substitutions in disease-associated connexin mutants from humans or animal models. Although mutations have been identified at several NT positions, the cellular and physiological behavior has been examined only for a few of these mutants. A D12V mutation was identified in zebrafish CX36.7 (55); fish carrying this mutation show disorganization of myofibrils and cardiac malformations. This residue (which aligns with Asp-12 in human CX37) may be important for gap junction plaque formation and function, because the CX36.7D12V mutant shows impaired trafficking to the plasma membrane in HeLa cells (55) and a different substitution in mouse CX43 (D12S) causes loss of Lucifer yellow transfer, although CX43D12SGFP does form gap junction plaques in baby hamster kidney cells (23). Many of the mutants within the NT associated with oculodentodigital dysplasia (CX43) or with cataracts (CX50) occur in amino acid residues beyond the predominant helical structure (after amino acid 16).

In summary, the CX37NT contains a substantial α-helical domain. The data from our site-directed mutants suggest that preservation of this α-helix is essential for maximal hemichannel and gap junction channel activity.

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