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Structural Studies of the N-terminus of Connexin 32 Using ^1H NMR Spectroscopy

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Abstract

The amino terminus of gap junction proteins, connexins, plays a fundamental role in voltage gating and ion permeation. We have previously shown with ^1H NMR that the structure of the N-terminus of a representative connexin molecule contains a flexible turn around glycine 12 (*Arch. Biochem. Biophys.* 381:181,2000) allowing the N-terminus to reside at the cytoplasmic entry of the channel forming a voltage sensor. Previous functional studies or neuropathies have shown that the mutation G12Y and G12S form nonfunctional channels while functional channels are formed from G12P. Using 2D ^1H NMR we show that similar to G12, the structure of the G12P mutant contains a more flexible turn around residue 12, whereas the G12S and G12Y mutants contain tighter, helical turns in this region. These results suggest an unconstrained turn is required around residue 12 to position the N-terminus within the pore allowing the formation of the cytoplasmic channel vestibule, which appears to be critical for proper channel function.

Keywords

NMR; atomic resolution structure; ion channels; voltage dependent gating; connexins; structure-function; Protein Structure and Function

INTRODUCTION

The vertebrate connexin gene family is comprised of 21 members that encode an integral membrane protein with four identified transmembrane domains (TM1-TM4), two extracellular loops (E1 and E2) and three intracellular domains; the amino terminus (NT), the cytoplasmic loop (CL) and the carboxyl terminus (CT). Cell specific hexameric assemblies of these proteins, termed “hemichannels” or “connexons”, associate by interactions of the extracellular loops to form an intercellular channel that aggregate to form

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morphologically distinct gap junctions. Gap junctions provide a regulatable, low resistance communication pathway between coupled cells that allows passage of ions, small signaling molecules and metabolites through pores that are estimated to be 15–20 Å in diameter [1–3]. Disruption of this pathway by mutations in connexin genes is known to be associated with a number of human heritable diseases, including but not limited to Connexin 32 (Cx32) - Charcot-Marie-Tooth [4], Connexin 26 (Cx26)- nonsyndromic sensorineural deafness [5] and Connexin 50 (Cx50)- congenital cataractogenesis [6]. Several members of the gene family may also function as unapposed hemichannels allowing the diffusion of ions and small molecules across the plasma membrane of single cells [7]. At least one disease associated Cx32 mutation, S85C can function as an unapposed hemichannel [8].

The amino terminus (NT) is known to be an important determinant of the structure-function relations of channels formed by Cx32 and Cx26. NMR studies established the presence of a turn within the first 15 amino acid residues of the NT of Cx26, initiated by a glycine residue at the 12th position. This flexible turn allowed the helical region, comprising the first 10 amino acids, to be positioned within the channel pore where they would form a vestibule at the cytoplasmic entry of the channel [9]. More recently, density maps of crystallized Cx26 channels [10,11] have led to the suggestion that six NT domains reside in the channel pore and may form a “gating-plug” at the cytoplasmic entrance of the channel.

Structural models, which place the NT within the channel pore, are supported by several functional studies. These include the electrophysiological studies of Verselis et al. [12], which demonstrated that the difference in the polarity of “V_j-gating” of channels formed by Cx32 and Cx26 results from a difference in the charge of the 2nd amino acid residue of the two connexins, and those of Purnick et al. [13] and Oh et al. [14], which reported that negative charge substitutions up to the 10th but not 11th residue can reverse the polarity of “V_j-gating” of Cx32 hemichannels. Thus, it appears that the NT of Cx26 and Cx32 forms at least a portion of a transjunctional (V_j) voltage-sensor, which must reside within the channel pore [15]. The conclusion that the first 10 residues of the NT forms a pore-lining vestibule at the cytoplasmic entry of Cx32 channels is further supported by functional studies demonstrating that charge substitutions at the 2nd, 5th, and 8th positions are determinants of unitary conductance [14], charge selectivity [16] and current rectification [14,17].

It should also be noted that mutations of 12 of the 22 residues that comprise the N-terminus of Cx32 underlie the etiology of a common hereditary neuropathy, X-linked Charcot-Marie-Tooth (CMTX) disease [18]. For the most part, Cx32-CMTX mutations are categorized as “loss of function mutations” indicating that some aspect of the structure-function relation of Cx32 channels has been disrupted [19]. The large numbers of mutationally sensitive sites in the N-terminus suggests that the structure of this domain is critical to channel function. Of the N-terminal CMTX mutations, Cx32G12S, has been shown not to form functional channels in pairs of *Xenopus* oocytes [9]. It has been suggested that this mutation as well as two other non-functional mutations at this locus, G12Y and G12V, change the structure and/or alter the intrinsic flexibility of the turn in the NT of Cx32 [9] resulting in a change in the structure of the channel vestibule and loss of channel function. Notably, the substitution of proline at this locus, Cx32G12P, which is expected to preserve the structure and/or flexibility of the turn, forms functional channels whose voltage dependence is similar to that

of wild type [9]. Here, we present the solution structure of four 22 amino acid peptides corresponding to the sequence of Cx32 and G12S, Y and P substitutions. Peptides of this length are believed to comprise the complete N-terminus of Cx32 channels [20]. The results indicate that peptides containing 12S and 12Y, but not 12P alter the structure of the turn in this region in a manner that would prevent the formation of the cytoplasmic channel vestibule that appears to be critical for proper channel function.

MATERIALS AND METHODS

Peptide synthesis

The Cx32 N-terminal peptide (MNWTGLYTLLSGVNRHSTAIGR) and mutants G12P and G12S were synthesized at the Laboratory for Macromolecular Analysis at Albert Einstein College of Medicine. The mutant G12Y was synthesized at New England Peptide Company. The synthesis method was standard Fmoc chemistry [21] and the sample was purified by HPLC and confirmed by electron ionization spectrometry. The peptides were not acetylated or amidated and contained free N and C termini. The peptides were dissolved in 100 mM KCL (ionic strength of 0.1 M) in 10% $^2\text{H}_2\text{O}$ / 90% H_2O (pH 7.0) at a concentration of 1.0–1.2 mM with 100 μM 3-(trimethylsilyl)propionic acid (TSP) as a chemical shift reference.

^1H 2D NMR

All NMR spectra were collected on Bruker DRX-600 MHz spectrometer, a Bruker 500 MHz spectrometer with a cryoprobe or a Varian INOVA 600 MHz spectrometer with a cryoprobe. Data sets in H_2O were collected at 283, 288, 293 and 298 K. Water suppression in all 2D experiments was accomplished with either the double gradient echo method of Hwang and Shaka [22] or with Watergate [23]. The data were processed using NMRPipe [24] and extended using linear prediction and zero filling. The data were analyzed using NMRView [25].

All proton resonances were assigned by standard procedures [26]. Both short (17ms) and long (75ms) mixing time TOCSY [27] experiments were used for proton assignments and proton-proton distance constraints were obtained from a 225 ms mixing time NOESY experiment at 283 K. Spin diffusion is not expected for a peptide of this size (22 amino acids) nor was there any evidence of spin diffusion observed. The intensity of the NOESY crosspeaks was classified into very strong (2.8 Å), moderately strong (3.4 Å), and weak (5.0 Å) ranges. Atoms that were not stereospecifically assigned were treated as pseudoatoms and given correction distances according to procedures in Wüthrich [26]. Amide proton temperature coefficients were calculated from the proton chemical shift change from TOCSY spectra collected in increments of 5° in the temperature range of 283–298 K. Any amide proton which shifted less than 6.0 ppb/K was considered likely to be hydrogen bonded. Initial structures were calculated using relevant NOE constraints and H_α chemical shifts by simulated annealing and torsional angle dynamics as applied in the program CNS 1.1 [28]. A hydrogen bond analysis was performed on all structures using the program MolMol [29]. A hydrogen bond was considered to occur if it was present in greater than 70% of the structures and if the donor proton had a reduced temperature coefficient. Final structures were then recalculated using CNS with hydrogen bonds, if present, used as

additional constraints. The 20 lowest energy structures were then energy minimized using CNS, evaluated with AQUA and PROCHECK-NMR [30] and displayed using MOLMOL [29].

RESULTS

The ^1H 1D NMR spectra of the wild-type and all mutant peptides exhibited well-resolved lines and no significant change in linewidth or chemical shift for peptide concentrations ranging from 0.1 to 4.5 mM. This suggests that in the concentration range tested there is no peptide aggregation and that the peptide is monomeric.

Figure 1 shows medium to long range NOE connectivities between W3 and Y7 aromatic protons and aliphatic protons of L6 and L9 in the NOESY spectrum of the G12S mutant. These interactions were also observed in the NOESY spectrum of the wild-type and other mutants as well. Some of these same interactions were also observed in the Connexin26 N-terminal peptide structure [9] and may be common to N-terminal peptides of Connexin molecules with similar sequences.

A summary of NOEs for the wild-type and mutant peptides is shown in Figures 2a–d. The NOE patterns for several of the structures indicate the presence of helices or turns. Moderately strong to lower intensity $d_{\text{NN}}(i,i+1)$ NOE connectivities (connectivities observed between consecutive NH protons) are observed in several areas of the wild-type, G12S and G12Y mutant structures. No $d_{\text{NN}}(i,i+1)$ NOE connectivities are observed in the G12P mutant. The more stringent signatures of helical structure are lower intensity $d_{\alpha\text{N}}(i,i+3)$, $d_{\alpha\beta}(i,i+3)$ and $d_{\alpha\text{N}}(i,i+4)$ NOE connectivities (noe crosspeaks between the αH proton and the NH proton 3 residues away, the αH proton and the βH proton 3 residues away and the αH proton and the NH proton 4 residues away respectively). Examination of Figure 2 shows distinct differences between the structures in the number and position of these low intensity NOEs along the sequence. The wild-type structure has only 3 $d_{\alpha\text{N}}(i,i+3)$, $d_{\alpha\beta}(i,i+3)$ or $d_{\alpha\text{N}}(i,i+4)$ NOEs. One is located near the N-terminus between residues W3-L6, the second is between G12-R15 and the third is at the C-terminus between residues A19-R22. The mutant G12P has only one of these weaker intensity NOEs at the C terminus between residues A19-R22. In contrast, the mutant G12S has 8 consecutive sets of these weaker intensity NOEs which span the sequence from W3-H16 and the mutant G12Y has 6 nearly consecutive sets of these NOEs which span the sequence from W3-T18. Specifically, the G12S and G12Y mutants have more of these signature helical NOEs within the region of the sequence L10-R15 (5 and 2 respectively) than that observed for the wild-type and G12P mutant (1 and 0) respectively. This suggests that there is a greater population of folded conformers with helical characteristics in the mutants G12S and G12Y than in the wild-type or G12P mutants.

The temperature coefficients are shown for the amide protons in Figure 3 for the wild-type and mutant peptides. They are largely consistent with the NOE data. The extent to which a temperature coefficient for an amide proton is lowered, from the solvent exposed value of $8 \times 10^{-3} \text{ ppm K}^{-1}$, is a measure of the degree of protection from the solvent and thus of the population of folded conformers [31]. A later study investigated the solvent exposed amide

temperature coefficients for each amino acid in a random coil conformation [32]. For the amino acids used in our study the random coil values for amide temperature coefficients range from -7.02 – -9.32 depending on the amino acid [32]. Temperature coefficients of less than 6×10^{-3} ppb K^{-1} are suggestive of hydrogen bonding [31]. Most of the structures have lowered temperature coefficients from residues 4–6 with that of G5 being particularly reduced from random coil values (T-7.40, G-7.02 and L-8.42). This is due to the hydrophobic interactions between W3 and L6 providing some solvent protection to these protons. The G12Y mutant has a lowered H temperature coefficient for His16 suggesting solvent protection and a more folded region of G12Y compared to the wild-type and G12P mutants. Noticeable differences in temperature coefficients between the wild-type and G12P mutant compared to those of the G12S and G12Y mutants occur within residues 11–15 (Figure 3b). In this region, the wildtype and the G12P peptides have NH temperature coefficients that are very close or even greater than their solvent exposed random coil values (7.02, 7.02, 8.35, 7.02 and 7.64 for SGVNR respectively, proline has no NH proton). In contrast, the temperature coefficients for residues 11–15 in the G12S (7.02 for S) and G12Y (9.32 for Y) mutants are significantly less than their random coil values and many of them are less than 6 ppb K^{-1} , which suggests the presence of a hydrogen bond within these residues and thus less flexibility. This data suggests less solvent exposure for residues 11–15 in the mutants G12S and G12Y and the presence of a more folded region in these peptides as compared to the wild-type and G12P mutants.

A total of 22 alpha proton chemical shifts, and 320 (wild-type), 192 (G12P mutant), 441 (G12S mutant) and 192 (G12Y mutant) meaningful distance constraints obtained by the NOESY experiments were used as input to calculate structures with the program CNS. The G12P and G12Y peptides were not as soluble as the wild-type and G12S peptides and showed slightly lower resonance intensity in the 1D spectra and fewer cross peaks in the NOESY spectra.

Hydrogen bond analysis of the G12S mutant structure (calculated without H bonds) showed that 70% of the structures had 2 H bonds (11 HN and 8 CO and 14 HN and 10 CO). The temperature coefficients for the NH proton in residues 11 and 14 were both below 6 ppb/K. Therefore, the final structure for the G12S mutant peptide was calculated with two hydrogen bond constraints (11 HN and 8 CO and 14 HN and 10 CO). The structural statistics for the 20 converged structures and atomic root-mean-square differences (RMSDs) for the wild-type and mutant structures are given in Tables 1–4. There were no NOE violations greater than 0.5 Å and no angle violations greater than 5°. Deviations from idealized covalent geometry are small and the van der Waals energy is negative, indicating no distortions and bad contacts exist in the converged structures.

The 20 lowest energy structures that best fit the NMR data for the wild-type and mutant peptides are shown in Figure 4. Superposition of the backbone atoms for residues 3–15 for the wild-type structure in Figure 4a gives an RMSD value of 1.244 Å. A turn within residues 11–15 is clearly visible and superposition of backbone atoms for these residues gives a RMSD of 0.785 Å (Figure 4). Superpositions of backbone atoms for the other mutants are also shown in Figure 4. The G12P mutant is shown in Figure 4 and gives a backbone RMSD of 1.783 Å for residues 3–15 and a RMSD of 1.084 Å for residues 8–15. The mutant G12S

is shown in Figure 4 and the RMSD for the backbone atoms for residues 3–15 is 0.913 Å. In contrast to the wild-type and G12P mutant peptide structure the G12S mutant shows a tighter helical turn from residues 11–15 with a RMSD for backbone atoms of 0.355 Å. The mutant G12Y, shown in Figure 4, has a RMSD for backbone atoms for residues 3–15 of 1.594 Å. Similar to the G12S mutant and also in contrast to the wild-type and G12P mutant, residues 11–15 show a tighter helical turn with a RMSD of 1.029 Å. These bundles indicate that the structures fit the NMR data well and that one conformation can be defined for each molecule. They also show that the structural turns around and including residue 12 are well defined by the NMR data.

Representative low energy structures for the wild-type and mutant peptide structures are shown in Figures 5 and 6. Figure 5a shows the wild-type structure and Figure 5b shows the mutant G12P structure. Figure 5a shows the hydrophobic interactions between the sidechains of the hydrophobic residues W3, L6, Y7, L9 and L10. Figure 1 shows representative NOE interactions between protons from these residues. This causes a large turn within residues 3–9 in the N-terminus in all the structures in the bundle. Residues 11–15 also form a loose turn leading to an unstructured region in the C-terminus from residues 16 to 22. The mutant G12P, Figure 5b, exhibits loose helical turns within residues 3–7 and also exhibits hydrophobic interactions between the sidechains of the hydrophobic amino acids. A larger, loose turn is shown within residues 8–15 which also leads to an unstructured C-terminus.

In contrast, the mutants G12S and G12Y, Figure 6a and b, show more defined secondary structure and smaller helical turns. Figure 6a shows interaction between the sidechains of the N-terminal hydrophobic residues mentioned above. The wild-type and all mutants show NOE interactions between the same hydrophobic residues although the exact distances of the interactions may vary somewhat. In addition, the dihedral angles for residues 8–10, in every structure, are consistent with a 3–10 helical turn. Also, in contrast to the wild-type and G12P models, the dihedral angles for residues 11–15 are consistent with an α -helical conformation or a tighter helical structure. Figure 6b shows the G12Y mutant structure. Interactions between the sidechains of the hydrophobic residues W3, L6, Y7, and L9 are shown in the N-terminal region as was observed in the other structures. Similar to the G12S mutant, residues within 10–15 are in an α - or 3–10 helical conformation in the majority of the models. In some models residues 10–17 are in a helical conformation. Comparing the wild-type and G12P mutants with the G12S and G12Y mutants it is obvious that residues surrounding residue 12 (9–15) are in one larger, looser turn in the wild-type and G12P mutants than those in G12S and G12Y which contain two helical turns within the same region.

DISCUSSION

Although there may be interactions of the N-terminus with other domains in the Cx32 protein, the functional studies [9] correlate well with the structures reported here indicating it is likely the NMR solution structures are similar to the conformation in the native channel. The amino terminus of the native channel resides in an aqueous environment (16) similar to that used in the determination of the NMR structures. Several studies have shown that

individual peptide segments of larger proteins have the same secondary structure as the corresponding region in the intact protein [33,34]. Indeed NMR structures of small peptides of the four-helix bundle in myohemerythrin showed the same secondary structure as those sequences exhibited in the intact protein [33]. Also, peptides of the loops interconnecting the helices in the integral membrane protein bacteriorhodopsin show the same solution structure by NMR as observed in the crystal structure of the intact protein [34].

Peptide structures have been shown to be stabilized by side chain hydrophobic interactions [35,36], salt bridge formation [37] and hydrogen bonds [38]. A large number of NOEs were observed for the wild-type and all mutant peptides between side chains of W3, L6, Y7 and L9 indicating close contact. Examination of the structures of the four peptides indicates interaction of these hydrophobic amino acid side chains, which could have a stabilizing effect on the structure near the N-terminus of the peptides. Both the wild-type (12G) and G12P mutant structures have temperature coefficients close to or above their random coil values in the turn regions consisting of residues 11–15 and 8–15 respectively. This suggests more solvent exposure and a lack of hydrogen bonding within the backbone protons in these turns and thus, these regions may form loose, flexible turns. Populations of turn conformations are often observed without significant hydrogen bonding [39,40] and NOES were observed between protons from residues within these turns indicating a significant population of conformers in these conformations. Residues present within both of these turns contain side chains, which can be hydrogen bond donors or acceptors such as T8, S11, N14 and R15. A hydrogen bond analysis of the wild-type structures indicated the possibility of a hydrogen bond between R15 H ϵ -S11 CO in several models. In two of the G12P structures, a hydrogen bond may exist between N14 sidechain protons and Tyr7 CO or the Thr8 sidechain hydroxyl group. Also in G12P the Ser11 sidechain hydroxyl proton is within hydrogen bonding distance of the V13 CO. These interactions may provide some stabilization for the turn observed in this region.

The mutant peptides G12S and G12Y exhibit much tighter helical turns, with many models showing either 3–10 or α -helical character within residues 11–15. In contrast to the wild-type and G12P structures, many of the backbone NH protons in these mutants have temperature coefficients less than 6 ppb K⁻¹ within residues 11–16. This indicates a more folded area of the structure and the possibility that the NH protons are involved in a hydrogen bond. Analysis of the G12S structures suggests the possible presence of a hydrogen bond between L10 NH – Y7 CO, S11 NH – T8 CO, N14 NH-L10 CO, R15 NH - S11 CO and a H16 ring proton HD1 with S12 sidechain OH in some of the models. The G12S peptide might be more helical because of the ability of the sidechain hydroxyl group to be a hydrogen bond donor or acceptor and stabilize the structure. The G12Y peptide also has temperature coefficients of less than 6 ppb K⁻¹ for some of the NH protons within residues 11–16. Similar analysis of these structures show the possibility of hydrogen bonds in some of the models between S11 NH – T8 CO, N14 NH – L10 CO and R15 NH – Y12 CO. These hydrogen bonds would stabilize the turns and suggests the region of residues 11–16 in the mutant peptides may be much less flexible than that of the wild-type (12G) and G12P mutant peptide structures. In addition, the spectra for the G12Y mutant peptide exhibited many NOES between the Y12 sidechain and hydrophobic sidechain protons of L9. The proximity of the two sidechains can be seen in the structure in Figure 6. These

interactions place the sidechains of V13 in closer proximity to L10 allowing the interaction between the hydrophobic sidechains of these two residues as well. Although tyrosine is not a good helix inducer, hydrophobic side chain interactions are known to stabilize helical structure [36]. The ability of tyrosine to form hydrophobic side chain interactions could explain the increased helical content in the G12Y peptide compared to the wild-type and G12P. Some support for our results is provided by the algorithm AGADIR which predicts helical behavior in peptides and helical propensities for each residue [41]. Helical propensity for residues 11–15 for the wild-type and G12P sequences are both 2.8 while that for the G12S and G12Y sequences are increased to values of 4.2 and 16.8 respectively. The prediction is not an exact agreement with the structures but generally supports the increased tendency for helicity in this region for the G12S and G12Y mutants.

Both structures with glycine or proline in position 12, exhibit larger, less constrained and more flexible turns around residues 11–15 and 8–15 respectively. Our previous structural study on the N-terminal peptide of the closely related Cx26 protein also found an open turn from residues 12–15, where residue 12 was also a glycine [9]. Glycine and proline are quite common in turns [40,42]. Glycine has the smallest side chain and can adopt conformations, which would be forbidden for residues with longer sidechains. This makes it more flexible than other residues [42]. Proline, having no NH proton for hydrogen bonding, is disruptive to normal secondary structure and amenable to forming turns [42]. For these reasons glycine and prolines are often found in areas of the molecule which need to move or hinge. This has been seen in the NMR structure of the sodium channel inactivation gate peptide [43]. The peptide forms a stable helical structure capped by an N-terminal turn in H₂O. Unstructured glycine- and proline-rich regions flank the folded core of the peptide, which is consistent with their proposed role as hinges in intact channels. Prior site-directed mutagenesis studies demonstrated that glycine and proline residues were required for fast inactivation of the channel [44]. Mutations of glycine or proline with alanine, an amino acid with a higher propensity for helical conformations, exhibited slower and impaired inactivation [44]. It is proposed that glycine and proline residues contribute to hinge regions, which allow movement of the inactivation gate during inactivation of Na⁺ channels [44].

In this study, we demonstrate a correlation between the structure of the N-terminal peptides and the ability of the channel to function. Both wild type (12G) and G12P peptides are characterized by a loose and unconstrained turn in the vicinity of residue 12 that can be related to the inherent properties of Gly and Pro residues. This turn would permit the positioning of the first 10 amino acids of Cx32 within the channel pore where they would form a vestibule at the cytoplasmic entry of the channel as required by structure-function studies. In contrast, G12S and G12Y peptides adopt a conformation in this region that is characterized by a constrained helical segment that is unlikely to allow the insertion of the NT into the channel pore. It is likely that the NT of G12S and G12Y extend into the cytoplasm. The solution structure of the WT and three mutant peptides described here provides a mechanistic explanation for the observed lack of function of the G12S and G12Y mutations, and near wild type voltage dependence of G12P channels as previously reported (9) and also explain the “loss of function” of G12S with respect to Charcot-Marie-Tooth disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The two-dimensional ^1H NMR spectrum of the G12S mutant peptide showing labeled NOE interactions between aliphatic and aromatic side chain protons.

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Figure 2.
A summary of NOE restraints for the wild-type and each mutant N-terminal peptide. The horizontal lines indicate backbone NOES with the thickness representing intensity.

Figure 3.

a) Plots of temperature coefficients vs. residue number for the wild-type and mutant N-terminal peptides. The proline residue (12) in the mutant G12P does not have an NH proton. Temperature coefficients were only calculated for residues whose NH proton was observable at all temperatures. **b)** Temperature coefficients of residues 11–15 for the wild-type, G12P, G12S and G12Y peptides. The error bars represent errors of 0.15 ppb/K.

Figure 4.

The 20 lowest energy conformers of the wild-type and the G12P, G12S and G12Y mutants are shown. Superpositions of the backbone atoms from residues 3–15 are shown in the larger bundles. The adjacent, smaller superpositions are for backbone atoms from residues 11–15 for the wild-type, G12S and G12Y mutants and for residues 8–15 for the G12P mutant.

Figure 5. Representative ribbon diagrams of the wild-type (left) and G12P (right) mutant peptide are shown.

Figure 6. Representative ribbon diagrams of the G12S (left) and the G12Y (right) mutant peptides are shown.

Table I

Wild-type

Structural Statistics	Values for 15 converged structures
Total Constraints	320
Intraresidue	36
Sequential	107
Medium range	147
long range	30
Largest distance constraint violation (Å)	0.30
RMSD	
Backbone (Å)	1.244 (res.3–15), 0.785 (res. 11–15)
Heavy atoms (Å)	2.038 (res.3–15), 1.990 (res.11–15)
Deviations from idealized geometry	
Bonds (Å)	0.0039
Angles (deg)	0.5370
Van der Waals Energy (kcal/mol)	–30
Ramachandran	33.2 % in most favorable 57.1 % additionally or generously allowed regions

Table II

G12P

Structural Statistics	Values for 15 converged structures
Total Constraints	192
intraresidue	53
sequential	77
medium range	59
long range	3
Largest distance constraint violation (Å)	0.33
RMSD	
Backbone (Å)	1.783 (res.3–15), 1.084 (res. 8–15)
Heavy atoms (Å)	2.687 (res.3–15), 2.416 (res.8–15)
Deviations from idealized geometry	
Bonds (Å)	0.0037
Angles (deg)	0.5296
Van der Waals Energy (kcal/mol)	−46
Ramachandran	35.9 % most favorable 59.4 % additionally or generously allowed regions

Table III**G12S Mutant**

Structural Statistics	Values for 15 converged structures
Total Constraints	441
intraresidue	96
sequential	138
medium range	191
long range	16
Largest distance constraint violation (Å)	0.37
RMSD	
Backbone (Å)	0.913 (res.3–15), 0.355 (res. 11–15)
Heavy atoms (Å)	1.907 (res.3–15), 1.518 (res.11–15)
Deviations from idealized geometry	
Bonds (Å)	0.0031
Angles (deg)	0.4589
Van der Waals Energy (kcal/mol)	–42
Ramachandran	48.6 % in most favorable 45.5 % additionally or generously allowed regions

Table IV**G12Y Mutant**

Structural Statistics	Values for 15 converged structures
Total Constraints	192
intraresidue	79
sequential	67
Medium range	41
long range	5
Largest distance constraint violation (Å)	0.35
RMSD	
Backbone (Å)	1.594 (res.3–15), 1.029 (res. 11–15)
Heavy atoms (Å)	3.261 (res.3–15), 2.639 (res.11–15)
Deviations from idealized geometry	
Bonds (Å)	0.0035
Angles (deg)	0.5261
Van der Waals Energy (kcal/mol)	–36
Ramachandran	37.8 % in most favorable 57.2 % additionally or generously allowed regions