A Novel N14Y Mutation in Connexin26 in Keratitis-Ichthyosis-Deafness Syndrome

Analyses of Altered Gap Junctional Communication and Molecular Structure of N Terminus of Mutated Connexin26

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Connexins (Cxs) are transmembranous proteins that connect adjacent cells via channels known as gap junctions. The N-terminal 21 amino acids of Cx26 are located at the cytoplasmic side of the channel pore and are thought to be essential for the regulation of channel selectivity. We have found a novel mutation, N14Y, in the N-terminal domain of Cx26 in a case of keratitis-ichthyosis-deafness syndrome. Reduced gap junctional intercellular communication was observed in the patient's keratinocytes by the dye transfer assay using scrape-loading methods. The effect of this mutation on molecular structure was investigated using synthetic N-terminal peptides from both wild-type and mutated Cx26. Two-dimensional ¹H nuclear magnetic resonance and circular dichroism measurements demonstrated that the secondary structures of these two model peptides are similar to each other. However, several novel nuclear Overhauser effect signals appeared in the N14Y mutant, and the secondary structure of the mutant peptide was more susceptible to induction of 2,2,2-trifluoroethanol than wild type. Thus, it is likely that the N14Y mutation induces a change in local structural flexibility of the N-terminal domain, which is important for exerting the activity of the channel function, resulting in impaired gap

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Gap junctions are involved in cell-cell attachment of almost all tissues, including the skin. Their most characteristic function is that of an intercellular channel. Gap junctions are made up of connexins (Cxs), transmembranous proteins that transverse the cell membrane four times, with their N and C termini located on the cytoplasmic side of the membrane. Cxs form tube-like hexamer structures, called connexons, that aggregate to the cell membrane and to connexons of opposite cells, forming gap junctional plaques. Through gap junctions, certain ions and second messengers less than 1 kd can pass from cell to cell. Thereby, gap junctions play important roles in cell-cell communication and tissue homeostasis.^{1,2}

The importance of gap junctional intercellular communication in the function of several tissues or organs is demonstrated by the presence of Cx gene mutations in several congenital disorders.^{1,2} For example, Cx26 mutations are a major cause of nonsyndromic congenital sensorineural deafness (DFNB1: MIM no. 220290). The Cx-related deafness is sometimes associated with congenital skin disorders, such as Vohwinkel's syndrome (MIM no. 124500)³ and keratitis-ichthyosis-deafness (KID) syndrome (MIM no. 148210).⁴ These syndromic deafness syndromes are autosomal dominant diseases in which it is assumed that the mutated Cx26 protein inhibits normal gap junction function by a dominant-negative effect.⁵

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Here, we report the case of a Japanese girl with KID syndrome. The mutation analysis of GJB2 (the coding region of Cx26 gene) revealed a novel missense mutation, N14Y. This mutation is in the N-terminal domain of Cx26 where other mutations in KID syndrome have previously been reported; therefore, it is assumed that the N-terminal domain of Cx26 should be necessary for the proper function of the protein. To understand the function of this domain, it was important to clarify the relation between the N14Y mutation and the altered channel function of the gap junction. For this, we performed the following experiments: 1) ultrastructural examination of gap junctions and immunohistological study for Cx26 expression in the patient's skin was performed; 2) we investigated the effect of N14Y mutation on gap junctional intercellular communication by a dye transfer assay; and 3) we studied the structural changes in the N-terminal domain of Cx26 by molecular structural analysis using nuclear magnetic resonance (NMR).

Materials and Methods

Skin Samples and DNA

Skin biopsies were taken from the skin lesion on the left foot of the patient after informed consent. Genomic DNA samples from peripheral blood were obtained from the family members including the patient and her parents after informed consent.

Mutation Analysis

Genomic DNA was extracted from peripheral blood and used as a template of gene amplification. The coding region of GJB2 (GenBank accession no. NM 004004) was amplified by polymerase chain reaction (PCR), as previously described.⁵ DNA sequencing of the PCR product was performed with an ABI Prism 3100-Avant genetic analyzer (Perkin Elmer-ABI, Foster City, CA).

Electron Microscopy

The skin sample was fixed in one-half strength Karnovsky's fixative or 2% glutaraldehyde solution, postfixed in 1% OsO₄, dehydrated, and embedded in Epon 812. The sample was ultrathin-sectioned at a thickness of 70 nm and stained with uranyl acetate and lead citrate. Photographs were taken using a Hitachi H-7100 transmission electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

Immunofluorescence Labeling

The patient's skin sample was snap-frozen in isopentane, and 6- μ m-thick sections were cut using a cryostat. The sections were washed with 0.01 mol/L phosphate-buffered saline (PBS) for 10 minutes and then incubated in rabbit polyclonal anti-Cx26 antibody—the epitope is a portion of the cytoplasmic loop of Cx26—(Zymed Labo-

ratories, San Francisco, CA) or mouse monoclonal anti-Cx43 antibody (clone 4E6.2; Chemicon International, Temecula, CA) solution for 1 hour at 37°C. Antibody dilutions were 1/10 for Cx26 antibody and 1/200 for Cx43 antibody. The sections were then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins for Cx26 and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins for Cx43 (Jackson ImmunoResearch Laboratories, West Grove, PA) solution for 30 minutes at room temperature, followed by 10 μ g/ml propidium iodide solution as a nuclear counterstain (Sigma Chemical Co., St. Louis, MO) for 10 minutes. The sections were extensively washed with 0.01 mol/L PBS between incubations. The stained sections were mounted using a glycerol-based mounting medium (Permafluor, Shandon, PA) and stored in the refrigerator in the dark. Immunostaining was detected as green (fluorescein isothiocyanate), and nuclear staining was observed as red (propidium iodide). Overlap of both fluorescein isothiocyanate and propidium iodide was demonstrated as yellowish color. Fluorescence images were observed using an Olympus IX70 confocal laser-scanning microscope. Image collection was performed by software Fluoview version 2.0 (Olympus America Inc., Melville, NY).

Cell Culture

Cell culture was performed with slight modifications to the methods previously described.⁶ A biopsy was taken from a hyperkeratotic plaque on the dorsum of the patient's left foot. Biopsy samples were kept in ice-cold PBS containing antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml gentamicin, 2.5 μ g/ml amphotericin B, and 0.4% neomycin). After trimming subepidermal connective tissue, the samples were placed (overnight, 4°C) in disperse in PBS. The epidermis was peeled off from the tissue and placed in trypsin-ethylenediaminetetraacetic acid solution (0.25% trypsin in 0.05% ethylenediaminetetraacetic acid). Single cells were suspended in keratinocyte seeding medium.⁷ Cells were seeded on mitomycin C-treated feeder layer of 3T3 cells in 35-mm-diameter tissue culture plates. Cultures were fed with Dulbecco's modified Eagle's medium, incubated at 37°C, 5% CO₂.

Dye Transfer Assay

The dye transfer assay was performed by scrape-loading methods introduced by el-Fouly and colleagues⁸ with slight modification. Briefly, epidermal keratinocytes from the patient, normal human epidermal keratinocyte (NHEK), and HaCaT cells (human keratinocyte cell line) were grown to confluency on 35-mm plastic plates in Dulbecco's modified Eagle's medium. After removal of medium, the cells were scraped using a plastic eraser, and 0.125% Lucifer yellow and 0.05% rhodamine dextran (Molecular Probes, Eugene, OR) dissolved in PBS were immediately added to the cells. Two minutes later, the dye solution was discarded, and the plates were rinsed with adequate amounts of PBS to remove detached cells

and background fluorescence. Cells were examined under an Olympus IX70 confocal laser-scanning microscope (Olympus America Inc., Melville, NY) at the time of 20 minutes after scrape loading. The degree of dye transfer was quantified by counting the Lucifer yellow-positive cells per unit length (50 μ m) of scratched plane. We counted only the Lucifer yellow-positive cells, and the cells that were both Lucifer yellow- and rhodamine-positive were excluded. The gap junction-mediated dye transfer was confirmed by the presence of red staining of rhodamine dextran at the edge of the scratched plane, because rhodamine dextran is 10 kd and cannot pass through the gap junction. Lucifer yellow, on the other hand, is 0.5 kd and can be transferred to other cells through gap junctions; therefore, it diffuses from the edge of the scratched plane to inner intact cells. Statistical analysis was performed using Student's *t*-test.

Peptide Synthesis

Wild-type and mutant peptides containing the 20 N-terminal amino acids of Cx26 (MDWGTLQTLGGVNHSTSI and MDWGTLQTLGGVYKHSTSI, respectively) were synthesized by Sigma Genosys (Japan).

NMR Spectroscopy

The peptides were dissolved to a final concentration of 2 mmol/L in 350 μ l of 90% H₂O/10% D₂O or 99.9% D₂O at pH 4.0. The pH was adjusted by adding μ l increments of HCl and NaOH. The NMR experiments were performed on JEOL ECA 600 or Bruker DMX 500 spectrometers. The NMR spectra, DQF-COSY,⁹ TOCSY,¹⁰ and NOESY¹¹ were recorded at 10°C, and some experiments were also recorded at 20 and 30°C to resolve ambiguities. TOCSY spectra with a MLEV-17 sequence were collected with spin-lock times of 90 ms, and NOESY spectra were obtained with mixing times of 100 to 300 ms. The chemical shifts were measured from the internal standard of sodium 2,2-dimethyl-2-silapentane-5-sulfate. All two-dimensional spectra were processed using NMRPipe software.¹²

Circular Dichroism (CD) Measurement

All measurements were performed on a Jasco J-725 spectropolarimeter (Tokyo, Jasco, Japan). Sample solutions were buffered with 10 mmol/L potassium phosphate buffer (pH 7.0) in various 2,2,2-trifluoroethanol (TFE) concentrations. Spectra were recorded at 25°C and at peptide concentrations of 0.2 mmol/L using a quartz cell with a path length of 1 mm. All of the spectra were baseline-corrected by subtracting buffer spectra.

Results

Patient

The patient was a 4-year-old Japanese girl with no family history of skin disorders or auditory dysfunction. From

Figure 1. Clinical figures of the patient at the age of 4. **A:** Hyperkeratotic plaques on her scalp. She wears a hearing aid on the left side because of sensorineural deafness. **B and C:** Hyperkeratosis of her palms and soles.

birth, impetiginous erythema had been observed on her neck, axilla, and perianal areas. At the age of 4 months, spiky white keratotic papules appeared on her palms and soles, and impetiginous plaques were also noted on her occipital area. At the age of 2 years, she was referred to the otolaryngological clinic, and profound sensorineural deafness was noted. At the age of 4 years, keratitis was found on both eyes. She was diagnosed with KID syndrome at this time. By the age of 4 years, hyperkeratotic plaques were scattered on her scalp and extremities (Figure 1A). Palms and soles were severely hyperkeratotic (Figure 1, B and C).

Mutation Analysis

Direct sequencing of the patient's genomic DNA, amplified by PCR, revealed that the patient was heterozygous for a novel missense mutation NML004004:c.40A>C resulting in the amino acid substitution asparagine to tyrosine (N14Y). This mutation results in the gain of a recognition site for the restriction enzyme Bsp1407I (Figure 2). N14Y was not found in her parents (Figure 2) and was

Figure 2. DNA sequences of GJB2. A heterozygous A to T transition at codon 40 is detected in the patient's gene (NML004004:c.40A>T). This mutation leads to the amino acid substitution N14Y. This missense mutation is *de novo* because it is not found in the parent's DNA. N14Y results in the gain of a Bsp1407I restriction site. PCR products from the patient (Pt) are digested into fragments of 733 and 943 bp. In contrast, the digested PCR products from the father (Fa) show only the 943-bp band.

Figure 3. Skin morphology and immunofluorescence study. **A:** H&E staining. Hyperkeratosis with focal parakeratosis and regular acanthosis with broad rete ridges are observed. Granular layer was lost and vacuolar change of cytoplasm was seen in the upper spinous layer. **B:** Electron microscopy in the patient's skin (granular layer of the epidermis). The gap junctions have a typical pentalaminar structure, 20 nm in width. Abnormal junctional structures were not found. **D:** Desmosome, **G:** gap junction. **C–F:** Cx26 and Cx43 expression of the patient's epidermis. Green (fluorescein isothiocyanate) indicates Cx26 expression. Red (propidium iodide) is nuclear staining. **C:** Cx26 was expressed in the keratinocytes in the widened rete ridge. **D:** The staining of the Cx26 in the keratinocytes was more cytoplasmic than membranous, although punctate membranous staining was also seen in the acrosyringium cells. Cx43 expression was observed in the keratinocytes in the middle and upper epidermal layers (**E**) and was mostly membranous (**F**). **G:** Cx26 staining in normal skin. There was no expression of Cx26 in the normal epidermis. The staining at the corneum seemed to be nonspecific. **H:** Cx43 staining in normal skin. The staining was observed at the membrane of epidermal keratinocytes. Scale bar = 100 nm.

thought to be a *de novo* mutation. In addition, this mutation was not found in 50 normal unrelated Japanese alleles (25 normal unrelated Japanese individuals) and was unlikely to be a polymorphism. Direct sequencing of the entire coding region and borders of GJB2 failed to detect any other pathogenic mutation in the patient's DNA.

Skin Morphology

Skin biopsy obtained from the lesional skin revealed hyperkeratosis with focal parakeratosis and regular acanthosis with broad rete ridges (Figure 3A). Granular layer was absent, and vacuolar change of cytoplasm was ob-

Figure 4. Dye transfer assay of cultured keratinocyte. **A:** NHEK. **B:** HaCaT. **C:** Keratinocytes of KID syndrome. The diffusion of Lucifer yellow of patient's keratinocytes was less than in NHEK and HaCaT cells. Rhodamine dextran (red) is observed at the edge of the scratched plane because the rhodamine dextran molecule is too bulky to pass through gap junctions (**D–F:** NHEK, HaCaT, and patient's keratinocyte, respectively). **G:** The number of Lucifer yellow-positive cells per unit length of scratched plane. The number of positive cells of the patient's keratinocytes was significantly smaller than that of NHEK and HaCaT, NHEK: 7.17 ± 1.14 (average $\pm$ SD); HaCaT: 7.43 ± 1.07 ; patient's cell: 4.34 ± 0.75 . * $P < 0.01$ by Student's *t*-test.

served in the upper spinous layer. Electron microscopy demonstrated gap junctions in all epidermal layers, with normal morphology showing typical pentalaminar structures 20 nm in width (Figure 3B). Immunofluorescence showed Cx26 expression in the upper layer of wide rete ridge in the patient's epidermis (Figure 3C), compared with the normal epidermis that does not express Cx26 (Figure 3G). The staining of Cx26 was more cytoplasmic than membranous in the keratinocytes of the upper layer, although punctate staining on cellular interface was also observed in the acrosyringium cells (Figure 3D). We also examined Cx43 expression in the patient's epidermis because Cx43 is the major Cx expressed in the epidermis. Cx43 staining was seen in the middle and upper epidermal layer, similar to the expression of Cx26 in the patient's skin (Figure 3E). The expression of Cx43 was almost completely membranous (Figure 3F) and seemed to be the same as in normal control skin (Figure 3H). The antibody used in the present study binds to both normal and mutant Cx26 peptides; thus, it was not clear whether the overexpressed Cx26 in the patient's epidermis was normal and/or mutated.

Dye Transfer

The transfer of dye between cells was observed in all cell types, although the degree of dye transfer of cultured patient's keratinocytes was less than in NHEK and HaCaT cells, indicating abnormality of gap junctional intercellular communication in the patient (Figure 4, A–C). The num-

Figure 5. Comparison of the fingerprint region from DQF-COSY spectra of wild type (**A**) and N14Y mutant (**B**). The mutation does not cause significant change in chemical shifts when compared to wild type.

ber of Lucifer yellow-positive keratinocytes per unit length of scratched plane was counted. The number of positive cells in cultured patient's keratinocytes was significantly smaller than that in NHEK and HaCaT cells ($P < 0.01$) (Figure 4G).

NMR Analysis

The chemical shift assignments of wild-type and mutant peptides were performed according to standard procedure of sequential assignment.¹³ An almost complete assignment of the proton NMR signals of wild-type and mutant peptides was obtained. A comparison between the DQF-COSY spectra of the fingerprint regions of wild type and mutant, with the results of assignment, is shown in Figure 5.

To compare clearly the structural properties of these two peptides, chemical shift differences of α -protons and amide protons are plotted in Figure 6. Figure 6A shows that the patterns of chemical shift deviation of the mutant peptide from random coil structure resemble that of wild-type peptide in both C α H and NH. In addition, continuous negative values of chemical shift deviations of C α H in the N-terminal region (Trp3-Leu10) suggest formation of helical conformation in both wild type and mutant. Thus, the three-dimensional structural properties of these two peptides are thought to be basically the same. This result is further confirmed by the data that large changes in shift between wild type and mutant are only observed for resonances around the mutated residue N14Y (Figure 6B). It is reasonable to think that the conformation of mutant peptide maintains its wild-type form because the chemical shifts of α -protons and amide protons are very sensitive to backbone conformation and secondary structure.

Figure 6. Chemical shift differences of amino acid residues in wild type and N14Y mutant. **A:** The differences in chemical shift defined as δ (wild-type or mutant peptide) - δ (random coil) are plotted. **B:** The differences in chemical shift defined as wild-type peptide - mutant are plotted.

Figure 7. NH-NH regions from NOESY spectra of wild-type (A) and N14Y mutant (B) peptides. Sequential NH-NH cross-peaks are annotated.

Unfortunately, the number of NOEs (nuclear Overhauser effects) observed in our experiments was not sufficient for structural calculation. However, the NOE data did suggest the formation of an α -helical conformation, especially in the N-terminal region. Figure 7 shows the NH-NH region from NOESY spectra of wild-type and mutant peptide. In both spectra, a number of sequential NH-NH NOEs were observed and these results indicated that these peptides have propensity to adopt α helical structure. Although the basic NOE pattern of the mutant peptide agreed with that of wild-type one, some novel NOEs were observed from Tyr14 (Figure 8). In particular, a number of medium-range NOEs from the side chain of Tyr14, such as between δ H of Tyr14 and α H of Gly12, ϵ H of Tyr14, and α H of Gly 12, ϵ H of Tyr14 and α H of His16 were observed. In contrast, no medium-range NOEs were observed in this C-terminal region in the case of wild-type peptide.

CD Measurement

The far-UV CD spectra were recorded for wild-type and mutant peptides derived from the N-terminal 20 amino acids of Cx26 (Figure 9). In water, the CD spectra of both wild-type and mutant peptides showed random-like conformational features (Figure 9A). The α -helix contents of these peptides are estimated to be considerably low judged from the value at 222 nm. On the other hand, in

Figure 8. Novel NOE signals in N14Y mutant.

the case of CD spectra in TFE solution, the α -helical contents increased with increasing TFE concentration (Figure 9B). Although the conformational changes were induced by TFE in both wild-type and mutant peptides, α -helical structure was induced more easily and strongly in the case of mutant one.

Discussion

Eighty different types of Cx26 mutations have been reported in congenital deafness disorders (refer to the Cx-deafness homepage at <http://davinci.crg.es/deafness/>). Cx26-related nonsyndromic deafness is inherited in an autosomal dominant or recessive manner whereas Cx26-related syndromic deafness shows only an autosomal dominant inheritance trait. The reported mutations in syndromic Cx26-related deafness are summarized in Figure 10.^{4,14–23} To date, five Cx26 mutations resulting in KID syndrome have been reported in the literature.^{4,15,16} Among them, two mutations, G12R and S17F, exist in the N-terminal domain of Cx26, similar to our case (N14Y).

The asparagine (N) at position 14 is highly conserved across the species and also across Cx families (GJB1 to 6), supporting the pathogenicity of this mutation.¹⁴ In addition, this mutation was not found in the normal control samples in this study, nor was it detected in another report that checked 192 control Japanese alleles.²⁴ N14K mutation in Cx26 was reported recently in a patient with Clouston syndrome-like phenotype with deafness.¹⁴ In this report the position of the mutated amino acid is identical to that of our study; however, the resultant clinical phenotype differs markedly from our patient. This patient had congenital sensorineural deafness but no keratitis, and the skin showed only mild erythrokeratoderma, mild hypotrichosis, and brittle nails. These clinical differences indicate that the chemical property of altered amino acid may be crucial for the phenotype in addition to the position of the mutation. Tyrosine (Y) has a benzene ring, and lysine (K) has an amino residue; thus the difference of the chemical characters of altered amino acid may influence Cx assembly and gap junction channel selectivity because of their molecular sizes, electronic charges, and hydrophilicity. It is interesting that another

Figure 9. Far-UV CD spectra of wild-type and mutant peptides. Wild-type (blue) and mutant peptide (red) were measured in water (A) and in various TFE concentrations (B, C). The numbers indicate the percentage of TFE.

mutation in the N terminus of Cx26 in KID syndrome, S17F (serine to phenylalanine), is similar to the N14Y mutation in that both mutations result in the replacement of a hydrophilic amino acid to one with a benzene ring.

Despite the mutation of Cx26 in our patient, the morphology of gap junctions observed by electron microscopy seemed to be normal, and disturbed adhesional structures were not observed in the specimens. In a report of erythrokeratoderma variabilis with Cx31 mutation, morphological changes of gap junctions were also not observed.²⁵ In our case, the mutation was in the cytoplasmic side; therefore, the coupling of connexons with those of opposite cells at the extracellular site might not be affected, resulting in normal morphology of gap junctions. Otherwise, the normal gap junction structures seen in the patient's skin sample may consist of Cx43, which was expressed normally in the patient's skin.

By immunofluorescence study, the expression of Cx26 was observed in the upper layer of the epidermis in our patient. In normal skin, Cx26 expression is not seen in the epidermis, although strong expression is observed in the hair follicle and the sweat duct and gland.²⁶ However, in some of the hyperkeratotic skin disorders such as psoriasis, Cx26 expression is seen in the upper layer of the epidermis.²⁷ Cx26 expression in the epidermis of patients with KID syndrome has also been previously report-

ed.⁴ The Cx26 staining in the epidermis of our patient confirmed *in vivo* expression of Cx26 in the patient's keratinocytes and supported the pathological significance of this protein for the hyperkeratotic changes of the epidermis.

The transfer of dye between cells was reduced in the patient's keratinocytes compared to NHEK and HaCaT cells. This result directly proved that the channel function of gap junctions was affected by the Cx26 mutation, N14Y. The dye transfer was not completely impaired in the patient's keratinocytes, probably because other Cx molecules, such as Cx43, can work partly independently of mutated Cx26. However, certain Cx26 mutations were reported to dominantly inhibit normal Cx43 function, demonstrated by coupling transfection of the mRNA of wild-type and mutant Cxs into *Xenopus* oocytes.²⁸ Therefore, a part of impairment of gap junctional intercellular communication may be because of trans-dominant inhibition of mutated Cx26 to functions of other intact Cxs.

Our NMR data of N-terminal peptides of human Cx26 in water suggested that both wild-type and mutated peptides have basically the same conformational feature. The chemical shift differences of CαH between the experimental shifts for the peptides and random coil shifts (Figure 6) clearly suggested that these peptides have a tendency to form α-helical structures, except for a few residues in the C termini. In addition, NOE data also showed formation of α-helix-like conformation in some N-terminal residues. However, the number of NOEs observed in our studies was not sufficient to confirm that these peptides form rigid α-helical conformation in water. In addition, CD data also suggested that helical contents of these peptides were low in water although α-helical structures were induced by considerably low TFE concentrations. Thus, these peptides are likely to have relatively high flexibility in water even though rigid helical structures are easily induced by nonpolar environments, such as in TFE solvent. In water, there were no significant differences between CD spectra of these peptides. However, the secondary structure of mutant peptide is susceptible to induction of TFE. Thus, the mutation may change the conformational flexibility of the peptide and the helical propensity.

Recently, sequence-specific ¹H NMR resonance assignment for the N-terminal 15-amino acid peptide derived from rat Cx26 have been reported.²⁹ The primary structures of the N-terminal domain of Cx26 are highly

Figure 10. The Cx26 mutations of syndromic sensorineural deafness reported in the literature. The mutations of KID syndrome are aggregated to the N-terminal side of Cx26, especially in the cytoplasmic portion.

conserved in mammals, and the difference between human and rat is only one residue (Thr8 in human to Ser8 in rat) in this N-terminal region. Our peptides synthesized from human Cx26 sequence are slightly longer than the peptide derived from rat (15 residues long). The results of NMR studies from rat Cx26 showed that the peptide has a more highly ordered structure than our human Cx26 peptide although the conformational features of rat Cx26 peptides basically agree with our human one. These peptides are highly homologous except in their length. Thus, the difference in flexibility may be attributable to the extension of C-terminal residues. Previous analysis of the rat Cx26 peptide suggested the importance of flexibility in the hinge region (Gly12 and Gly13) in the placement of the N-terminal residues within the channel pore. In the present study, some NOEs to these glycine residues, which were not observed in the wild-type peptide, appeared in the N14Y mutant. Thus, it is likely that the N14Y mutation induced a change in local flexibility and that the motion of the N-terminal residues, which are important for the channel function of the protein, was altered profoundly.

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