Defective trafficking and cell death is characteristic of skin disease-associated connexin 31 mutations

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Distinct germline mutations in the gene (GJB3) encoding connexin 31 (Cx31) underlie the skin disease erythrokeratoderma variabilis (EKV) or sensorineural hearing loss with/without peripheral neuropathy. Here we describe a number of functional analyses to investigate the effect of these different disease-associated Cx31 mutants on connexon trafficking and intercellular communication. Immunostaining of a biopsy taken from an EKV patient harbouring the R42P mutation revealed sparse epidermal staining of Cx31, and, when present, it had a perinuclear localization. Transfection and microinjection studies in both keratinocytes and fibroblast cell lines also demonstrated that R42P and four other EKV-associated mutant Cx31 proteins displayed defective trafficking to the plasma membrane. The deafness/neuropathy only mutant 66delD had primarily a cytoplasmic localization, but some protein was visualized at the plasma membrane in a few transfected cells. Both 66delD- and R32W-Cx31/EGFP proteins had significantly impaired dye transfer rates compared to wild-type Cx31/EGFP protein. A striking characteristic feature observed with the dominant skin disease Cx31 mutations was a high incidence of cell death. This was not observed with wild-type, R32W 66delD Cx31 proteins. In conclusion, we have identified some key cellular phenotypic differences with respect to disease-associated Cx31 mutations.

INTRODUCTION

Gap junctions provide a mechanism of synchronized cellular response to a variety of intercellular signals by regulating the diffusion of small molecules (<1 kDa) such as metabolites and ions between the cytoplasm of adjacent cells (1). Connexins are the major proteins of gap junctions and are encoded by a large gene family (2). All connexins have four transmembrane domains and two extracellular loops with the N- and C-termini located in the cytoplasm. Each connexin assembles into hexameric hemichannels (termed connexons) in the endoplasmic reticulum and are then transported into the lipid bilayer of the plasma membrane. A connexon then docks with a connexon of an adjacent cell to form a dodecameric aqueous channel. These intercellular channels, cluster in the cell to form the gap junctions. Connexons can form either homotypic or heterotypic channels, with various channel types having distinct molecular permeabilities (3,4).

The epidermis is a stratified squamous epithelium comprising predominantly keratinocytes. The keratinocyte undergoes a process of terminal differentiation resulting in a stratum corneum, the critical component for barrier function. There are abundant gap junctions and connexin proteins in the epidermis (5). We have recently shown that at least 10 connexin proteins are expressed in the human epidermis, with the junctional composition and cellular localization changing during keratinocyte differentiation (6). Compelling evidence for a key role for connexins in epidermal biology has come from the association of mutations in four connexins with hyperproliferative skin disorders (7–10). These genetic studies demonstrate that intercellular communication via gap junctions is an important mechanism by which the normal epidermis develops and differentiates.

One such skin disease is erythrokeratoderma variabilis (EKV), in which a number of mutations in the gene encoding Cx31 have been demonstrated (7,11,12). EKV presents as a diffuse thickening of the palmoplantar epidermis, symmetrically distributed fixed hyperkeratotic plaques and transient erythematous areas (13). Both the hyperkeratosis and erythematous patches can be triggered by trauma to the skin, temperature
changes, UV exposure and emotional stress. Like Cx26 and Cx30 (reviewed in 14), dominant Cx31 mutations have also been described associated with hearing loss with no epidermal manifestations (15). Adding further complexity to the biology of Cx31 and its role in disease is the recent identification of another dominant GJB3 mutation, 66delD, segregating in a family with peripheral neuropathy and sensorineural hearing loss (16). Though recessive GJB3 mutations have been associated with hearing loss (17), more recently the L34P mutation in GJB3 has been shown to underlie a recessive form of the skin disease EKV (18). Thus the spectrum of GJB3 mutations described to date results in a variety of genetic disorders.

Human genetic disease studies suggest that different GJB3 mutations have distinct effects on the Cx31 protein in terms of epidermal differentiation, auditory transduction and peripheral neuronal function. Here, we demonstrate genotype–phenotype differences at the cellular level and identify cell death as a common mechanism linked with EKV-associated Cx31 mutations.

RESULTS

Perinuclear localization of Cx31 in EKV-affected epidermis

Cx31 is expressed in late keratinocyte differentiation, localizing at gap junctions in the stratum granulosum of epidermis (Fig. 1A) (6). To look at the effect of an EKV-associated mutation on Cx31 localization, immunocytochemistry using a polyclonal Cx31 antibody was performed on frozen tissue sections taken from an EKV patient heterozygous for the R42P mutation in GJB3 (11). The majority of Cx31 staining in the patient’s epidermis was perinuclear, with little punctate staining observed (Fig. 1C). These in vivo data indicate that R42P effects the trafficking of Cx31 to the plasma membrane and also possibly reduces the expression level of Cx31 in keratinocytes. These two observations also indicate that R42P can dominantly disrupt the trafficking of wild-type Cx31, which is in agreement with its autosomal dominant mode of inheritance. From the antibodies available, the R42P in Cx31 does not appear to effect the expression and localization of other epidermally expressed connexins, including Cx26, Cx30, Cx30.3 and Cx43 (data not shown).

Localization of mutant Cx31-EGFP

To investigate the localization of other mutant forms of Cx31, we examined the cellular localization of wild-type (WT) Cx31/EGFP and various mutant Cx31/EGFP 24 hours post-transfection (Table 1). Cells expressing WT-Cx31/EGFP showed punctate staining and aggregation at the plasma membrane, especially in regions of cell–cell contact (Fig. 2A, B and I). In contrast, none of the mutant clones formed membrane-associated aggregates. As with the keratinocytes endogenously heterozygous for R42P, the R42P-Cx31/EGFP was cytoplasmic and had a perinuclear protein localization in expressing cells (Fig. 2C and J). Similar to R42P-Cx31/EGFP, the other mutant Cx31 proteins associated with EKV—C86S-Cx31/EGFP, G12R-Cx31/EGFP and G12D-Cx31/EGFP—also had a cytoplasmic localization (Fig. 2D, E and F). Immunocytochemistry for the keratin and actin filaments in transfected NEB1 cells using keratin LP34 antibody and a β-actin antibody confirmed a cytoplasmic location for these mutant proteins and also revealed an intact keratin and actin filament network among those cells expressing either wild-type or mutant Cx31/EGFP. Another intriguing phenotype was observed for all of the EKV-associated Cx31/EGFP mutants: many of the transfected cells became rounded, detached and died. This suggested that these skin disease-associated mutant Cx31 proteins were inducing cell death.

A slightly different cellular distribution was observed for the mutant Cx31 associated with peripheral neuropathy and hearing impairment. Though the majority of 66delD-Cx31/EGFP was cytoplasmic (Fig. 2G), some punctate staining at the plasma membrane was observed. Another distinct localization was observed for R32W-Cx31/EGFP, a rare polymorphic variant of GJB3 with no identified disease association, which showed a punctate staining pattern, but with no detectable aggregation at the plasma membrane between R32W-Cx31/EGFP-expressing cells (Fig. 2H). In contrast to the EKV-associated Cx31/EGFP mutants, cells transfected with these mutant constructs exhibited the normal wild-type cell culture phenotype with no increased levels of cell death.

Dye transfer studies of WT-Cx31/EGFP, R32W-Cx31/EGFP and 66delD-Cx31/EGFP

Prior to performing the microinjection dye transfer studies, immunocytochemistry using a panel of connexins—Cx26, Cx30, Cx31, 32, 40, 43 and 45—was performed on both human NEB1 keratinocyte-derived cells and the murine NIH 3T3 fibroblast cells. Immunostaining analysis in NEB1 cells showed low levels of Cx26, 30, 32, 40, 43 and 45, but abundant Cx31 expression with an irregular distribution. NIH 3T3 cells have previously been shown to express Cx43 (19), and, in addition, we found the expression of a number of other connexins, including Cx31 (data not shown). This indicates that both cell lines used in this study express a number of connexins endogenously.

WT-Cx31/EGFP-expressing NEB1 cells exhibited an enhanced rate of dye transfer compared with non-transfected cells. Dye transferred most rapidly through adjoining fluorescent cells when compared to their non-fluorescent neighbours (Figs 3A–D and 6). However, the extent to which dye transferred between adjoining fluorescent cells varied widely between experiments, possibly reflecting a difference in expression levels of endogenous protein or channel activity states between cells in the local population. Indeed, a similar variation in dye transfer efficiency could also be seen in the uninjected cell population, with some cells exhibiting a significant ability to transfer dye while others showed only poor transfer rates. These experiments were also performed in NIH 3T3 fibroblasts. Though these cells express endogenous Cx31 and other connexins, basal dye transfer levels were poor, with dye rarely propagating further than those cells directly in contact with the injected cell. In dramatic contrast, however, dye rapidly propagated through the WT-Cx31/EGFP-expressing population (Fig. 6). The steady rate at which dye transferred through Cx31-expressing cells suggests that channels exhibited similar activity states and that endogenous
protein levels were sufficiently low not to influence the effects of the exogenous protein.

Unlike WT-Cx31/EGFP, the expression of 66delD-Cx31/EGFP did not significantly enhance the rate of dye transfer in either NEB1 cells (Figs 3I-L and 6) or NIH 3T3 fibroblasts (Fig. 6), indicating that the mutant form cannot form functional channels. In addition, R32W-Cx31/EGFP also did not form functional channels. Expression of R32W-Cx31/EGFP in NEB1 cells did not elevate dye transfer rates above basal levels (Figs 3E–H and 6), and little or no dye transfer was observed in fibroblasts (Fig. 6). R32W in GJB3 has been reported as a polymorphism with no proven disease association (20,21). However, we have hypothesized that it may have a modifying effect with respect to epidermal disease severity or hearing loss when associated with other connexin mutations. This has been debated further (22). Our data show that R32W does not form

Figure 1. Cx31 localization in normal and EKV epidermis. (A) Palm epidermis from an individual with wild-type GJB3. Note the evenly distributed punctate membranous pattern of Cx31 in the stratum granulosum. (B) Control immunostaining of normal palm epidermis without specific primary Cx31 antibody. Non-specific staining is seen in the stratum corneum. (C) Lesional epidermis from an individual with EKV heterozygous for R42P in GJB3. Note the sparse and perinuclear Cx31 immunostaining in the stratum granulosum. (D) Haematoxylin and eosin staining of lesional epidermis from the same EKV patient as in (C).
functional intercellular channels and has impaired dye transfer, suggesting that it is a non-functional variant of GJB3.

Microinjection studies of R42P-Cx31/EGFP- and C86S-Cx31/EGFP-induced cell death

From our transfection studies, expression of R42P-, C86S-, G12R- and G12D-Cx31/EGFP in NEB1 cells resulted in increased cell death. This phenomenon did not appear in cells transfected or microinjected with WT-, 66delD- and R32W-Cx31/EGFP constructs. Using a combination of microinjection and digital time-lapse microscopy, we investigated further the cellular phenotypes for two of the skin disease-associated Cx31 mutants, R42P-Cx31/EGFP and C86S-Cx31/EGFP. Interestingly, R42P-Cx31/EGFP exhibited two distinctive cellular phenotypes. The first was an immediate change in cell morphology, including extensive cell blebbing and nuclear shrinkage, phenotypes associated with cell death (Fig 4A and B). Cells poorly adhered to the substrate and were difficult to microinject. In cases where microinjection was successful, dye did not transfer to neighbouring cells but merely leaked out from the cell. Cells also showed positive nuclear labelling with propidium iodide, suggesting a disrupted plasma membrane (Fig. 4C and D). As with the other localization studies described previously, R42P-Cx31/EGFP had a perinuclear localization. The second phenotype was similar to that of un.injected cells, with some protein present at the plasma membrane, and no cell death (Fig. 6). As with R42P-Cx31/EGFP, C86S-Cx31/EGFP-expressing NEB1 cells also exhibited two major phenotypes; those that showed profound blebbing and nuclear shrinkage, and those that were similar to the uninjectected cell population. The blebbing phenotype corresponded with positive nuclear labelling with propidium iodide (Fig. 4E and F). Initially, dye transfer was occurring through fluorescent cells, but as the cells changed morphology, the dye leaked out of the cell, as for R42P-Cx31/EGFP. In the phenotype where membrane integrity remained intact throughout, the protein showed both vesicular localization and, to some degree, punctate membrane localization, although not as striking as is seen with the wild-type protein. Indeed, dye transfer did seem to propagate preferentially through the C86S-Cx31/EGFP-expressing cells associated with this second phenotype when compared to the surrounding non-fluorescent cell population (Fig. 6). This ‘normal’ phenotype was not observed in NIH 3T3 cells for either R42P-Cx31/EGFP or C86S-Cx31/EGFP (Fig. 6).

DISCUSSION

From immunocytochemical analysis of affected epidermis from an EKV patient harbouring the R42P mutation in Cx31 and studies using a R42P-Cx31/EGFP construct, a cytoplasmic localization of the mutant protein was revealed. Previously, we had described no obvious structural abnormalities in the skin biopsy from this patient (11). In particular, gap junctions were still present in different epidermal layers, with no observable gross abnormalities by light microscopy and electron microscopy. We speculated that one possible mechanistic effect of the R42P mutation in GJB3 on connexin 31 would be to alter gating polarity, resulting in defective channel formation and regulation. Another hypothesis was that the substitution of arginine 42 by proline would have a wider structural destabilization effect on connexon formation. The functional data described in this study would support the latter mechanism, due to a cytoplasmic localization of the protein with undetectable Cx31 protein at the plasma membrane. Surprisingly, all the other EKV-associated Cx31 mutant proteins assayed also displayed trafficking defects with retention in the cytoplasm, suggesting that this is a common cellular phenotype. It is possible that these particular Cx31 mutant proteins may also disrupt the assembly of other connexon types in the Golgi and endoplasmic reticulum (ER). From immunocytochemistry of epidermis from an EKV patient harbouring the R42P mutation in Cx31, no obvious alterations in the localization and distribution of other connexins were observable.

All the other EKV-associated Cx31 mutants also displayed the same trafficking defects as R42P and had a cytoplasmic localization. Previously, specific mutations in Cx32 have also been shown to have trafficking defects accumulating in the cytoplasm and also within the Golgi apparatus (23,24). Interestingly, one of these Cx32 mutations is G12S. This is the same conserved residue mutated in Cx31 (G12R and G12D), and these Cx31 mutants also display similar trafficking defects. These data suggest that this residue in the N-terminus of connexins is important for the correct folding of the connexin and/or its assembly into connexons. A disruptive conformational change for the EKV mutations R42P and C86S in Cx31 that lie adjacent to or within transmembrane domains, respectively, may also lead to retention in the cytoplasm and possibly the Golgi apparatus/ER.

The other characteristic feature associated with the EKV Cx31 mutants was increased cell death, particularly striking in the NIH 3T3 cells. Although the mechanism of cell death is not known, it could possibly relate to increased intracellular Ca²⁺ and/or plasma membrane disruption. Evidence from the microinjection experiments indicated that the plasma membrane was very ‘leaky’ in the two EKV-associated Cx31 mutants investigated. From the microinjection studies for R42P- and C86S-Cx31/EGFP in the keratinocyte cell line, two cellular phenotypes were observed. Predominantly, these mutants induced blebbing and cell death. However, some microinjected cells did not die. This difference in the cellular processing of the microinjected mutant Cx31 constructs could

Table 1. Genotype and phenotype of mutated Cx31

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Phenotype</th>
<th>Primers for SDM⁴</th>
<th>References</th>
</tr>
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<tr>
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<td>EKV</td>
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<tr>
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</tr>
<tr>
<td>G12D</td>
<td>EKV</td>
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<tr>
<td>C86S</td>
<td>EKV</td>
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<td>7</td>
</tr>
<tr>
<td>66delD</td>
<td>Neuropathy and hearing loss</td>
<td>GCACCAACTGGTCTGAC</td>
<td>7</td>
</tr>
<tr>
<td>R32W</td>
<td>Of unknown disease significance</td>
<td>GGTGTCTTCGTCGTGGTGTC</td>
<td>20, 21</td>
</tr>
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⁴The mutated site is bold and mutated or deleted codon underlined.
be related to the inherent variability in different cell populations. For example, in the NEB1 keratinocytes, it could relate to the variable expression of endogenous connexins and/or the differentiation status of different cell populations. Further studies are in progress to understand why these two different in vitro cell phenotypes occur and if this variable processing is dependent on the cellular background. These studies may help to explain further the genotype–phenotype correlation of Cx31 and other connexin mutations.

The 66delD mutation is associated with a dominant syndrome of hearing loss and peripheral neuropathy (16). The localization studies and dye transfer experiments support the deleterious nature of this mutation with respect to Cx31 channel formation (Fig. 2G and 6). Interestingly, 66delD in Cx31 affects the same residue mutated in Cx26, D66H, which associates with Vohwinkel’s syndrome: palmoplantar keratoderma and mild–moderate hearing loss (8,20). Like 66delD-Cx31/EGFP, D66H-Cx26 has defective trafficking with primarily a cytoplasmic localization (25).

Though R32W-Cx31/EGFP could traffic to the plasma membrane, it never formed large aggregates of protein that are indicative of connexon channels, and was unable to transfer dye efficiently. Previously, it has been shown that, for Cx43, the formation of plaques is a requirement for intercellular communication as measured by dye-coupling experiments (26). R32W in GJB3 results in a substitution of a highly conserved arginine residue in the first transmembrane domain of the Cx31 protein and appears to be a relatively rare coding polymorphism (22). We have investigated further its prevalence in a number of ethnic control populations. R32W was found in a heterozygous form in 3% of Irish, 0.7% of Afro-Caribbeans and 2.5% of Asians. In all control samples tested (n=364), the overall allele frequency was 1.9%. As no R32W homozygotes were detected, it is still plausible that it is a recessive GJB3 allele. However, it has been demonstrated that L34P in GJB3 and M34T in GJB2, which both occur in the first transmembrane domain, are recessive alleles underlying EKV and mild–moderate hearing loss, respectively (18,27,28). As our functional data suggest that R32W has a

Figure 2. Localization studies in NEB1 keratinocytes transfected with (A and B) WT-Cx31/EGFP, (C) R42P-Cx31/EGFP, (D) C86S-Cx31/EGFP, (E) G12R-Cx31/EGFP, (F) G12D-Cx31/EGFP, (G) 66delD-Cx31/EGFP, (H) R32W-Cx31/EGFP, (I) WT-Cx31/EGFP and (J) R42P-Cx31/EGFP. Green fluorescence indicates the EGFP fusion connexin protein. Red fluorescence shows β-actin (A–H) and keratin filaments I, J. Note the conspicuous aggregation of wild-type Cx31 at the plasma membrane between the cells. In contrast, the majority of the mutant Cx31/EGFP proteins, e.g. R42P-Cx31/EGFP, were cytoplasmic, with less apparent membrane localization. Note the appearance of an intact keratin filament network.
deleterious effect on Cx31 plaque formation and dye transfer, we postulate that homozygosity for R32W may result in an unknown, recessive disease phenotype. Further genetic studies and analyses need to be performed to assess a possible disease association of this Cx31 variant.

In summary, our in vivo and in vitro data demonstrate that R42P in Cx31 prevents the trafficking of the protein to the membrane. This abnormal localization appears to lead to cell death. Our in vitro data indicate that trafficking defects and cell death are common mechanisms of mutant Cx31 associated with EKV. It is not clear how these two cellular phenotypes associate with the hyperkeratosis seen in the patients. It is possibly related to the loss of Cx31-mediated intercellular communication during terminal keratinocyte differentiation and/or the induction of cell death invoking a wound-healing response in surviving keratinocytes, leading to hyperproliferation. In addition, the transient nature of the erythematous plaques may correspond to the differential effects of the EKV-associated mutations in different keratinocyte populations. These data suggest that trafficking defects represent a common feature of epidermal disease-associated connexin mutations and constitute a step towards understanding the intriguing genotypes-phenotypes produced due to β-connexin mutations. The nature of the mechanism of cell death associated with the skin disease Cx31 mutants remains to be elucidated.

Figure 3. Dye transfer analysis using Alexa 568 in NEB1 cells microinjected with Cx31/EGFP fusion constructs. Images show green (EGFP)/red (Alexa568) fluorescence overlays without phase contrast (left column) and with phase contrast (right column) to indicate surrounding un.injected cells. NEB1 cells are expressing WT-Cx31/EGFP (A)-(D), R32W-Cx31/EGFP (E)-(H), and 66delD-Cx31/EGFP (I)-(L). Images (A), (B), (E), (F), (I) and (J) represent the first time point of the time-lapse sequences, and the remaining images were acquired 33 min later. Note the efficient dye transfer between WT-Cx31/EGFP-expressing cells but reduced transfer between cells expressing R32W-Cx31/EGFP and 66delD-Cx31/EGFP. Asterisk indicates the cell microinjected with Alexa 568 dye. Scale bar 50 μm.

Figure 4. Illustration of the induction of cell death following the microinjection of R42P-Cx31/EGFP and C86S-Cx31/EGFP constructs in NEB1 cells. (A and B) show Green (EGFP)/red (Alexa568) fluorescence overlays without phase contrast (left column) and with phase contrast (right column). (C–F) Green (EGFP)/red (propidium iodide) fluorescence overlays without phase contrast (left column) and with phase contrast (right column). NEB1 cells express R42P-Cx31/EGFP in (A)–(D) and C86S-Cx31/EGFP in (E)–(F). Note the blebbing phenotype (examples of blebs indicated by arrows), the nuclear shrinkage and the intense nuclear propidium iodide staining showing cell death. Scale bar 50 μm.
MATERIALS AND METHODS

Tissue processing and cell culture

Skin biopsy material was obtained and processed from a consenting individual with EKV who is heterozygous for an arginine-to-proline substitution in codon 42 (R42P) in connexin 31 (Cx31) (11). The biopsy was immediately snap-frozen in liquid nitrogen and stored at −70°C for immunocytochemical analysis. This study has ethical approval.

NEB1 cells, which were immortalized from normal keratinocytes by HPV16 (29), were cultured in 3 : 1 Dulbecco’s modified eagle’s medium (DMEM) F12 medium, supplemented with 10% fetal calf serum (FCS), 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 10 μg/ml transferrin, 2 μg/ml streptomycin, and 5 μg/ml penicillin in 50 mM Tris-HCl, pH 8.5. Images were recorded using a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss) and processed using Adobe Photoshop 6.

Immunofluorescence staining and confocal microscopy

Immunofluorescence staining was performed in frozen tissue sections (6 μm thickness) and fixed cells using methods previously described (6). The following connexin antibodies were used: monoclonal anti-mouse Cx26 (dilution 1 : 100; Zymed, San Francisco, CA, USA), polyclonal rabbit anti-mouse Cx30 (dilution 1 : 100; Zymed), polyclonal rabbit anti-human Cx31 (dilution 1 : 100 (6)), monoclonal anti-rat Cx32 (dilution 1 : 100; Zymed), polyclonal rabbit anti-mouse Cx40 (dilution 1 : 100; Alpha Diagnostic, San Antonio, TX, USA), monoclonal anti-rat Cx43 (dilution 1 : 100; Zymed) and polyclonal rabbit anti-mouse Cx45 (dilution 1 : 50; Alpha Diagnostic). Keratin filaments were stained with monoclonal pan-keratin antibody LP34 (30), and actin filaments were stained with monoclonal anti-mouse β-actin (Sigma, Poole, Dorset, UK). Briefly, cells were fixed with 4% paraformaldehyde for connexin antibody staining, 100% methanol for β-actin staining and 50 : 50 acetone/methanol for the pan-keratin antibody. Sections were then rinsed with phosphate-buffered saline (PBS) and incubated in PBS containing 3% rabbit or goat serum and 0.1% Triton X-100 (Sigma) for 10 min at room temperature. After rinsing, the slides were incubated in a humidified chamber at 37°C for 2 h with primary antibody. Following several rinses with PBS, sections were incubated in the dark for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (Molecular Probes, Oregon, USA) diluted 1 : 100. After rinsing with PBS, the sections were mounted in mowiol reagent containing 10% mowiol D-488 (Calbiochem, Nottingham, UK), 25% glycerol and 2.5% 1,4-diazabicyclo[2.2.2] octane (Sigma) in 50 mM Tris-HCl, pH 8.5. Images were recorded using a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss) and processed using Adobe Photoshop 6.

Construction of chimeric Cx31/EGFP

PCR was carried out on wild-type (WT) human genomic DNA. The open reading frame of WT-Cx31 was fused in-frame to the N-terminus of enhanced GFP (EGFP) by a two-step cloning procedure. First, the authentic connexin stop codon was changed from TGA to GGA by PCR using the primers with a restriction site sequence SacII, 5’-GCCGCCGATGGAAGACTGGAACG-3’ for forward and 5’-GCCCCGCCTTCCGATGGGGCTAC-3’ for reverse, and was cloned directly into a TA cloning vector, pGEM-T (Promega, Southampton, UK). Second, the SacII fragment of Cx31 from pGEM-T/Cx31 was further cloned into pEGFP-N3 vector (Clontech, Hampshire, UK). R42P mutant Cx31 gene was generated from patient genomic DNA using the primers with restriction site sequences HindIII and SalI, 5’-GGCAGAATTCATGGACTGGAAGACACTC-3’ for forward and 5’-GGCTATGGACGCTCGTCG-3’ for reverse. The PCR product was directly cloned into pEGFP vector. In this orientation, the EGFP was at the C-terminus of the WT-Cx31 and R42P-Cx31 protein and the stop codon was altered to allow ‘read through’ and expression of the EGFP protein. All positive clones were identified by restriction enzyme analysis and automated DNA sequencing to determine that there were no ‘introduced’ nucleotide errors in both clones.

Site-directed mutagenesis (SDM)

The five other Cx31 mutations were independently introduced into WT-Cx31/EGFP by SDM. Their abbreviations, clinical phenotypes and the primers used for SDM are listed in Table 1. The WT-Cx31/EGFP sequence was converted using the Quickchange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. Briefly, PCR reactions using overlapping primers containing the base pair change were carried out using the supercoiled double-stranded DNA from pWT-Cx31/EGFP as a template. Then, the products were directly transformed into competent JM109 E. coli (Clontech) and several colonies were selected and sequenced. Colonies with the correct base change and no additional mutations were selected for subsequent study.

Figure 5. Processing of red fluorescence images (A and C) for calculations of normalized mean distance of Alexa 568 dye transfer in NIH 3T3 cells expressing WT-Cx31/EGFP. A and B represent time point 0 min; C and D 30 min. B and D show post-processed pseudocoloured images used for mean dye distance calculations. Scale bar 50 μm. Minimum intensity in the pseudocolour scale bar is represented in blue, and maximum intensity in white.
Transfection with Cx31/EGFP fusion constructs

NEB1 cells were transfected with the pWT-Cx31/EGFP and each of the mutated pCx31/EGFP constructs using Transfast (Promega). Cells (0.5–1 × 10^5/10 mm coverslip) were transfected with 0.5 mg of plasmid DNA in 1:2 Transfast reagent. Twenty-four hours post-transfection, cells were fixed with 4% buffered paraformaldehyde for the intracellular localization analysis and immunofluorescence staining.

Nuclear microinjection of Cx31/EGFP fusion constructs

NEB1 keratinocytes and NIH 3T3 fibroblasts were seeded in MatTek dishes and allowed 3 days to grow to near confluency. Culture medium for microinjection and imaging was supplemented with 20 mM HEPES. A transjector and micromanipulator (Eppendorf) mounted on a Zeiss Axiovert 135 TV inverted microscope (Carl Zeiss) were used for microinjection of plasmid DNA–EGFP fusion constructs. Groups of adjacent cells in a culture were injected with one of the constructs at a concentration of 0.05 mg/ml to create tight patches of fluorescent cells. Cells were allowed 4–5 h to express the proteins.

Measurement of dye transfer rate

For all dye transfer experiments, patches of fluorescent cells were identified and single cells close to the patch perimeter were microinjected in the cytoplasm with 2 mM Alexa 568 dye (Molecular Probes). The occurrence of dye transfer between adjacent cells was determined using digital time-lapse

Figure 6. Summary of dye transfer and cell death in NEB1 and NIH 3T3 cells expressing different Cx31 mutations. Top left plot shows time courses of normalized mean dye transfer distances represented by mean values (points) and standard errors of sample mean (bars) for groups of experiments with NEB1 cells expressing WT-Cx31/EGFP (open box), C86S-Cx31/EGFP (open star), R42P-Cx31/EGFP (filled star), R32W-Cx31/EGFP (filled diamond), and NIH 3T3 cells expressing WT-Cx31/EGFP (filled box), R32W-Cx31/EGFP (filled triangle) and 66delD-Cx31/EGFP (open triangle). The top right bar chart presents means and standard errors of relative incidence of viability and death found in NEB1 cells expressing the EKV-associated Cx31 mutations R42P and C86S. Propidium iodide-negative cells with absence of nuclear shrinkage were scored as viable, and propidium iodide-positive cells as dead. The bottom table overviews the numerical representation and statistical significances of the effects of different Cx31 mutations on both the rate of dye transfer and cell survival in NEB1 and NIH 3T3 cells. The mean dye transfer rate for each treatment is calculated as the mean slope of the linear regression of the dye transfer distance time courses and expressed in micrometres per hour. The P-values represent significances of differences in dye transfer rates or incidence of cell death between cells expressing the relevant Cx31 mutation as compared to the WT protein.
Fluorescence images of the Alexa 568 dye were cropped, binned to a pixel size of 2.6 × 2.6 μm and automatically thresholded (Fig. 5). The site of dye microinjection was interactively marked using a mouse pointer, and normally thresholded. The distance of dye transfer was calculated as NMD(t) = \sum I_{x,y} d_{x,y} / \sum I_{x,y} for each frame in the sequence. I_{x,y} is the intensity of an image pixel (x,y) at time t, and d_{x,y} is the distance of the pixel (x,y) from the site of dye microinjection. NMD(t) data evaluated for the initial 10 min of a recording were subjected to a linear regression analysis, and the slope of the regression function was taken as the rate of the dye transfer for the particular sequence. Significance of differences in the rate of the dye transfer between groups of sequences were evaluated by two-sided t-test. Image processing, regression analysis and statistical tests were performed in Mathematica (Wolfram Research) using specially developed software.

**Determination of cell death**

Cells microinjected with the expression Cx31/EGFP fusion constructs were incubated for 5 h and then treated with 0.5 μg/ml of the membrane-impermeable dye propidium iodide. After 10 min, images were acquired in phase-contrast, red and green fluorescence. Dead cells (propidium iodide positive) and viable cells (propidium iodide negative and absence of nuclear shrinkage) were counted. Data were expressed as percentages of total cells scored and tested for significant differences by analysis of variance (ANOVA).

**REFERENCES**


