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Molecular interaction of connexin 30.3 and connexin 31 suggests a dominant-negative mechanism associated with erythrokeratodermia variabilis

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Connexins are homologous four-transmembrane-domain proteins and major components of gap junctions. We recently identified mutations in either GJB3 or GJB4 genes, encoding respectively connexin 31 (Cx31) or 30.3 (Cx30.3), as causally involved in erythrokeratodermia variabilis (EKV), a mostly autosomal dominant disorder of keratinization. Despite slight differences, phenotypes of EKV Mendes Da Costa (Cx31) and EKV Cram–Mevorah (Cx30.3) show major clinical overlap and both Cx30.3 and Cx31 are expressed in the upper epidermal layers. These similarities suggested to us that Cx30.3 and Cx31 may interact at a molecular level. Indeed, expression of wild-type Cx30.3 in HeLa cell resulted only in minor amounts of protein addressed to the plasma membrane. Mutant Cx30.3 was hardly detectable and disturbed intercellular coupling. In sharp contrast, co-expression of both wild-type proteins led to a gigantic increase of stabilized heteromeric gap junctions. Furthermore, co-expressed wild-type Cx30.3 and Cx31 coprecipitate, which demonstrates a physical interaction. Inhibitor experiments revealed that this interaction begins in the endoplasmic reticulum. These results not only provide new insights into epidermal connexin synthesis and polymerization, but also allow a novel molecular explanation for the similarity of EKV phenotypes.

INTRODUCTION

Gap junctions are clusters of intercellular plasma membrane channels connecting the cytoplasm of neighboring cells and allowing free transfer of small molecules (less than 1 kDa). Gap junctions are essential for intercellular communication and thus for control of cell fate and tissue homeostasis. The intercellular channels are formed by the docking of two connexons consisting of six connexins. All the connexins within a connexon can be the same (homomeric) or different (heteromeric) and the two connexons docking together can be identical (homotypic junctions) or different (heterotypic junctions).

Connexins (Cx) compose a large and highly homologous gene family encoding plasma membrane proteins. Connexins contain four transmembrane domains linked by one cytoplasmic and two extracellular loops; N and C termini are located on the cytoplasmic side. Transmembrane domains bear conserved amino acids, whereas the cytoplasmic loop and the C terminal region are the most variable parts of connexins. Twenty connexins with different selectivity properties have been described (1,2) and each tissue expresses a specific subset (3). The importance of the physiological functions of connexins is illustrated by the identification of connexin mutations as the molecular cause of various human diseases (4), such as X-linked Charcot–Mary–Tooth peripheral neuropathy, cataract or hearing loss.

In skin, at least nine different connexins are expressed (5). In the basal cell layer, Cx26 is the predominant connexin, while in the spinous and granular cell layers the most expressed connexins are Cx43, Cx31 and Cx37 (3,6–8). Cx30.3 is expressed in kidney, placenta and skin (9). Cx31 and Cx30.3 appear to be coexpressed in the suprabasal compartment of mouse and human epidermis (8). Cx31 is preferentially expressed in the stratum corneum and the stratum granulosum (7). The precise localization of Cx30.3 has not been reported yet. Different skin diseases are caused by connexin mutations (3,10). For instance mutations of Cx26 lead to Vohwinkel syndrome (palmoplantar keratoderma and hearing loss) (11) and KID syndrome (erythroker-

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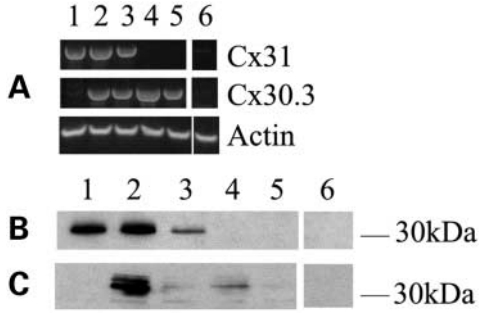

Figure 1. Expression of connexins in the stable cell lines. (**A**) RT–PCR amplification of RNA from HeLa cells expressing WT-Cx31 (lane 1), WT-Cx31 and WT-Cx30.3 (lane 2), WT-Cx31 and F137L-Cx30.3 (lane 3), WT-Cx30.3 (lane 4), F137L-Cx30.3 (lane 5) and normal HeLa cells (lane 6) using primers specific for either Cx31 or Cx30.3. Western blot analysis of the same stable cell lines with anti-V5 antibody for Cx31 (**B**) and anti-myc antibody for Cx30.3 (**C**). The numbers above each lane correspond to those mentioned in (A).

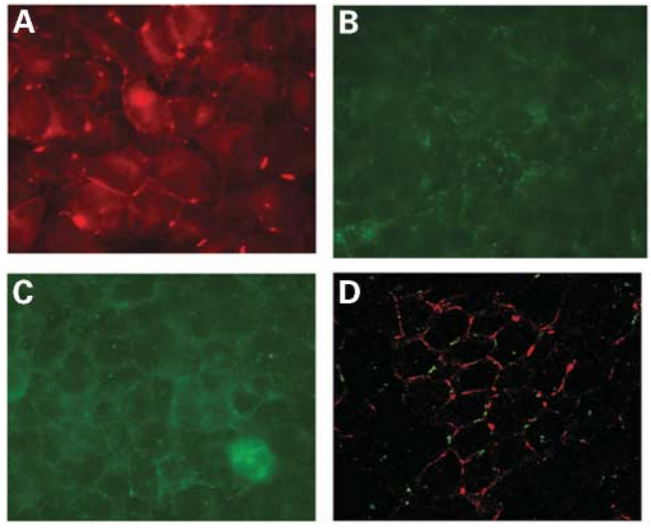

Figure 2. Plasma membrane localization of WT-Cx31 and WT-Cx30.3 in stable HeLa cell lines. (**A**) Immunofluorescence on cells expressing WT-Cx31 (A), WT-Cx30.3 (**B**), F137L-Cx30.3 (**C**) and WT-Cx31/WT-Cx30.3 (**D**) with anti-V5 antibody (A, in red), anti-myc antibody (B, C and D, in green) and N-Cadherin antibody (D in red).

atoderma, deafness and keratitis) (12,13) or mutations of Cx30 are responsible for Clouston syndrome (hearing loss and hidrotic ectodermal dysplasia) (14). Recently, we have identified mutations of Cx31 (11,16–20) and Cx30.3 (8,21) to cause autosomal dominant erythrokeratodermia variabilis (EKV; MIM 133200) characterized by fixed hyperkeratotic plaques and transient erythematous areas. There is similarity in the clinical pictures of patients with EKV due to Cx31 and Cx30.3 mutations, despite minor differences, with EKV Cram–Mevorah (Cx30.3) being more annular in appearance (8,21) than EKV of the Mendes Da Costa type (Cx31). Furthermore, Cx30.3 and Cx31 are expressed in the upper differentiating epidermal layers (6–8). In addition to epidermis, Cx31 is expressed in the cochlea, and some mutations have been associated with hearing loss or impairment without obvious cutaneous phenotypes (10,19,22). The similarities of the clinical phenotypes of EKV which are due to mutations of Cx30.3 and Cx31 suggested to us that Cx30.3 and Cx31 might cooperate structurally or functionally at a molecular level. Therefore, we set out to analyze the coexpression of Cx31 and Cx30.3 and to study the influence of the Cx30.3 mutation F137L (F137L-Cx30.3) (21), which affects a highly conserved amino acid oriented towards the channel pore formed by the third transmembrane domain of the protein (23).

RESULTS

Expression in stable cell lines

HeLa cells, which are deficient in connexin expression, were transfected with plasmids driving the expression of one or more wild-type (WT) or mutant connexins and stably transfected cells were selected. RT–PCR was performed to assess the expression of transgenes in stable cell lines (Fig. 1A). A Cx31 transcript was detected in cells transfected with WT-Cx31, WT-Cx31/WT-Cx30.3 and WT-Cx31/F137L-Cx30.3. Four cell lines expressed a transcript for Cx30.3 (WT-Cx31/WT-Cx30.3, WT-Cx31/F137L-Cx30.3, WT-Cx30.3 and F137L-Cx30.3). The presence of the mutation F317L in Cx30.3 was checked by restriction enzyme digestion. Connexin proteins were detected by western blotting (Fig. 1B and C). The expression pattern of immunolabeled proteins was fully in agreement with that of the corresponding transcripts. Interestingly the levels of Cx31 were markedly decreased when mutant Cx30.3 was coexpressed (Fig. 1B). No connexins were detected in non-transfected HeLa cells (Fig. 1A, B and C, lane 6).

Localization of wild-type and mutated connexins

Immunolabeling (Fig. 2A and B) with specific antibodies revealed that WT-Cx30.3 and WT-Cx31 localized at the plasma membrane as small plaques at points of contact between adjacent cells. This membrane localization was confirmed by colocalization with N-Cadherin (Fig. 2D). Nevertheless, expression of WT-Cx30.3 alone resulted in a protein with a localization different to that of WT-Cx31 as judged by a faint and more diffuse immunostaining (Fig. 2B). When WT-Cx31 and WT-Cx30.3 were coexpressed, the staining was much stronger and large plaques were seen at areas of cell–cell contact (Fig. 3A, C and E), where the two connexins were precisely colocalized in these cells (Fig. 3A, C and E). In contrast, coexpression of F137L-Cx30.3 and of WT-Cx31 resulted in a diffuse staining with only a few detectable dots of WT-Cx31 at the cell membrane (Fig. 3B, D and F). Consequently, we supposed that there was a cooperation between both connexin which enhanced the formation of gap junction and that the F137L mutation of Cx30.3 had a dominant negative effect.

When cells expressing WT-Cx31 were co-cultured with cells expressing WT-Cx30.3, the staining was similar to that of the cells cultured separately (data not shown), indicating that collaboration of the two proteins occurs during the formation of heteromeric connexons and not in the heterotypic docking of two connexons of adjacent cells, and that this heteromerization is essential for the trafficking of Cx30.3.

Table 1. The F137L mutation of Cx30.3 decreases gap junctions and coupling in HeLa cells expressing WT-Cx31

Cell line	Dye coupling				Electron microscopy			
	No. of cells injected	No. (%) of cells exchanging Lucifer Yellow[a]			No. of cells scored (% with gap junctions)	No. (%) of cells showing gap junctions[a]		
		0	2–3	>3		Minute	Medium	Large
WT-Cx31/WT-Cx30.3	20	1 (5)	2 (10)	17 (85)	110 (38)	12 (21)	24 (41)	22 (38)
WT-Cx31/F137L-Cx30.3	17	5 (29)	10 (59)	2 (12)	109 (29)	36 (60)	21 (34)	4 (6)

[a]$P > 0.001$ WT-Cx31/F137L-Cx30.3 versus WT-Cx31/WT-Cx30.3, as compared by a χ^2 test.

Integration of connexins into gap junctions

Incorporation of connexins into gap junction plaques correlates with a loss of solubility in Triton X-100 (24–27). Protein extracts from different HeLa cells were thus separated into soluble and insoluble fractions. Connexins in soluble fractions, corresponding to dispersed connexons, were observed at similar levels in HeLa cells whether WT-Cx30.3 or F137L-Cx30.3 was coexpressed with WT-Cx31. Connexins in the TritonX-100 insoluble fraction were also detected in both cell lines. However, the abundance of connexins in this fraction was markedly decreased in cells expressing the mutant connexin, as judged by immunolabeling of Cx31 (Fig. 3G), indicating that less gap junctions are formed in the presence of F137L-Cx30.3.

The F137L mutation of Cx30.3 decreases coupling and gap junctions of HeLa cells expressing Cx31

Microinjection of Lucifer Yellow revealed limited coupling in HeLa cells expressing the wild-type form of Cx31 (data not shown). The incidence and extent of this coupling was significantly increased between cells coexpressing this connexin together with WT-Cx30.3 (Table 1 and Fig. 4). In contrast, cells coexpressing WT-Cx31 and F137L-Cx30.3 showed a much decreased ($P < 0.001$) coupling (Table 1 and Fig. 4). This difference correlated with different sizes of gap junction plaques, as evaluated on freeze-fracture replicas of membranes of transfected HeLa cells. Thus, whereas cells coexpressing the wild-type form of WT-Cx31 and WT-Cx30.3 featured a sizable number of very large gap junction plaques, cells expressing WT-Cx31 and F137L-Cx30.3 showed less abundant and much smaller gap junctions and appeared unable to concentrate large numbers of connexons into individual plaques (Table 1 and Fig. 4).

Interaction between both connexins

To test whether Cx31 and Cx30.3 interact, we immunoprecipitated a total extract of protein of HeLa cells stably coexpressing WT-Cx31 and WT-Cx30.3, using antibodies against the Cx30.3-Myc tag fusion protein. The precipitated fraction was then analyzed on western blots with antibodies directed against the Cx31-V5 tag protein (Fig. 5A). Cx31-V5 was detected in the precipitated fraction, indicating a physical interaction between Cx31 and Cx30.3. This interaction was also observed in cells coexpressing WT-Cx31 with F137L-Cx30.3, indicating that the Cx30.3 mutation F137L does not disturb the oligomerization of both connexins into connexons. Co-immunoprecipitation was also performed after incubation of the cells with brefeldin A (BFA), which disrupts the Golgi apparatus. This interaction can be detected after treatment with BFA, meaning that this interaction takes place before the passage through the Golgi apparatus (Fig. 5B).

Turnover of cell surface connexins in Brefeldin A treated HeLa cells expressing WT-Cx31 and WT-Cx30.3

The observation of very large gap junctional plaques in presence of both wild-type proteins could be the sign of a slow degradation of the proteins. Thus, we analyzed cells by inmmunofluorescence after treatment with BFA to block the transport of newly synthesized connexins to the cell surface and to allow us to monitor the disappearance of WT-Cx31/WT-Cx30.3 gap junction plaques. The intense staining at appositional membranes in untreated cells was rapidly lost in BFA (5 μg/mL) treated culture (Fig. 6A–C). The plaques became smaller and the intracellular staining, corresponding to newly synthesized connexin (28), increased. After 6 h almost all gap junctions had been degraded (Fig. 6C), leading to the conclusion that the time needed to degrade large plaques formed in presence of both wild-type connexins is normal (29). The mechanism of disappearance of membrane staining was inhibited when cells were simultaneously treated with MG132 a proteasome inhibitor (Fig. 6D), indicating that the degradation of gap junctional plaques is due to the proteasome.

Cx31 and Cx30.3 degradation

We treated cultured cells with either proteasome or lysosome inhibitors, two pathways implicated in the destruction of integral membrane proteins (27), to study the degradation of Cx31 and Cx30.3. Treatment of cells coexpressing Cx31 and Cx30.3 with MG132 led to an accumulation of connexins at plasma membrane in presence of WT-Cx30.3 (Fig. 7E) and in the cytoplasm in presence of F137L-Cx30.3 (Fig. 7F), leading to the conclusion that the proteasome plays a role in normal degradation of connexin integrated into gap junction and in degradation of mutant protein at the exit of the endoplasmic reticulum (ER). The effect is confirmed by a cotreatment of cells coexpressing WT-Cx31 and WT-Cx30.3 with BrefeldinA and MG132, indicating the implication of the proteasome in the degradation of gap junctional plaques but not in degradation of newly synthesized connexins (Fig. 6D). Treatment with chloroquine did not change the staining of cells coexpressing WT-Cx31 and WT-Cx30.3 (Fig. 7C). On the

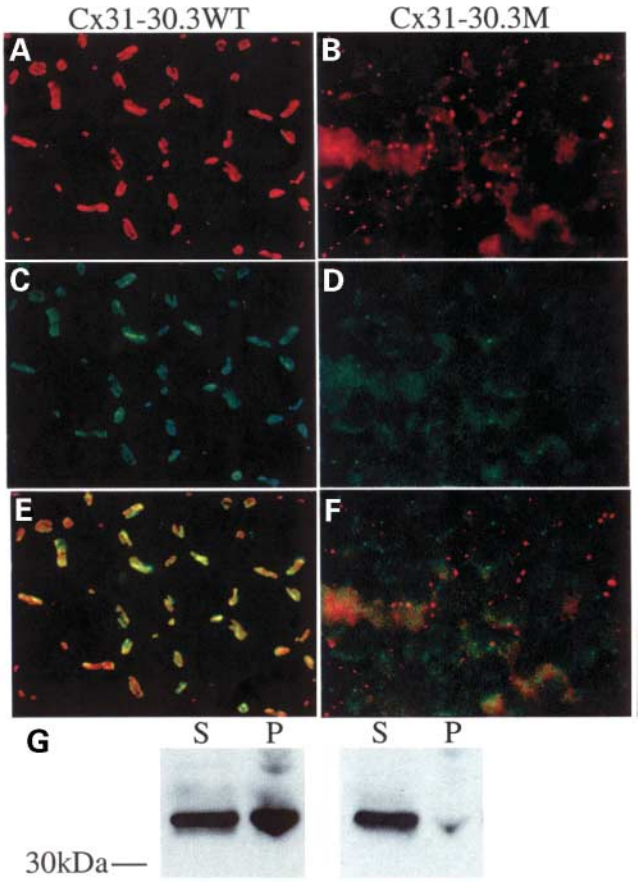

Figure 3. Immunofluorescence analysis of HeLa cells stably expressing WT-Cx31 and WT-Cx30.3 (**A**, **C**, **E**) or WT-Cx31 and F137L-Cx30.3 (**B**, **D**, **F**) with anti-V5 antibody (Cx31) in (A, C, E) or anti-myc antibody (Cx30.3) in (B, D, F). The superposed images are shown in (E) and (F). (**G**) The TritonX-100 insoluble form of Cx31 is markedly decreased in presence of F137L-Cx30.3. Cx31 was detected with an anti-V5 antibody in insoluble (P) or soluble (S) fractions.

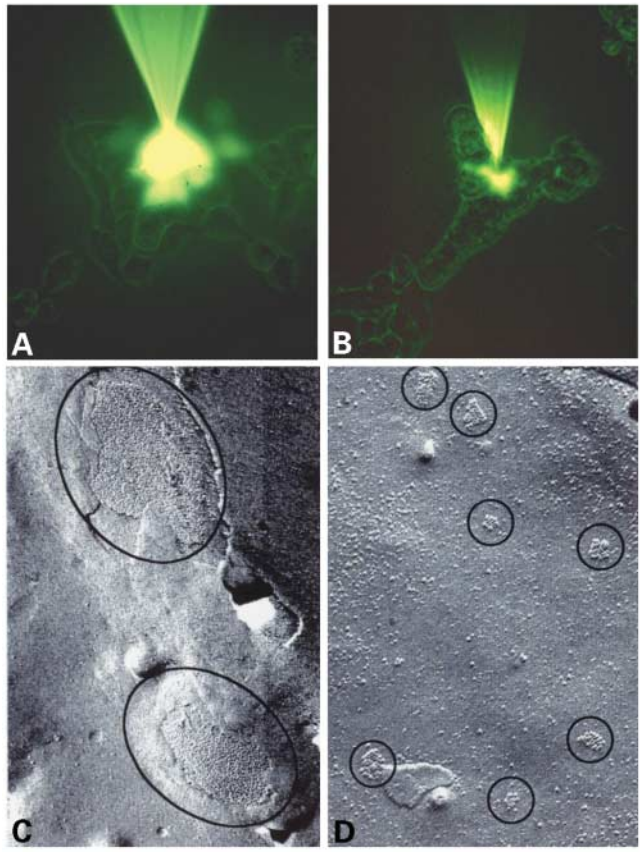

Figure 4. The F137L mutation of Cx30.3 decreases coupling and gap junctions of HeLa cells expressing WT-Cx31. After a 5 min injection, most HeLa cells co-expressing the wild-type forms of Cx31 and Cx30.3 exchanged Lucifer Yellow with several of their neighbors (**A**). Lucifer Yellow transfer was markedly decreased between HeLa cells co-expressing the wild-type form of Cx31 and the F137L mutation of Cx30.3 (**B**). Freeze-fracture analysis of the plasma membrane of cells expressing both WT connexins revealed that most of these cells were joined by large gap junction plaques (**C**). When WT-Cx31 and F137L-Cx30.3 were coexpressed, freeze-fracture analysis showed that most of these cells were linked by minute gap junctions (**D**).

contrary, the cytoplasmic staining was more intense than in the control when WT-Cx31 and F137L-Cx30.3 were coexpressed (Fig. 7D). Chloroquine blocks a lysosomal pathway of degradation which occurs at the exit of the ER for protein misfolded or with a problem in oligomerization. We can also note that, even when the degradation of F137L-Cx30.3 is blocked, connexins are not exported to the plasma membrane (Fig. 7D), indicating that the mutation leads to the degradation of the protein at the exit of the ER.

DISCUSSION

The formation of functional gap junctions results in a series of intracellular processes that assemble connexin subunits into connexon hemichannels before their delivery to the plasma membrane (30). Connexins are maturated in the ER where they are then oligomerized into connexons (31). Connexons are then exported via the Golgi apparatus to the membrane of the cells. During gap junction formation, connexons align and dock with partners in neighboring plasma membranes to generate gap junction intercellular channels. Mutations in connexin may modify or disrupt those events that lead to channel formation and operation. Two genes of the same family of proteins cause the two distinct forms of erythrokeratodermia variabilis and these two genes are expressed during the process of epidermal differentiation. These two facts led us to hypothesize that Cx31 and Cx30.3 could interact structurally or functionally at the molecular level and that this interaction is important for epidermal homeostasis. The high majority of mutations of Cx31 and Cx30.3 concern the transmembrane domains of the proteins, i.e. the most conserved parts of connexins. The F137L mutation in Cx30.3 affects a highly conserved amino acid of the third transmembrane domain. By analogy to Cx32, we know that this amino acid faces the lumen of the gap junction channel (23).

In this study, we have used stable mammalian cell lines in order to obtain reproducible conditions to analyze the interaction of WT-Cx31 and WT-Cx30.3 as well as the F137L-Cx30.3 mutation associated with EKV. We decided to use HeLa cells for expressing our connexins as they are deficient in connexins and do not form gap junctions. Thus we observed the interaction of both connexins without the intervention of

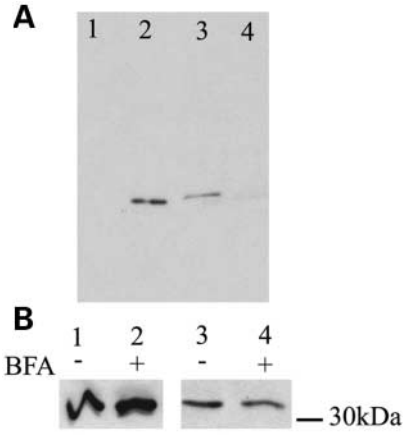

Figure 5. Cx31 and Cx30.3 interact before entering Golgi apparatus. (**A**) Total extract of HeLa cells expressing WT-Cx31 (lane 1), WT-Cx31/WT-Cx30.3 (lane 2), WT-Cx31/F137L-Cx30.3 (lane 3) and WT-Cx30.3 (lane 4) were precipited with an anti-myc antibody and detected on western blot with an anti-V5 antibody. (**B**) HeLa cells expressing WT-Cx31/WT-Cx30.3 (lanes 1 and 12) and WT-Cx31/F137L-Cx30.3 (lanes 3 and 4) were immunoprecipitated as described above (lanes 1 and 3) or immunoprecipitated after treatment with BFA for 6 h (lanes 2 and 4).

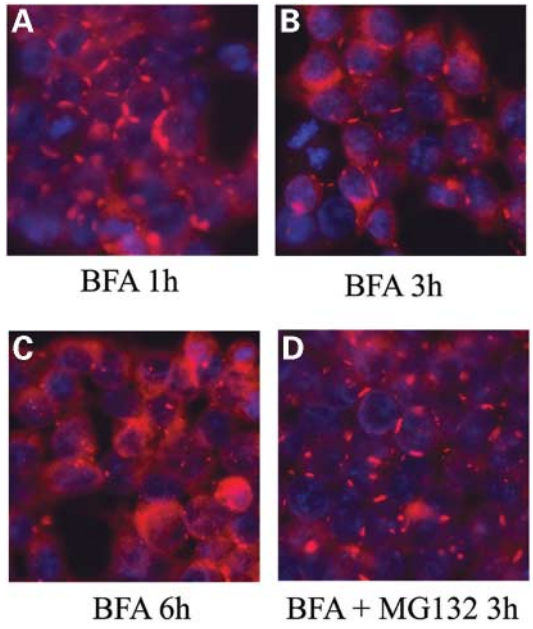

Figure 6. Immunofluorescent detection of the loss of cell surface WT-Cx31 in BrefeldinA treated WT-Cx31/WT-Cx30.3 cells and inhibition of this mechanism by a proteasome inhibitor. Cells were treated with Brefeldin A for the indicated times (**A**, **B**, **C**) or with BFA and MG132 (**D**) prior to immunofluorescence analysis with anti-V5 antibody (in red).

other connexins, that is to say in a model different from the natural situation, but allowing the study of a precise interaction. In contrast, skin is rather complex for the study of connexin as keratinocytes express a large panel of connexins and Cx31 and Cx30.3 are not predominant. Cx30.3 is expressed in various tissues (skin, kidney, placenta) (9), but the F137L mutation leads only to EKV, indicating that the effect of this connexin mutation may be cell-specific. Nevertheless, since mutations of connexins weakly expressed in the epidermis lead to disease, this indicates that their function is of major importance. Intercellular communication mediated by both Cx31 and Cx30.3 affects epidermal differentiation significantly.

Our results indicate that Cx31 and Cx30.3 oligomerize to form heteromeric connexons. First, simultaneous immunostaining experiments showed that both connexins colocalized to the same junctional plaques at interfaces between cells. Secondly, the formation of plaques is greatly enhanced in the presence of both wild-type connexins compared to the single connexin expression, and this is not due to a slow degradation as the turnover of the protein is classical. Furthermore, this cooperation of both connexins occurs during the oligomerization of heteromeric connexons and not during the formation of heterotypic channels. The expression of WT-Cx30.3 in a cell neighboring a cell expressing WT-Cx31 does not lead to the formation of large plaques. It seems that Cx30.3 needs to heteromerize with another connexin to be efficiently exported to the plasma membrane as very small dots were observed when it was expressed alone. Since only Cx31 and Cx30.3 have so far been implicated in EKV, we did not look for their interaction with other connexins also expressed in the skin, such as Cx26, Cx30 or Cx43. Cx30.3 could interact with some of these connexins. Similar to our present observations, Cx26 and Cx30 have been described to interact and Cx26 mutations to have a dominant negative effect on Cx30 (32). However, the reported coexpression of Cx26 and Cx30 did not enhance the formation of gap junction at cell membrane (32). To our knowledge, this is the first report providing experimental evidence for molecular interaction of connexins with heterodimeric stabilization. Furthermore, this molecular interaction provides a novel explanation for the similarity of EKV phenotypes Mendes da Costa (Cx31) and EKV Cram–Mevorah (Cx30.3).

Mutations of connexins can disturb different stages in their life cycle (9). The F137L mutation does not lead to a premature stop codon and does not interfere with protein oligomerization as we have been able to coprecipitate Cx30.3 and Cx31 even in the presence of the mutation. In presence of the F137L mutation of Cx30.3, very few gap junctions are formed (TritonX-100 solubility and electron microscopy) and consequently only a few dots were observed at the membrane by immunofluorescence, and the coupling between adjacent cells was sharply decreased compared with the cell coexpressing both wild-type connexins. As our protein was not detected at plasma membrane, a disturbance of connexon docking or an alteration of gap junction permeability was excluded. The stage disturbed by our mutation is the protein folding, leading to the degradation of the protein at the exit of the endoplasmic reticulum. This kind of trafficking defect was already observed with mutant connexin31 (5,33) or connexin32 (34), for example. There is a selective recognition, retention and degradation of incorrectly folded proteins known as quality control process (27). Mutations of Cx31, including F137L-Cx31 (35), have also been described to lead to cell death (5,36). In contrast, F137L-Cx30.3 did not lead to this phenomenon in either our HeLa cells nor in HaCat cells (35), consistent with the lack of obvious cell death in EKV.

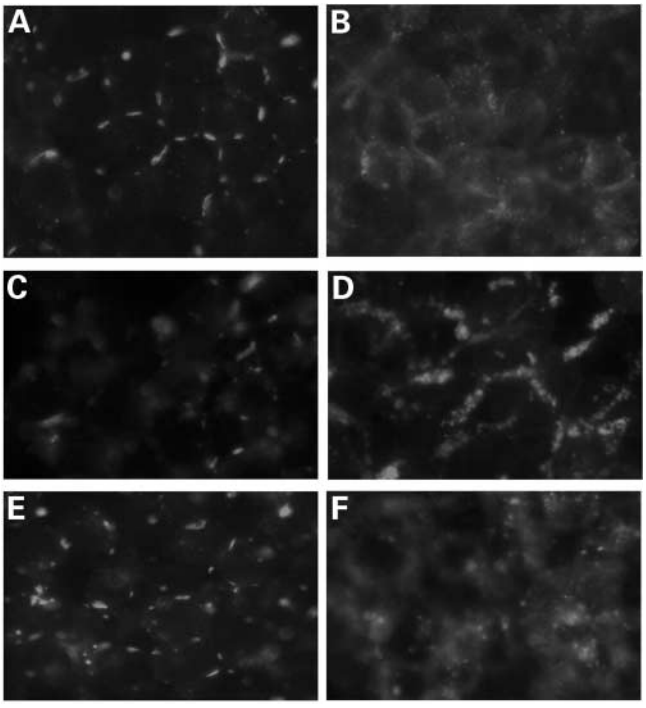

Figure 7. Influence of inhibitors of proteasome and lysosome on the degradation of connexins in cells coexpressing WT-Cx31 with either WT-Cx30.3 (**A**, **C** and **E**) or F137L-Cx30.3 (**B**, **D** and **F**). Cells were cultured without protease inhibitor (A, B) or treated with chloroquine (C, D) or MG132 (E, F) prior to immunolabelling with anti-V5 antibody.

In summary, Cx30.3 mutation F137L prevents the trafficking of Cx31 and itself to the membrane of the cells and leads to a decrease in cellular coupling. Thus communication of epidermal cells is sharply disturbed in presence of this mutation. Our study is the first clue indicating an interaction between Cx31 and Cx30.3 in the formation of gap junctions.

MATERIALS AND METHODS

Cell culture

HeLa and 293T cells were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal calf serum, 100 U/ml Penicillin and 0.1 mg/mL Streptomycin. Zeocin (Invitrogen) was added at a concentration of 600 μg/ml for selection of stably transfected cells. Cells were grown on cover slips for immunostaining.

Cells were incubated with 5 μg/ml BrefeldinA (Sigma) for 1, 3 and 6 h. They were also incubated for 3 h with different protease inhibitors: 40 μM MG132 (Calbiochem) and 200 μM Chloroquine (Fluka BioChemica).

Antibodies

The following antibodies were used: rabbit anti c-myc antibody (1 : 3000; Santa Cruz Biotechnology Inc.); mouse anti V5 antibody (1 : 5000; Invitrogen); anti-Rabbit IgG, HRP-linked antibody (1 : 3000; Amersham Bioscience); anti-mouse IgG, HRP-linked antibody (1 : 5000; Amersham Bioscience); rabbit immunoglobulins-FITC (1 : 40; DAKO); biotinylated anti-mouse IgG antibody (1 : 100; Vector, Switzerland); Streptavidin–Texas Red (1 : 400; Amersham Life Science); Alexa488 goat anti rabbit (1 : 200; A11070 Juro Supply).

Construction of chimeric Cx31-V5 and Cx30.3-Myc

The coding sequence of wild-type Cx31 and Cx30.3 and that of F137L-Cx30.3 were amplified with specific primers containing restriction site sequences. Cx31 was cloned in *Sfu*I sites of pBudCE4 plasmid (Invitrogen) with primers TTCGAAGGCG CCATGGACTGGAAGACA (forward) and TTCGAAGATGG GGGTCAGGTTGGGTGC (reverse). Cx30.3 was cloned using a forward primer containing a *Hind*III site (AAGCTTGCACC ATGAACTGGGCATTT) and a reverse primer containing an *Xba*I site (TCTAGATGGATACCCACCTGCATCCAC). Cloning was designed so that the inserts were fused with C-terminal tags. Constructions were assessed by sequencing and enzymatic digestion.

Transfection and selection

HeLa cells were transfected with lipofectamine (Invitrogen) or lipofectamine2000 (Invitrogen). After 48 h, medium was replaced by the selection medium containing Zeocin (Invitrogen). Single colonies were isolated and grown separately to expand monoclonal cell lines.

Extraction of proteins and immunoblotting

Cells were harvested in lysis buffer containing 10 mM Tris–HCl (pH 7.5), 5 mM EDTA, 50 μg/ml PMSF and complete antiprotease mixture (Roche).

Protein extracts were heated for 5 min at 95°C. After separation on SDS polyacrylamide gels, proteins were transferred onto nitrocellulose membrane, stained with Ponceau and then incubated with antibodies.

For TritonX-100 solubility assay, cells were harvested in a buffer containing 1% Triton-X100, 145 mM NaCl, 10 mM Tris–HCl pH 7.4, 5 mM EDTA, 2 mM EGTA and 1 mM PMSF. After 30 min centrifugation, soluble and insoluble fractions were separated by western blotting.

Co-immunoprecipitation

Cells were harvested by scraping in a buffer containing PBS, 1% Triton X-100, 0.5% CHAPS, 0.1% SDS, and protease inhibitors (37) (IP buffer). The myc antibody was used to precipitate Cx30.3. The antigen–antibody complexes were collected using protein G-Sepharose beads, washed first with IP buffer without protease inhibitors supplemented with 0.5 M Sucrose and 0.5% BSA and then with IP buffer and analyzed by SDS–PAGE, using an anti-V5 antibody.

Immunofluorescence

Cells were grown on cover slips and fixed in methanol acetone (1 : 1). After blocking in 1% BSA–PBS (1 h at 37°C), cells were incubated for 2 h at 37°C with primary antibodies and then exposed to secondary and ternary antibodies if necessary (30 min each). Cover slips were mounted in fluorescent mounting medium (DAKO Diagnostic AG). Images were acquired using a Zeiss Axioskop microscope (Carl Zeiss, Germany) and processed with an RT color SPOT CCD Camera (Diagnosis Instruments Inc.) and Adobe Photoshop 6. Green and red fluorescence were collected separately. There was no signal from the green channel in the red one and vice versa.

Dye coupling

For assessment of junctional coupling, individual cells were microinjected by iontophoresis within monolayer cultures, using microelectrodes containing 4% Lucifer Yellow CH (Sigma Chemical Co., St Louis, MO, USA) in 150 mM LiCl.

The percentage of microinjected cells that exhibited cell-to-cell transfer of Lucifer Yellow was determined on photographs taken immediately after each microinjection.

Electron microscopy

For assessment of gap junction plaques, cells were fixed for 60 min in a 2.5% glutaraldehyde solution in 0.1 M phosphate buffer, infiltrated with 30% glycerol, frozen in Freon22 cooled in liquid nitrogen, and processed for freeze-fracture using a Balzers BAF (Balzers High Vacuum, Balzers, Liechtenstein). Replicas were examined in a Philips EM 300 microscope.

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