Multiple Epidermal Connexins are Expressed in Different Keratinocyte Subpopulations Including Connexin 31

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Recent genetic studies have demonstrated the importance of epidermal gap junctions with mutations in four β-connexins associated with autosomal dominant epidermal disease. One of these disorders, erythrokeratoderma variabilis, is associated with germline mutations in the genes encoding connexins (Cx) Cx31 and Cx30.3. Towards understanding the functional mechanism of Cx31 mutations in epidermal disease, we have developed and characterized a polyclonal antibody raised against human Cx31. Using this antibody to immunostain normal epidermis, Cx31 protein was found to be expressed predominately in the stratum granulosum with a punctate pattern of staining at the plasma membrane. In addition, we used reverse transcriptase polymerase chain reaction and, where reagents were available, immunocytochemistry to investigate which other connexins are expressed in the epidermis. Surprisingly, this analysis revealed that there are at least 10 connexins expressed with an overlapping distribution and localization to distinct keratinocyte subpopulations. These data provide additional evidence for multiple gap junction channel types in the human epidermis. Elucidation of this complexity of channel types with respect to specific permeabilities and function of each wildtype and mutant channel type in epidermal biology will require further investigations. ***Key words: gap junction/intercellular communication/keratoderma. J Invest Dermatol 117:958–964, 2001***

Gap junctions are intercellular channels allowing the passage of low molecular weight substances (<1000 Da) such as second messengers, ions, and nutrients between cells. In the epidermis this direct cytoplasmic exchange of metabolites and ions is thought to coordinate epidermal homeostasis. The importance of gap junctions in epidermal differentiation is supported by the discovery that mutations in gap junction proteins are the underlying cause of several inherited skin disorders (Kelsell *et al*, 2001).

The major proteins of gap junctions are connexins (Cx). Connexins are membrane proteins composed of four hydrophobic transmembrane domains oriented such that both the N and C termini of the molecule are cytoplasmic (Unger *et al*, 1999). Six connexin molecules assemble to form a ring structure known as a connexon. Connexons interact with similar structures in neighboring cells to form functional channels. There are at least 14 different human connexin molecules differentially expressed in a tissue- and state-specific manner. The contribution of different connexin molecules to the mature gap junction is unclear but heterotypic interactions between connexons have been demonstrated to form functional channels (Cao *et al*, 1998).

There is evidence for the expression of a number of connexins in human epidermis, including Cx26 and Cx43 (Guo *et al*, 1992; Salomon *et al*, 1994; Lucke *et al*, 1999), Cx31, Cx31.1 (Richard *et al*, 1998a), Cx30 (Lamartine *et al*, 2000), and Cx30.3 (Macari *et al*, 2000). Cx43 and Cx26 have been most widely studied. Cx43 is found in both interfollicular epidermis and epidermal adnexae whereas Cx26 is restricted to the adnexae where it shows codistribution with Cx43 (Salomon *et al*, 1994). Low levels of Cx26 have also been reported to occur in the basal cells of plantar skin (Lucke *et al*, 1999). Cx43 and Cx26 are differentially expressed during stratification of keratinocytes in culture (Wiszniewski *et al*, 2000) and Cx26 has been shown to be upregulated in keratinocytes of psoriatic lesions (Labarthe *et al*, 1998; Lucke *et al*, 1999). Both of these connexins are upregulated by retinoic acid (Masgrau-Peya *et al*, 1997).

Several connexin gene mutations have been identified as the cause of a number of epidermal disorders. Mutations in the gene encoding Cx26 have been found to cause syndromes of deafness and palmoplantar keratoderma (Richard *et al*, 1998b; Maestrini *et al*, 1999; Kelsell *et al*, 2000). Mutations in both Cx31 and Cx30.3 give rise to the skin disease erythrokeratoderma variabilis (EKV) (Richard *et al*, 1998a; Wilgoss *et al*, 1999; Macari *et al*, 2000) and mutations in Cx30 are the cause of Clouston's hidrotic ectodermal dysplasia (Lamartine *et al*, 2000). In addition to causing skin disease, different mutations in Cx26, Cx30, and Cx31 can result in nonsyndromic hearing loss (Kelsell *et al*, 1997; Xia *et al*, 1998; Grifa *et al*, 1999; Liu *et al*, 2000). In particular, mutations in Cx26 are a major cause of recessive hereditary hearing loss (http://www.iro.es/CX26deaf.html). Mutations in Cx31 can also give rise to a syndrome of deafness and neuropathy (López-Bigas *et al*, 2001). Thus, different mutations in the same gene can result in a variety of phenotypes affecting different tissues.

The most parsimonious explanation for the different phenotypic manifestations of mutations in connexin genes is that other

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Abbreviations: Cx, connexin; EKV, erythrokeratoderma variabilis.

Table I. Antibodies used in the study

Antibody	protein domain	Dilution for staining	Source	Reference
monoclonal antimouse Cx26	C terminus	1:100	Zymed	Lucke *et al*, 1999
polyclonal rabbit antimouse Cx30	C terminus	1:50	Zymed	Nagy *et al*, 1999
polyclonal rabbit antihuman Cx31	C terminus	1:100	In house	
monoclonal antirat Cx32	Cytoplasmic loop	1:100	Zymed	Kojima *et al*, 1999
polyclonal rabbit antimouse Cx40	C terminus	1:50	Alpha diagnostic	Harold, 2000
monoclonal antirat Cx43	near C terminus	1:100	Zymed	Nagy *et al*, 1997
polyclonal rabbit antimouse Cx45	near C terminus	1:22	Alpha diagnostic	van Veen *et al*, 2000

Table II. Primers for RT-PCR

Connexins	Accession No.	Forward primers (5′–3′)	Reverse primers (5′–3′)	Annealing temperature	Fragment length (bp)
Cx26	M86849	TCTTTTCCAGAGCAAACCGC	GACACGAAGATCAGCTGCAG	60	285
Cx30	HSA005585	TCAACAAACACTCCACCAGC	CAATCCCACATTTCAACACC	55	477
Cx30.3	AL121988	TACCCACCTGCATCCACTGG	GGTGGACGTACTTGCTGAGC	60	409
Cx31	AF052692	AATTCTCGCAGGTAGGCACG	CCAGAGAGTGTGCAGCAGGT	60	495
Cx31.1	AF052693	GTGGACATATGTCTGCAGCC	CTATGAGAGATGCTAGAGC	60	492
Cx32	X04325	GACAGGTTTGTACACCTTGC	CGTCGCACTTGACCAGCCGC	60	499
Cx37	M96789	GTTGCTGGACCAGGTCCAGG	GGATGCGCAGGCCACCATCT	62	415
Cx40	L34954	GTACACAAGCACTCGACCGT	GCAGGGTGGTCAGGAAGATT	60	506
Cx43	X52947	CAATCACTTGGCGTGACTTC	GTTTGGGCAACCTTGAGTTC	60	408
Cx45	U03493	GGAGCTTTCTGACTCGCCTG	CGGCCATCATGCTTAGGTTT	60	487
Cx36	NM020660	GCAGCACTCCACTATGATCG	CGTCGCACTTGACCAGCCGC	60	459

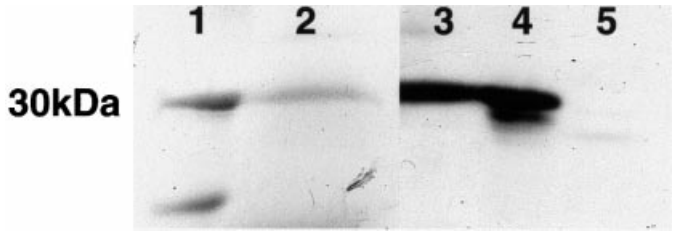

Figure 1. SDS-PAGE analysis for Cx31 antibody. Cx31 protein was translated *in vitro* (TNT system) or *in vivo* (HeLa cells) as described in *Materials and Methods* and subjected to SDS-PAGE: *lane 1*, ^{14}C-labeled molecular weight marker; *lane 2*, ^{35}S-methionine-labeled Cx31 protein (the image of *lanes 1* and *2* was taken by autoradiography); *lane 3*, unlabeled Cx31 protein translated using the TNT system; *lane 4*, HeLa cell lysate transfected with pcDNA3/Cx31; *lane 5*, the lysate of untransfected Hela cells. *Lanes 3*, *4*, and *5* were transferred to nitrocellulose membrane after electrophoresis and blotted with the Cx31 antibody.

connexins are able to compensate for the mutated proteins. In addition to the position and nature of the mutation in the connexin molecule, the phenotype of the disease may also depend on which other connexins are expressed in a given tissue. Evidence to support this hypothesis comes from mouse studies in which Cx32 and Cx40 have been shown to be able to compensate, at least partially, for Cx43 (Plum *et al*, 2000). Therefore to understand the contribution of a particular connexin to gap junction function it is necessary to characterize the expression and distribution of other connexins within a given tissue and cell type.

A major problem with such studies is the current lack of characterized human connexin antibodies. To start to address this issue we have generated a new antibody to Cx31 and used this to study Cx31 expression in normal epidermis. We have also used reverse transcriptase polymerase chain reaction (RT-PCR) to identify expression of other cutaneous connexins and immunocytochemistry to identify and compare expression of other connexins in the epidermis. We have identified transcripts for 10 different connexins in human skin and, from immunocytochemistry, seven that are expressed in the epidermis with distinct but overlapping expression.

MATERIALS AND METHODS

Tissue and keratinocyte culture Fresh normal human palm was obtained from consenting volunteers. Interfollicular epidermis was obtained from excess tissue derived from either breast or abdominal reduction surgery and follicular epidermis from excess tissue derived from face-lift surgery. Biopsies for immunofluorescence staining were immediately snap-frozen in liquid nitrogen and stored at –70°C until needed, and those for primary keratinocyte culture were collected in transfer medium and prepared by enzymatic dispersion (Leigh and Watt, 1994). HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After reaching confluence, the cells were freed from the flasks with the addition of trypsin and plated on dishes for transfection.

Cx31 antibody Polyclonal antibody was raised in rabbits against the human Cx31 peptide, PDPGNNKLQASAPNLTPI (position 253–270aa: GenBank accession number CAA06165), conjugated with bovine serum albumin (BSA). The immunization schedule was as follows. The Cx31 peptide–BSA conjugate emulsified in complete Freund's adjuvant (1:1) was injected subcutaneously at multiple sites in two rabbits. Approval for this study was given (MUREX project 4033). Two weeks after the first injection, a booster injection in incomplete Freund's adjuvant was given subcutaneously. This was repeated five times at 2 wk intervals, and then rabbits were exsanguinated and the antiserum was collected. The antiserum was affinity-purified, first on a HiTrap rProtein A column, and second on a Sephadex G-25 column (Amersham-Pharmacia Biotech, U.K.). The procedures were performed following the manufacturer's recommended protocol.

Immunofluorescence staining and confocal microscopy Frozen tissue sections of about 6 μm thickness were cut using a cryomicrotome. Sections were then rinsed with phosphate-buffered saline (PBS) and incubated in PBS containing 3% rabbit or goat serum and 0.1% Triton

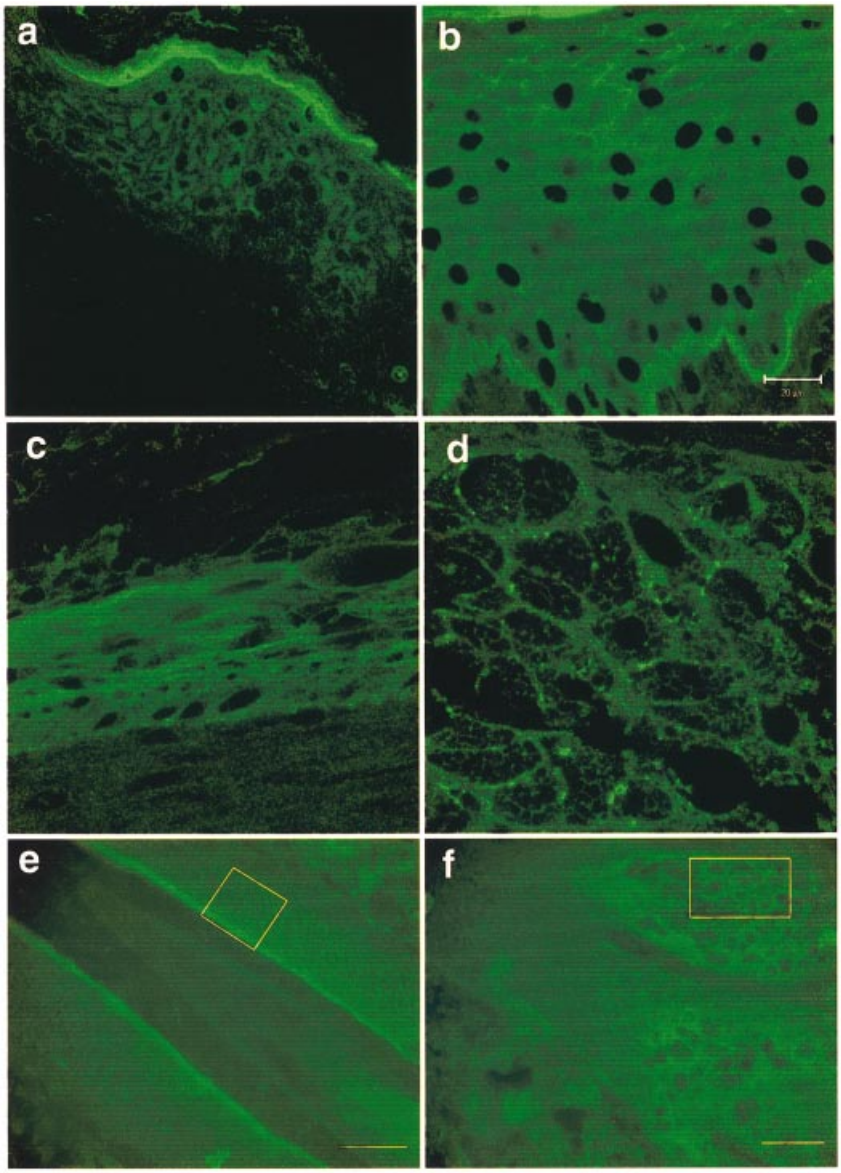

Figure 2. Connexin 31 portein in the human epidermis. The expression pattern of Cx31 protein in (*a*) normal human interfollicular epidermis (*scale bar*: 20 μm), (*b*) normal palm epidermis (*scale bar*: 20 μm), (*c*) the outer root sheath of the hair follicle (*scale bar*: 20 μm) (taken from the boxed area in (*e*), *scale bar*: 10 μm), and (*d*) sebaceous gland (*scale bar*: 20 μm) (taken from the boxed area in (*f*), *scale bar*: 10 μm).

X-100 (Sigma, U.K.) for 30 min at room temperature. After rinsing, the slides were incubated in a humidified chamber at 37°C for 2 h with one of the primary antibodies listed in **Table I**. After several rinses with PBS, sections were incubated in the dark for 1 h at room temperature with fluorescein isothiocyanate (FITC) conjugated rabbit antimouse IgG or goat antirabbit IgG (Molecular Probes, Oregon) diluted 1:100. For Cx31 staining, after incubation with primary antibody, the section was incubated with 1:200 swine antirabbit IgG–biotin conjugate (Dako, Denmark) for 1 h at room temperature and then incubated with 1:100 FITC–streptavidin (Dako) for 30 min. After rinsing with PBS, the sections were mounted in mowiol reagent containing 10% mowiol D-488 (Calbiochem, U.K.), 25% glycerol, and 2.5% 1.4-diazabicyclo[2.2.2] octane (Sigma, U.K.) in 50 mM Tris/HCl, pH 8.5. The images of expression and distribution of the connexin protein were recorded using a Ziess LSM510 laser scanning confocal microscope (Carl Zeiss, Germany) and were processed using Adobe Photoshop 5.

Cx31 construct Full-length Cx31 gene was cloned into the pcDNA3 express vector (Invitrogen, U.S.A.). PCR was carried out on wildtype human genomic DNA using Cx31-specific primers incorporating ECOR V or Xba I restriction sites (underlined in primer sequence), GCG-GATATCACCATGGACTGGAAGACACTC (5′–3′) for forward primer and GCGTCTAGATCAGATGGGGGTCAGGTT (5′–3′) for reverse primer. The resulting PCR product was cloned into the ECOR V/Xba I sites of pcDNA3.

***In vitro* transcription/translation of Cx31** 1 μg of pcDNA3/Cx31 DNA was subjected to transcription and translation using the TNT T7 transcription/translation system (Promega, U.K.) with ^{35}S-methionine (Amersham-Pharmacia Biotech) or nonradioactive methionine following the manufacturer's recommended protocol. The synthesized proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography or immunoblotting.

SDS-PAGE and immunoblotting HeLa cells transfected with pcDNA3/Cx31 were harvested and lyzed in SDS loading buffer containing 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, a protease inhibitor cocktail (Roche, Germany), and 50 mM dithiothreitol in 50 mM Tris/HCl, pH 6.8. The lysates were sonicated three times for 10 s with 15 s intervals and centrifuged at 12,000*g* for 5 min. Cell lysate and synthesized proteins from the TNT system were separated by 12% SDS-PAGE. After electrophoresis, the gel containing ^{35}S-methionine-labeled proteins was fixed, dried, and subjected to autoradiography. The gel containing HeLa cell lysates and nonradioactive methionine synthetic proteins was transferred onto a nitrocellulose membrane and probed using the affinity-purified rabbit anti-Cx31 (1:200) antibody. The blots were further incubated with a secondary antirabbit IgG–horseradish peroxidase conjugate (Dako), and were developed using the enhanced chemiluminescence plus system (Amersham-Pharmacia Biotech).

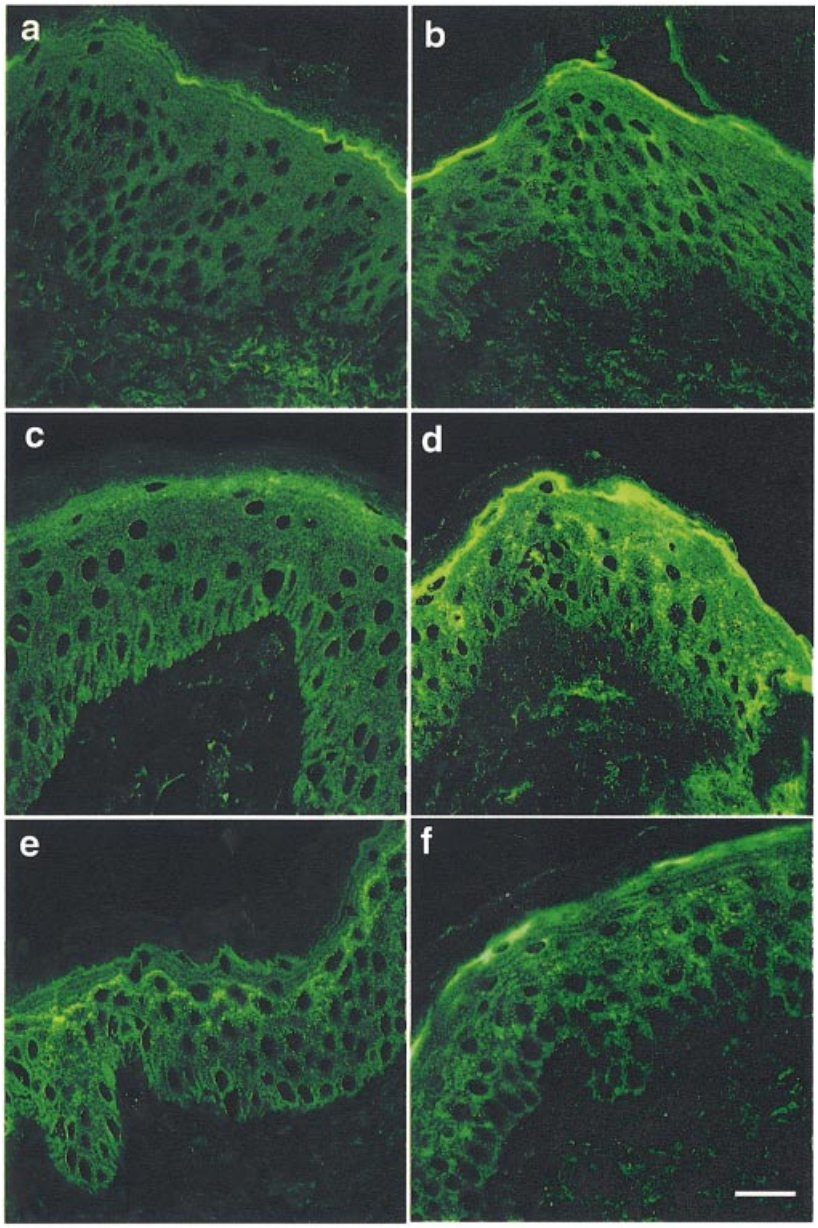

Figure 3. The expression pattern of connexin proteins in normal human interfollicular epidermis. Immunofluorescence staining using connexin antibodies: (*a*) Cx26; (*b*) Cx30; (*c*) Cx32; (*d*) Cx40; (*e*) Cx43; (*f*) Cx45. *Scale bar*: 20 μm.

RT-PCR Total RNA was separated using Trizol reagent (Life Technologies, U.K.), and mRNA was extracted and purified using oligo(dT)-cellulose (Amersham-Pharmacia Biotech). The procedures were performed according to the manufacturer's instructions. 50 ng mRNA was reverse transcribed to first-strand cDNA by Moloney murine leukemia virus reverse transcriptase with random hexamer primer (Perkin Elmer, U.K.) in a 20 μl reaction mixture, and 3 μl of reverse transcribed product was amplified by PCR using the primers listed in **Table II**. The PCR program was 94°C for 2 min, followed by 35 cycles as follows: 94°C for 30 s, 55°C or 60°C or 62°C for 30 s, 72°C for 45 s, completed with a final extension of 72°C for 10 min. As all paired primers lie within a single exon (most connexins are encoded in a single exon), a control for each RT-PCR was run using the same conditions but without reverse transcriptase to eliminate the possibility of genomic DNA contamination. RT-PCR products were separated on a 2% agarose gel containing 0.5 mg per ml ethidium bromide and visualized using a UVP transilluminator.

***In situ* hybridization for Cx31** The pCRII-TOPO/Cx31 DNA was linearized with restriction enzymes Hind III (sense) and Not I (antisense). cRNA sense and antisense probes were generated using the DIG RNA labeling kit (Boehringer Mannheim, Germany) with DIG labeled UTP. Frozen sections of about 10 mm thickness of human palm were used for *in situ* hybridization. The procedure was as described previously (Machesney *et al*, 1998). Images after *in situ* hybridization were recorded using a Leica DC200 microscope (Milton Keynes, U.K.) under the same brightness and contrast settings.

RESULTS

Characterization of Cx31 antibody For Cx31 protein localization, a polyclonal antibody raised against a peptide sequence corresponding to the intracellular C-terminus tail of human Cx31 was characterized. This peptide sequence is part of the intracellular region of the C-terminus of the protein and is specific to human Cx31 based on sequence homology alignments with all the known connexins. In addition, BlastP analysis of this peptide sequence against the translated nucleotide GenBank database (via http://www.ncbi.nlm.nih.gov/) did not detect significant homology with any other predicted or known peptide sequence other than Cx31. Specificity of the Cx31 antibody was assessed following SDS-PAGE and immunoblotting. The results showed that the affinity-purified Cx31 antibody recognized a single 31 kDa after Cx31 was synthesized *in vitro* (TNT system) or when

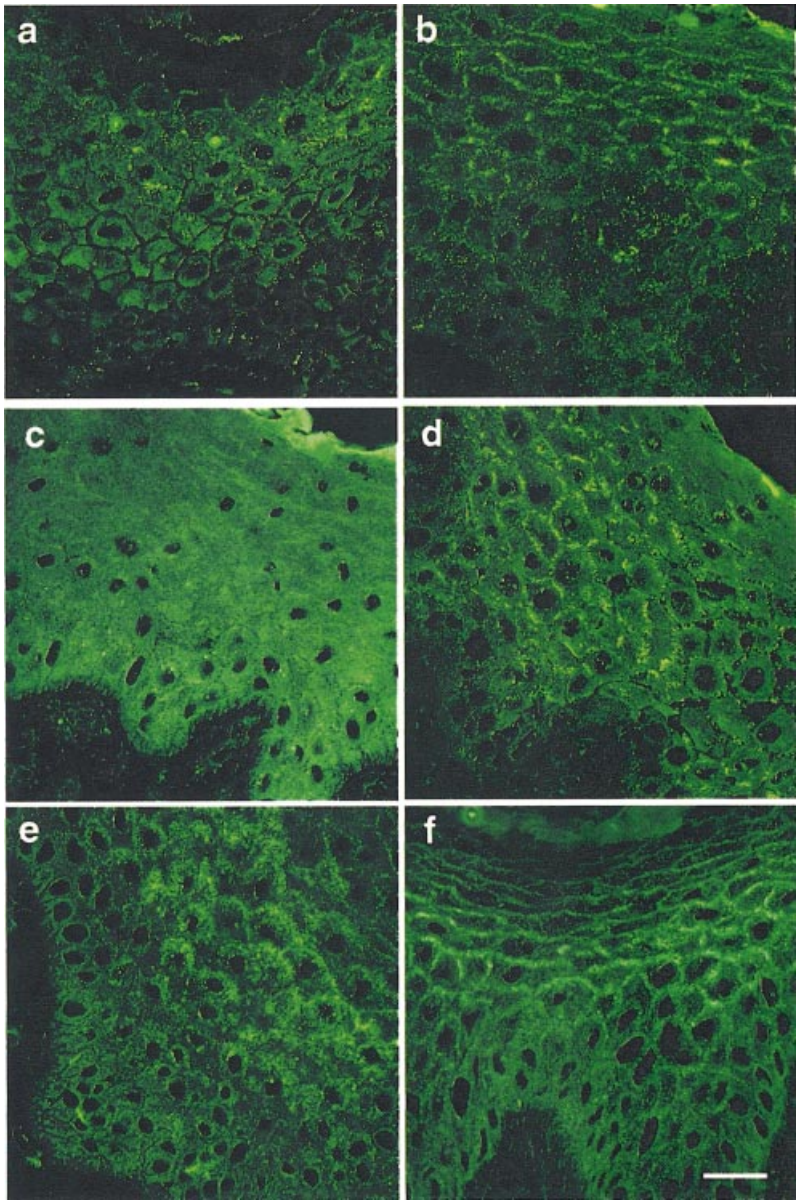

Figure 4. The expression pattern of connexin proteins in normal palm epidermis. Immunofluorescence staining using connexin antibodies: (*a*) Cx26; (*b*) Cx30; (*c*) Cx32; (*d*) Cx40; (*e*) Cx43; (*f*) Cx45. *Scale bar*: 20 μm.

expressed *in vivo* (transfected HeLa cells) by pcDNA3/Cx31. The weaker lower band may represent a dephosphorylated form of Cx31. The 31 kDa band comigrated with the 31 kDa band of ^{35}S-methionine-labeled protein (**Fig 1**). In contrast, there was no 31 kDa band (or the weaker lower band) in the nontransfected HeLa cells. Immunocytochemical analysis of normal palm and interfollicular skin using this Cx31 antibody indicated that Cx31 was present in the suprabasal layer of the epidermis with higher protein levels in the stratum granulosum than in the stratum spinosum (**Fig 2*a*, *b***). The pattern of staining was punctate and localized to the plasma membrane presumably representing Cx31 gap junction plaques between adjoining keratinocytes. From immunocytochemistry of hair-bearing skin, Cx31 also stained positively in the sebaceous gland and the innermost layer of the outer root sheath (**Fig 2*e*, *f***).

Distribution of other connexins in human epidermis (protein data) Immunocytochemical analysis using six commercially available connexin antibodies (Cx26, Cx30, Cx32, Cx40, Cx43, and Cx45) illustrated the distribution and morphology of these connexin proteins in human interfollicular epidermis (**Fig 3**) and palm epidermis (**Fig 4**). All connexin proteins showed a punctate staining at the plasma membrane suggesting the protein localized at gap junction plaques between adjoining keratinocytes. Observationally, it appeared that expression levels of connexins were higher in palm than in interfollicular epidermis.

Cx26 in palm had a patchy distribution and was detectable in regions of the stratum basale, rarely detectable in the stratum spinosum, and detectable again in the stratum granulosum. Cx26 in interfollicular epidermis was rarely detectable though there was a strong punctate staining in sweat duct (image not shown). These Cx26 localization data are similar to those described in previous studies using the same monoclonal anti-Cx26 antibody (Lucke *et al*, 1999). In the palm, Cx30 and Cx45 were detected in the suprabasal layers with the highest levels of protein expression in the granular cells. They both had a similar pattern of expression in interfollicular epidermis but the Cx30 protein expression levels were much less than that in palms. Cx40 was present in the suprabasal layers in both palm and skin with uniformly protein levels between granular cells and spinous cells. A low level of Cx32 protein was detectable,

particularly in the palm. As described previously, Cx43 was detected in the suprabasal layers with, subjectively, the highest protein expression levels in the granular cells (Salomon *et al*, 1994).

Evidence for other connexins expressed in human skin (RT-PCR) To determine if other connexins were present in human skin, we investigated how many of the known human connexins were actually expressed in normal palm and interfollicular skin. Amplification of mRNA from human palm and interfollicular skin by RT-PCR revealed that there were at least 10 connexins expressed in interfollicular skin. In addition to the connexins identified by immunocytochemistry (Cx26, Cx30, Cx31, Cx32, Cx40, Cx43, and Cx45), transcripts for Cx30.3, Cx31.1, and Cx37 were also found to be present (**Fig 5**). The smaller PCR product generated for Cx37 represents a nonspecific PCR artifact. Cx36 was the only tested connexin that was negative in all skin mRNA tested (data not shown). The lens-specific connexins Cx46 and Cx50 were not tested. For Cx31 mRNA analysis, tissue *in situ* hybridization of human palm skin was also performed and revealed that Cx31 mRNA was uniformly distributed predominately in basal keratinocytes and in the stratum spinosum (**Fig 6**). This contrasts with the Cx31 protein distribution revealed by immunocytochemistry. The difference could be due to the Cx31 protein only being translocated to the plasma membrane in the stratum granulosum.

DISCUSSION

In this study, we have examined the expression of Cx31 in human epidermis by immunocytochemistry, RT-PCR, and tissue *in situ* hybridization. To perform the former analysis, we have characterized a new polyclonal antibody raised against the intracellular region of the C terminus of the human Cx31 protein. This domain of the protein is specific to human Cx31. Immunocytochemical analysis with this antibody revealed that Cx31 was expressed strongly in the stratum granulosum of both interfollicular and palmoplantar epidermis with a punctate pattern of staining localized at the plasma membrane. No Cx31 protein was detected in cultured keratinocytes by immunocytochemistry or by immunoblot (data not shown). These protein expression and localization data suggest that Cx31 has a more prominent role in gap junction intercellular communication in later stages of keratinocyte differentiation. In addition to studying mutant Cx31 in EKV lesional epidermis and keratinocytes, this new antibody will be useful to characterize Cx31 expression in other cell types including those affected by specific dominant Cx31 mutations such as peripheral neurons and the epithelia of the inner ear (Xia *et al*, 1998; López-Bigas *et al*, 2001).

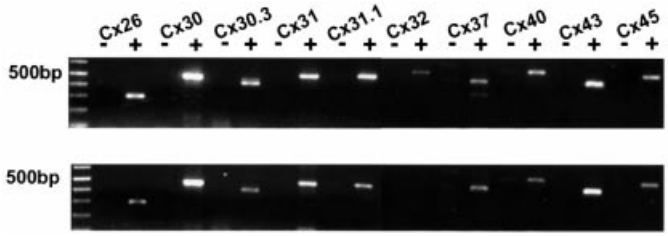

Figure 5. Connexin mRNA expressed in human interfollicular and palm skin. mRNA transcripts were amplified from human skin (*upper image*) and palm (*low image*) by RT-PCR. (–) indicates that the amplifications in the absence of reverse transcriptase were used as controls.

Previous studies have shown that the major epidermal connexin proteins are Cx43 and Cx26. From expression studies in rodents (Butterweck *et al*, 1994) and the identification of human epidermal disease-associated connexin mutations (Richard *et al*, 1998a; Lamartine *et al*, 2000; Macari *et al*, 2000), however, other connexins such as Cx31, Cx30.3, and Cx30 must also be expressed in the epidermis. At least 14 human connexin genes have now been identified and we have investigated their expression profile in human skin. RT-PCR analysis of interfollicular and palmoplantar skin identified transcripts for 10 connexins. Immunocytochemistry using antibodies representing epitopes for Cx31 (as discussed previously), Cx26, Cx43, Cx32, Cx40, Cx30, and Cx45 confirmed that at least seven of these connexins are also expressed at the protein level in the epidermis localizing at the plasma membrane with a characteristic punctate pattern. Previously, it has been reported that Cx32 and Cx40 were absent in human epidermis (Salomon *et al*, 1994). In our study, however, RT-PCR and immunocytochemistry revealed that these connexin proteins are indeed components of the epidermis, though Cx32 is only expressed at low levels. In rodent skin, Cx45, Cx40, Cx37, Cx31.1, Cx31 in addition to Cx26 and Cx43 have previously been shown to be present, suggesting there is a similar complex pattern of connexin expression in human and rodent epidermis (Butterweck *et al*, 1994; Goliger and Paul, 1994).

Mutations in four connexins have been associated with autosomal dominant inherited epidermal disease: Cx26 and Vohwinkel's syndrome or palmoplantar keratoderma associated with different types of hearing impairment; Cx31/Cx30.3 and EKV; and Cx30 and Clouston's hidrotic ectodermal dysplasia (Richard *et al*, 1998a; Maestrini *et al*, 1999; Kelsell *et al*, 2000; Lamartine *et al*, 2000; Macari *et al*, 2000). As mutations in Cx31 and Cx30.3 underlie clinically similar forms of EKV, it is tempting to speculate that Cx30.3 and Cx31 colocalize in the epidermis, and to address this we are currently developing antibodies raised against human Cx30.3. The expression pattern of Cx26 and Cx31 in normal human epidermis reflects, to some degree, the extent of epidermal disease with Cx26 expression restricted to the palmoplantar epidermis and Cx31 expressed in both palmoplanter and interfollicular epidermis. As the clinical onset and severity can vary in a patient's lifetime and between individuals harbouring the same connexin mutation, however, other epidermal connexins must be able to compensate for the mutant connexin protein and maintain a ''normal'' epidermal state. As recessive mutations in Cx26 and Cx31 underlie nonsyndromic hearing loss (Kelsell *et al*, 1997; Liu *et al*, 2000), it is likely that epidermal connexins can compensate for

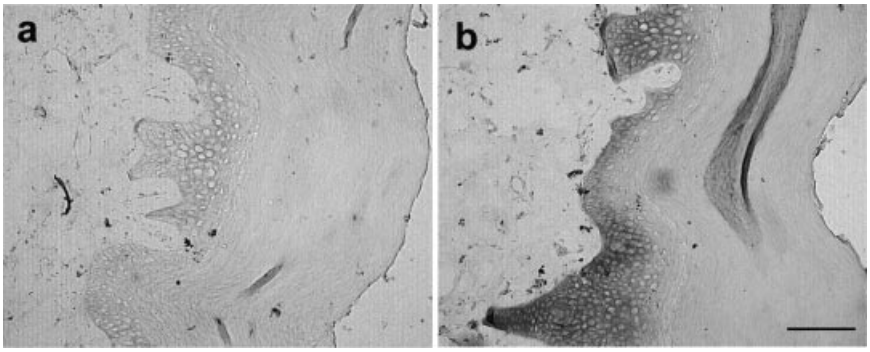

Figure 6. Distribution of Cx31 mRNA in palm. The Cx31 mRNA was detected by *in situ* hybridization: (*a*) sense; (*b*) antisense. *Scale bar*: 10 μm.

the ''loss'' of these connexin proteins. Another issue in connexin disease biology is that some dominant connexin mutations in the same protein cause skin disease and others cause hearing loss or both phenotypes.

Functional studies using the paired Xenopus oocyte assay and mammalian cell culture systems and have shown that some connexin mutations are processed differently (Richard *et al*, 1998b; White *et al*, 1998; Martin *et al*, 1999). These systems have only assayed mutant proteins individually, however, and not with respect to the full complement of other connexins expressed in the affected tissue. The characterization of the connexin content of the epidermis and developing antibodies raised against each connexin will enable the study of mutant connexins in the cell type affected such as keratinocytes. This will allow investigation of the effect of mutation on keratinocyte differentiation and on other endogenous connexins. These studies may shed light on the genotype-phenotypes of specific connexin mutations.

In summary, we have shown that in the human epidermis multiple connexin proteins are expressed in distinct keratinocyte subpopulations but with an overlapping distribution. The question that then arises is why there are so many. It has been demonstrated that specific homomeric and heteromeric connexons form selective channels with different affinities for the transport of ions and metabolites and having distinct gating properties (Goldberg *et al*, 1999; Niessen *et al*, 2000). Thus it is likely that, in the epidermis, specific channel types will have distinct properties of gap junctional intercellular communication and further studies are required to dissect their specific permeabilities to ions and metabolites. It is also plausible that not all the epidermal connexins will have a role in gap junctional intercellular communication as there is evidence that some connexons form hemichannels that regulate extracellular calcium ion concentration (Quist *et al*, 2000) and some may associate with specific tight junction proteins (Giepmans and Moolenaar, 1998; Kojima *et al*, 1999).

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