Skin-Specific Expression of ank-3_{93}, a Novel Ankyrin-3 Splice Variant

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Ankyrins represent a protein family whose members are associated with membrane proteins and the actin cytoskeleton. The principal ankyrin domain structure comprises an amino-terminal membrane-binding, a spectrin-binding, and a regulatory domain, and can be modulated by alternative splicing. In order to investigate the role of ankyrin-3 in skin, we have isolated three complete ankyrin-3 cDNA clones of 5.8 kb, 5.2 kb, and 2.5 kb by reverse transcription–polymerase chain reaction of mouse skin RNA. DNA sequencing confirmed the isolated clones to be splice variants of ankyrin-3. Of these, the smallest cDNA represents a novel ankyrin named ankyrin-3_{93}. Surprisingly, this novel ankyrin subtype lacks not only all ankyrin repeats, but also the first 75 amino acids of the spectrin-binding domain. Immunofluorescence analysis of mouse skin showed that ankyrin-3 is expressed in all living layers of mouse epidermis. Here, it predominates along the basal and lateral membranes of the basal layer in addition to an even cytoplasmic distribution. In primary mouse keratinocytes grown at elevated Ca^{2+} levels, ankyrin-3_{93} was localized along the plasma membrane and throughout the cell in a Golgi-like fashion. Depending on fixation conditions, nuclear staining became apparent in many cells. In agreement with previous data, northern blotting revealed a widespread distribution of the two larger ankyrin splice variants. In contrast, the mRNA coding for ankyrin-3_{93} was restricted to mouse skin. Reverse transcription–polymerase chain reaction of mouse skin RNA strongly suggested additional ankyrin isoforms in skin. Our data on ankyrin-3_{93}, which lacks a part of the spectrin-binding domain that regulates the affinity to spectrin, suggests a new function for this member of the ankyrin family. ***Key words: ankyrin-3/mouse/skin. J Invest Dermatol 116:216–223, 2001***

Ankyrins are regarded as a family of proteins that provide the connection of integral membrane proteins to the underlying spectrin-based cytoskeleton. Besides maintaining the structural integrity of the cell, they are supposedly involved in the formation of polarized membrane domains by accumulating and maintaining distinct integral proteins to specific subcellular sites (Bennett, 1982). Binding partners of ankyrin belong to the families of ion channel and cell adhesion proteins, for example the Cl^-/HCO_3^- anion exchanger from erythrocytes (Bennett and Stenbuck, 1980), the voltage-gated sodium channel from brain (Srinivasan *et al.* 1988), the Na^+/K^+-ATPase from kidney (Koob *et al*, 1990), the neurofascin/L1 NrCAM family (Davis and Bennett, 1994), and the hyaluronic acid receptor CD44 (Lokeshwar *et al*, 1994).

All ankyrins share a protein structure with three independently folded domains. The N-terminal 89–95 kDa membrane-binding domain contains 24 tandem repeats, so-called ankyrin (ANK) repeats, of a 33 amino acid sequence and is folded into four subdomains of six repeats each. This domain enables ankyrin to associate with transmembrane proteins. The ANK repeat is one of the most common protein sequence motifs, and has been recognized in more than 400 proteins in a highly variable number, including cyclin-dependent kinases (CDK), transcriptional regulators, cytoskeleton-associated proteins, developmental regulators, and toxins (Sedgwick and Smerdon, 1999).

The central 62 kDa spectrin-binding domain is divided into a N-terminal acidic region of high sequence variability and a C-terminal basic region. This domain provides the linkage to β-spectrin (also named fodrin), which is part of the subplasmalemmal cytoskeleton based on spectrin spokes and actin hubs. The C-terminal 55 kDa rod domain is supposed to regulate the activity of the two other domains and is poorly conserved between ankyrins (De Matteis and Morrow, 1998).

So far, three ankyrin genes have been identified in mammals: *Ank1*, *Ank2*, and *Ank3*. All ankyrin genes are subject to tissue-specific alternative splicing, thereby modulating the affinity to different binding partners and conveying different functional roles (Kordeli *et al*, 1998; De Matteis and Morrow, 1998).

Ankyrin-1 is expressed in erythrocytes and in a subset of spinal cord and hippocampal neurons in large isoforms of 210 and 220 kDa (Bennett and Stenbuck, 1979; Lambert and Bennett, 1993). Two very small ankyrin-1 isoforms of 28 and 30 kDa have been detected in muscle (Gallagher and Forget, 1998), which contain only the C-terminal part of the regulatory domain. In addition, a Golgi-associated isoform of 195 kDa in MDCK (Madin–Darby canine kidney) epithelial cells has been preliminary identified by immunologics criteria (Beck *et al*, 1997). Ankyrin-2 is the major ankyrin of the nervous system giving rise to two different

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Abbreviation: UTR, untranslated region.

splice variants: a 440 kDa isoform found in fetal unmyelinated axons and dendrites, containing a 220 kDa insert between the spectrin-binding and the regulatory domain, and a 220 kDa isoform found in adult neuron cell bodies, dendrites, and glia (Kunimoto *et al*, 1991; Otto *et al*, 1991).

The third member of the family, ankyrin-3, shows the most widespread tissue distribution and is subjected to intense alternative splicing, leading to numerous ankyrin-3 isoforms. Ankyrin-3 is the predominant ankyrin of epithelia. In kidney, isoforms of 215, 200, 190, 170, 120, and 105 kDa are present (Peters *et al*, 1995). An additional 190 kDa isoform was described for the kidney and lung (Thevananther *et al*, 1998). In brain, two large isoforms of 270 and 480 kDa were localized to the axon initial segment and the node of Ranvier (Kordeli *et al*, 1995). Both these isoforms have a brain-specific insert between the spectrin-binding and the regulatory domain. A Golgi-associated 119 kDa isoform of ankyrin-3 (ank_{G119}) has been detected in the kidney and muscle (Devarajan *et al*, 1996). Finally, two small isoforms of 100 and 120 kDa have been found associated with lysosomes of macrophages (Hoock *et al*, 1997). These three small isoforms all miss part or all of the repeat domain and in the case of ank_{G119} even part of the regulatory domain.

Thus, besides the expression of multiple isoforms of the same ankyrin gene in a cell, ankyrin isoforms of different ankyrin genes can be expressed in the same cell with distinct localizations indicating related but distrinct functions: in the neuron, for example, ankyrin-2 is found in the cell bodies and dendrites, and ankyrin-3 at the axon initial segments and nodes of Ranvier.

The epidermis is a particularly attractive tissue to investigate the regulatory mechanisms underlying cell proliferation, differentiation, and migration. In the epidermis, these processes lead from a basal, proliferating cell layer to a highly differentiated layer of suprabasal cells that no longer divide. Whereas the role of keratin intermediate filaments in epidermis has been investigated in great detail, not the least due to their involvement in blistering skin disorders (Uitto and Christiano, 1993; Fuchs and Weber, 1994; Corden and McLean, 1996), the functional significance of the microfilament system and its associated proteins is largely unclear. Previous work has identified an unspecified ankyrin in all layers of human epidermis (Shimizu *et al*, 1996).

In order to gain a first insight into the function of ankyrin-3 in skin, we started to analyze the expression and subcellular distribution of ankyrin-3 isoforms in mouse skin.

MATERIALS AND METHODS

Southern blot analysis Mouse genomic DNA was isolated according to Strauss (1994). For southern blot analysis, 10 μg of genomic DNA/lane were digested with *Sal*I, *Xba*I, *Bam*HI, *Eco*RI, *Hind*III, *Cla*I, and *Pst*I and separated on a 0.8% agarose gel. Gels and blots were processed as previously described (Porter *et al*, 1996). The hybridization probe was derived from the membrane-binding domain (positions 1026–1431 of the published ankyrin-3 sequence (Peters *et al*, 1995) (**Fig 1*b***) and random prime labeled (Roche Diagnostics, Mannheim, Germany). Hybridization was performed under stringent conditions (Magin *et al*, 1998) with 20 min washes done three times in 0.1% sodium dodecyl sulfate (SDS)/0.1 × sodium citrate/chloride buffer (SSC) at 68°C.

RNA preparation Total RNA of newborn mouse skin and adult mouse kidney, brain, liver, and heart was isolated with TRIZOL reagent (Gibco BRL, Karlsruhe, Germany) according to the protocol of the supplier. Polyadenylated RNA [poly(A)$^+$-RNA] was isolated on Oligo dT Cellulose columns (Amersham Pharmacia Biotech, Freiburg, Germany).

Northern blot analysis Ten micrograms of poly(A)$^+$-RNA of each organ was separated on a 0.8% formaldehyde/agarose gel, transferred to a nylon membrane (Genescreen, DuPont, Germany) and hybridized with a [^{32}P]-deoxycytidine triphosphate random prime labeled ankyrin-3 probe derived from the regulatory domain of ankyrin-3 (positions 4781-6012) (**Fig 1*b***). Final washes were done at 68°C in 0.1 × SSC/0.1% SDS, then the blot was subjected to autoradiography at –80°C. For loading controls, the blot was reprobed with a mouse glyceraldehyde-3-phosphate dehydrogenase probe.

For the confirmation of the new splice variant, the same northern blot was stripped and reprobed with a riboprobe spanning positions 44 to 122 of ank-3_{93} (**Fig *2***). The probe was generated by polymerase chain reaction (PCR), cloned into a pCR 2.1-Topo vector (Invitrogen, Groningen, The Netherlands) and *in vitro* transcribed with T7 RNA polymerase in the presence of [^{32}P]-labeled rCTP. After hybridization overnight at 65°C, the blot was washed three times at 72°C in 0.1×SSC/0.1% SDS.

Reverse transcription–PCR for the isolation of ankyrin-3 cDNAs For the isolation of ankyrin-3 cDNA clones, we performed reverse transcription–PCR. Total RNA from mouse skin was prepared as described above. Primers were deduced upstream of the 5′-start codon and downstream of the 3′-stop codon of a published Ank3-sequence (Peters *et al*, 1995) (EMBL/GenBank/DDBJ accession no. L40631): sense primer (primer 1): 5′–GATCTCAAGGAAAGATGAGT-3′ (positions 43–62); anti-sense primer (primer 4): 5′–CTGCTGATCTCTGGTGAGTT–3′ (positions 6012–5993) (**Fig 1*a***). In the reverse transcription reaction, 1 μg of total RNA and 20 pmol of hexanucleotide primers (Roche) were incubated at 85°C for 1 min, followed by a 10 min incubation at 65°C. After a short incubation on ice, 10 mM dithiothreitol, 1 mM deoxyribonucleoside triphosphate (sodium salts), 20 units RNAsin (Promega, Mannheim, Germany), and 50 units of Expand™ Reverse Transcriptase (Roche) were added to reach a total volume of 20 μl. The reaction was incubated for 10 min at 30°C, 45 min at 42°C and then stopped on ice. Immediately after reverse transcription, PCR was performed with the Expand Long Template PCR-System (Roche) in a 50 μl reaction mixture with buffer 3, containing 5 μl of template cDNA, 0.5 mM deoxyribonucleoside triphosphate 0.4 μM of each primer, 8% dimethylsulfoxide and 2.5 units of Expand Long Template enzyme mixture. Amplification was carried out according to the supplier's protocol using an annealing temperature of 50°C. Reaction products were subcloned into the pCRII-Topo vector (Invitrogen) and analyzed by restriction with several enzymes. For automated DNA sequencing (ABI prism; PE Biosystems, Weiterstadt, Germany), DNA was purified by phenol/chloroform, followed by ethanol precipitation and a final wash in 70% ethanol.

Confirmation of novel isoform by reverse transcription–PCR For the confirmation of the small isoform we used two primer pairs: primer 1 (sense primer), positions 43–62; primer 2 (anti-sense primer), positions 3089–3076; primer 3 (anti-sense primer), positions 4992–4971 (**Fig 1*a***). PCR conditions were as described above. The product of the reverse transcription–PCR with primers 1 and 2 was sequenced to confirm the splice consensus sequence.

Preparation of an ankyrin-3 anti-serum A domain-specific ankyrin-3 anti-serum, named ank-3/a, was raised against the sequence EEEGSTRSEPKQGE of the regulatory domain (amino acid positions 1930–1943) (**Fig 1*c***) (Eurogentec, Herstal, Belgium). An additional anti-serum, named ank-3/b, was raised against the peptide RKRKGKKHLTFTRE, which corresponds to the new splice site described here for ank-3_{93} (amino acid positions 15–28). The peptides were coupled to KLH (key limpet hemocyanin) and each were injected into two rabbits. The ankyrin-3 anti-serum was affinity purified on the appropriate peptide. Preimmunsera stainings were negative in western blotting and immunofluorescence analysis.

***In vitro* transcription/translation** *In vitro* transcription and translation was performed with the TNT Quick Coupled Transcription/Translation System (Promega, Heidelberg, Germany) according to the manufacturer's protocol with biotin-labeled lysine-tRNA (Transcend tRNA). One microgram of the pCRII-Topo vector containing the cDNA for the new splice variant was used, and the reaction was incubated for 90 minutes at 30°C. Two microliters of the lysat were mixed with 20 μl of 1 × SDS sample buffer and boiled for 2 min at 95°C. Ten microliters of the sample were loaded on a 10% SDS–polyacrylamide gel. After the run, proteins were electrotransferred to a polyvinylidene difluoride membrane (Schroder *et al*, 1999). For detection of the *in vitro* translated proteins, the membrane was blocked in Tris-buffered saline/0.1% Tween-20 for 30 min, followed by the incubation with Streptavidin–horseradish peroxidase in a 1 : 30,000 dilution for 1 h. For detection, we used Pierce Super Signal Ultra (KMF, St Augustin, Germany). Alternatively, unlabeled translation products were detected by western blotting (see below).

SDS–polyacrylamide gel electrophoresis and western blot Total proteins were extracted from neonatal mouse skin, dermis, epidermis, and mouse keratinocytes in 1 × SDS sample buffer (Schroder *et al*, 1999).

Dermis and epidermis were separated by incubating decapitated neonatal mice in water at 60°C for 40 s. Gel electrophoresis was performed by standard procedures. The proteins were electrotransferred to polyvinylidene difluoride membranes as described above. Membranes were blocked in 0.1% Tween-20 according to Porter *et al*, (1996). The ank-3/a anti-serum was used in a dilution of 1 : 5000, the ank-3/b antiserum in a dilution of 1 : 2500. For the competition experiment 30 μg per ml of ank-3/b peptide were added to the primary antibody incubation. For detection, we used a horseradish peroxidase-conjugated rabbit anti-mouse anti-serum in a 1 : 30,000 dilution.

Keratinocyte cell culture Primary mouse keratinocytes from wild-type mice were received from David Melton (Edinburgh, personal communication). For immunofluorescence studies, cells were cultivated as reported elsewhere (Melton *et al*, in preparation). Twenty-four hours before fixation, the calcium ion concentration was raised to 2 mM.

Immunofluorescence microscopy Back skin was taken from newborn mice, frozen in liquid nitrogen, and stored at –80°C. Cryosectioning was performed at –20°C. Slides were air dried for 2 h and then fixed for 10 min at –20°C in acetone and air dried. Sections were encircled with a wax pen (Dako, Hamburg, Germany). For ankyrin and desmoplakin staining, mouse keratinocytes were washed two times in prewarmed (37°C) phosphate-buffered saline and then fixed for 3 min (10 min) in methanol (–20°C), followed by 20 s in acetone (–20°C). For actin staining, cells were fixed for 10 min in 4% paraformaldehyde (room temperature), washed in phosphate-buffered saline and incubated in acetone (–20°C) for 3–5 min. Primary antibodies ank-3/a and ank-3/b were used in a dilution of 1 : 100. After incubation in a humidified chamber for 1 h, the slides were washed three times in phosphate-buffered saline (pH 7.4) and then incubated for 30 min with 25 μl of the secondary antibody (Alexa-conjugated anti-sera, 1 : 400 dilution, Molecular Probes, Leiden, The Netherlands) or phalloidin-actin 1 : 40 (Molecular Probes). The washing was repeated as described, followed by brief rinses in water and ethanol. Coverslips were mounted with Mowiol containing 5% Dabco (Calbiochem, Bad Soden, Germany). Tissue sections and keratinocytes were analyzed with a fluorescence photomicroscope (Axiophot2, Zeiss, Oberkochen, Germany).

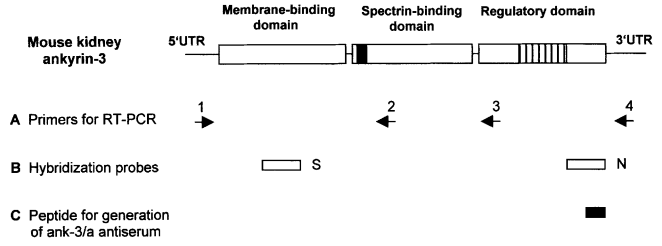

Figure 1. Analysis of ankyrin-3 expression in mouse skin. (*A*) Primers deduced for reverse transcription–PCR based on the 7 kb ankyrin-3 isoform mRNA detected in the mouse kidney (Peters *et al*, 1995). Primers 1 and 4 were used for the isolation of complete ankyrin-3 cDNA clones. Primers 2 and 3 were used in combination with primer 1 for a reverse transcription–PCR to confirm the existence of the isolated 2.5 kb isoform. (*B*) Hybridization probes used in southern (S) and northern (N) blotting. (*C*) Localization of the epitope used for the ank-3/a anti-serum synthesis. For exact nucleotide positions see *Materials and Methods*.

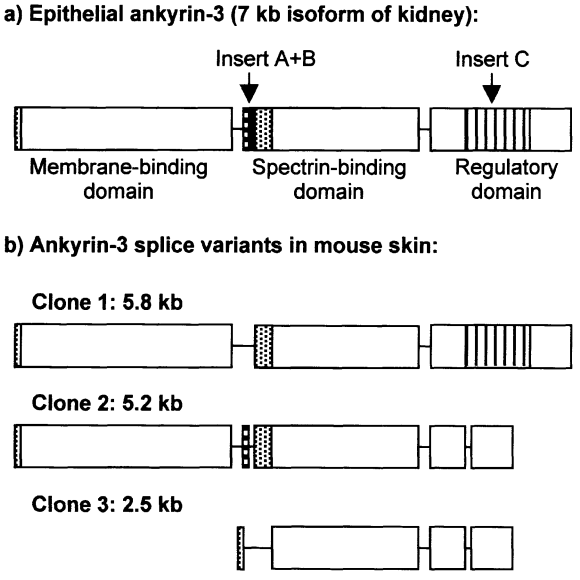

Figure 2. Protein structure of the ankyrin-3 splice variants isolated from mouse skin. The splice pattern of clone 1 and clone 2 is identical to the one described for ankyrin-3 isoforms detected in mouse kidney: (with inserts (*A*) cross-stiped, (*B*) black, and (*C*) vertically striped (Peters *et al*, 1995). Clone 3 exhibits a new splice pattern lacking not only all ANK repeats, but in addition the N-terminal part of the spectrin-binding domain (dotted).

Analysis of DNA sequence data The analysis of the DNA and protein sequences was performed using the Heidelberg Unix Sequence Analysis Resources (HUSAR) program based on the Genetics Computer Group (GCG) programs.

RESULTS

cDNA cloning of ankyrin-3 from mouse skin So far, most ankyrin-3 splice variants have been analyzed in single layered epithelia. Based on cross-reacting anti-sera and RNA hybridization data, previous work has hinted towards the presence of an unspecified ankyrin in epidermis (Peters *et al*, 1995; Shimizu *et al*, 1996). In order to investigate the role of ankyrin-3 in skin, we initiated the cDNA cloning of ankyrin-3 from total RNA isolated from newborn mouse skin. Based on primers deduced upstream of the start codon, and downstream of the stop codon of an ankyrin-3 cDNA from mouse kidney epithelium (Peters *et al*, 1995), we performed reverse transcription–PCR optimized for long trranscripts (**Fig 1**). This led to the isolation of three ankyrin-3 cDNAs from mouse skin RNA.

The largest cDNA clone (5766 bases, counted from the start to stop codon) exhibited 99.1% identity (18 exchanges, of which one is conservative) by sequence comparison with the published 7 kb isoform in the kidney. Inserts A and B, typical of kidney ankyrin-3 were lacking (**Fig 3**). The size difference between the isolated clone and the published 7 kb isoform results from the 5′ and 3′ untranslated regions, which are not isolated by our reverse transcription–PCR approach. These add approximately 1.2 kb to each of the cDNAs.

The second cDNA clone (5244 bases) corresponded to another splice variant of the 7 kb clone exhibiting 99.3% sequence identity with 14 exchanges of which one is conservative. It also codes for insert A, but lacks the acidic insert C in the regulatory domain. In both clones, amino acid 115 in the third ankyrin repat is exchanged, but replaced by different amino acids. At present, we cannot decide whether this is due to a polymorphism or an error in one of the sequences.

The smallest ankyrin-3 cDNA encoded an open reading frame of 2517 bases and represents a novel isoform with unique features (**Figs 2** and **3**). The first 21 amino acids of the N-terminal membrane-binding domain were identical to those of ankyrin-3 in the kidney, but the typical ankyrin repeats were absent. In addition, the first 75 amino acids of the N-terminal part of the spectrin-binding domain were missing, resulting from a splice pattern that has not been reported before. Starting from amino acid position 22, the cDNA was identical to the published 7 kb ankyrin-3 isoform starting at position 949 and missing insert C. Only one difference to the published ankyrin-3 sequence was found: amino acid residue 81 of the new splice variant was changed from isoleucine to valine. The ankyrin encoded by this cDNA, which we named ank-3_{93}, represents a new splice variant.

The HUSAR program PEPSTATS predicted a protein of 838 amino acids with a predicted mass of 93 kDa and a calculated isoelectric point of 8.32 for ank-3_{93}. This protein was detected in western blotting after coupled *in vitro* transcription/translation of

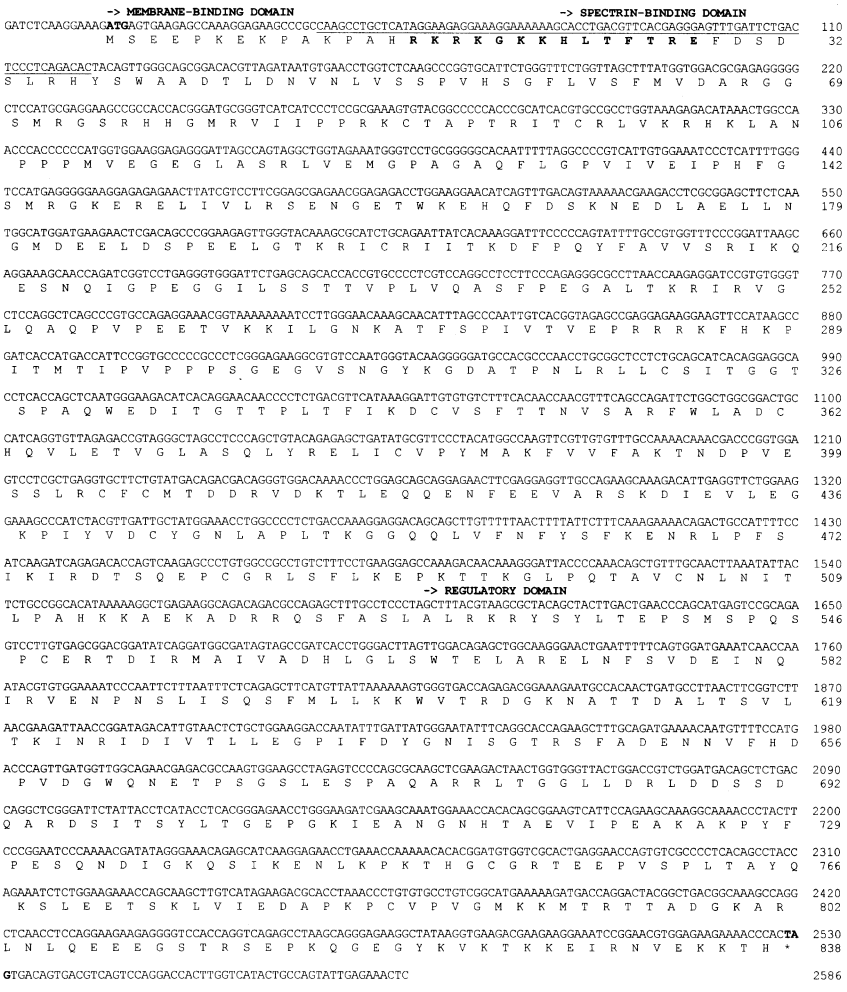

Figure 3. Nucleotide and deduced amino acid sequence of the novel ankyrin-3 splice variant ank-3_{93}. The start of each domain is marked by an *arrow*. Compared with the 7 kb ankyrin-3 isoform described in kidney the 24 ANK repeats and the first 75 amino acids of the spectrin-binding domain, all together a 2.8 kb insert, are absent. The hybridization probe used in northern blotting for the confirmation of ank-3_{93} is underlined. The epitope used for synthesis of the ank-3_{93} antiserum is underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number AF212924.

the corresponding cDNA both by streptavidin–horseradish peroxidase and the newly synthezised ank-3_{93} anti-serum (see below). The protein migrated at about 98 kDa (**Fig 4**). In addition, two-dimensional gel electrophoresis of the *in vitro* translated protein confirmed its calculated isoelectric point (8.32) (data not shown). In order to test whether ankyrin-3 isoforms, including ank-3_{93}, were the products of a single gene, we performed southern blot analysis (**Fig 5**). Mouse genomic DNA was digested with several restriction enzymes and hybridized with a probe corresponding to ANK repeats 9–14 of the membrane-binding domain of ankyrin-3. Under stringent conditions only one fragment of each restriction digest was recognized, indicating that all ankyrin-3 splice forms were the products of a single copy gene.

Confirmation of the new splice variant In order to get additional evidence for the presence of the mRNA for ank-3_{93}, we designed a reverse transcriptase–PCR experiment that would detect our novel splice variant. The positions of the two primer pairs are outlined in **Fig 1**. The amplified products should have a length of 267 bases (primers 1/2) and 2122 bases (primers 1/3), respectively, to be specific for the new isoform. In two separate approaches, we were able to amplify the expected fragments reproducibly (**Fig 6*b***). Beside the two expected fragments, a 600 bp fragment was amplified, indicating an additional ankyrin-3 isoform that has not been characterized further by cDNA cloning. Sequencing of the amplified 267 bases product confirmed the splice pattern of the complete 2.5 kb clone.

Tissue-specific expression of ankyrin-3 Next, we used a probe derived from the regulatory domain of ankyrin-3, which does not cross-react with ankyrin-1 or ankyrin-2 to study the expression of all ankyrin-3 splice variants. In mouse skin, mRNA of 8.6, 7.0, 6.4, 5.2, 4.5, and 3.7 kb were detected (**Fig 7*a***). Of these, the 7.0 and the 6.4 kb mRNA correspond to those

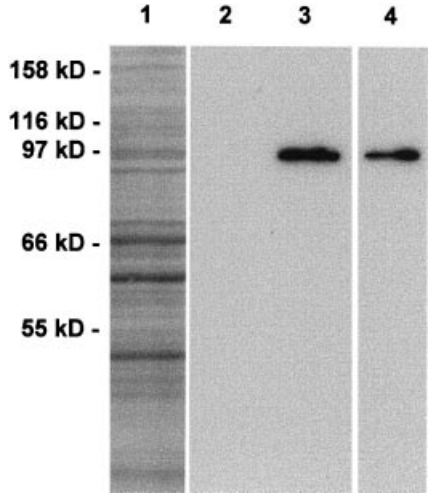

Figure 4. Ank-3_{93} encodes a protein of about 98 kDa. Clone 3 was translated *in vitro* with a biotin-coupled amino acid and after electrophoresis analyzed. The new ank-3_{93} anti-serum and streptavidin–horseradish peroxidase both gave a single strong signal of about 98 kDa. *Lane 1*: Coomassie staining of *in vitro* translated protein; *lane 2*: negative control (no plasmid); *lane 3*: detection with ank-3/b; *Lane 4*: detection with streptavidin–horseradish peroxidase. On the left side, the protein marker sizes are denoted.

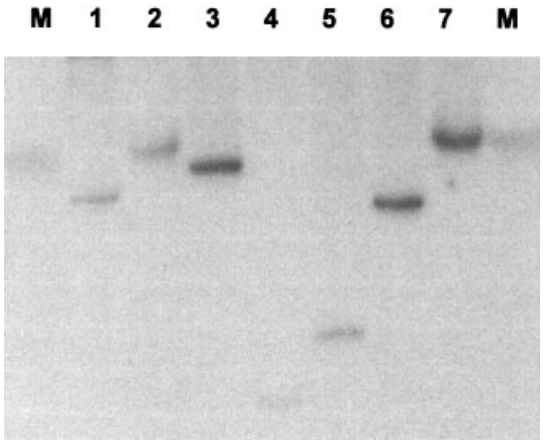

Figure 5. Ankyrin-3 is the product of a single copy gene. Southern blot of mouse genomic DNA. Restrictions were as follows: *Sal*I (1), *Xba*I (2), *Bam*HI (3), *Eco*RI (4), *Hind*III (5), *Cla*I (6), and *Pst*I (7). The hybridization probe, corresponding to the ANK repeats 9–14 of the membrane-binding domain, recognized a single fragment of each restriction digest, indicating ankyrin-3 splice variants to be the product of a single copy gene.

previously reported in kidney epithelium (Peters *et al*, 1995) and cloned in this report. These mRNA give rise to ankyrin isoforms of 214 and 190 kDA (for western blotting, see below). In addition to mRNA identified by cDNA cloning, our northern blot data predict at least three additional ankyrin-3 isoforms in mouse skin.

As ankyrins are widely expressed in most tissues, northern blot analysis was extended to mouse kidney, brain, heart, and liver (**Fig 7*a***). This revealed the prevalance of splice variants of about 5 kb in skin, but also in liver. In contrast, large isoforms of ankyrin-3 are the major mRNA in brain and heart: isoforms of more than 10 kb dominate in brain, isoforms of about 7 kb in heart. Smaller isoforms could only be detected in minor amounts. Only in kidney, prominent mRNA of about 4–5 kb and 6–7 kb were seen with additional minor mRNA ranging from 3 to 9 kb.

In order to detect the mRNA for ank-3_{93} selectively by northern blotting, we used a riboprobe covering 40 bases upstream and 40 bases downstream of the potential new splice site (**Fig 2**). Given that ank-3 mRNA contains 5′- and 3′-untranslated regions of 1.2 kb, we predicted a mRNA of 3.7 kb for the newly discovered ank-3_{93}. In addition, this probe should detect potential additional isoforms exhibiting the same splice pattern. This riboprobe was first characterized on *in vitro* transcribed RNA of the 5.8 kb and 2.5 kb cDNA and proved to be specific for the new splice variant (data not shown). When the tissue northern blot (**Fig 7*a***) was reprobed with this riboprobe (**Fig 7*b***), we detected a single mRNA of 3.7 kb exclusively in mouse skin. Therefore, we conclude that the ank-3_{93} splice variant is skin specific.

In order to correlate mRNA and protein expression, we raised an anti-serum against ankyrin-3 (named ank-3/a) based on the results of the sequencing of our cDNA clones. This anti-serum was supposed to recognize all published ankyrin-3 isoforms. The peptide was derived from the C-terminus of the regulatory domain (**Fig 1**). The affinity purified anti-serum ank-3/a was used in western blotting of total protein extracts of mouse skin, mouse dermis, epidermis, and mouse keratinocytes (**Fig 8**). Among multiple proteins, polypeptides of 214, 190, and 93 kDa were detected in mouse skin, corresponding to the proteins predicted by cDNA cloning and northern blotting. Ank-3_{93} appeared only as a minor product. Therefore, the results of the Western blotting were in agreement with the results of our northern blot experiment and strongly suggest the presence of ank-3_{93} in mouse skin.

Localization of ankyrin-3 in mouse skin We analyzed the localization of ankyrin-3 in mouse skin by immunofluorescence

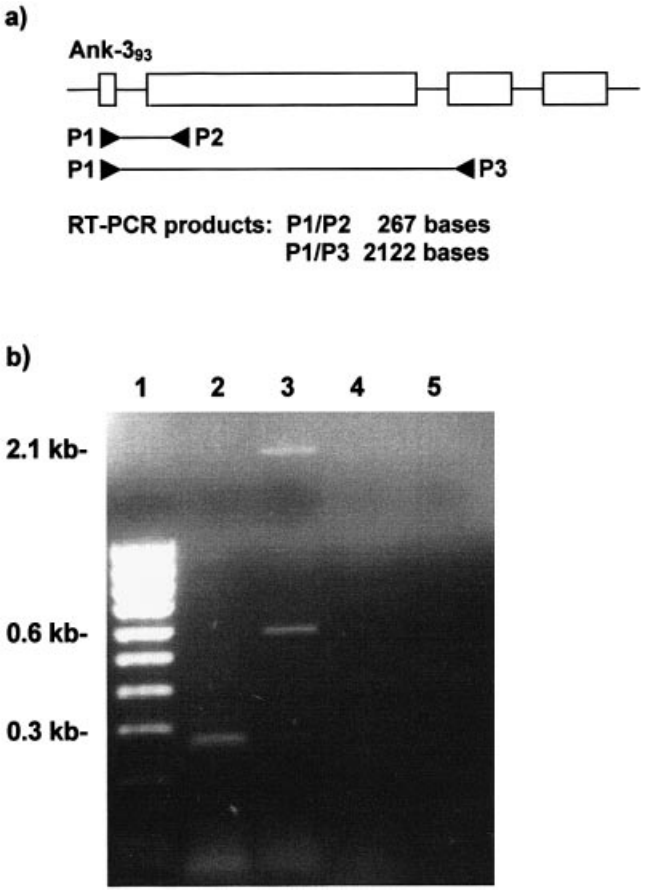

Figure 6. The splice consensus of ank-3_{93} is confirmed by reverse transcription–PCR. With two primer pairs (primers 1 and 2, primers 1 and 3) we analyzed for the presence of the mRNA coding for the small isoform in total RNA of mouse epidermis. (*a*) Position of the primers and size of the expected products. The PCR should generate one fragment of 267 bases (primer pair 1/2) and one of 2122 bases (primer pair 1/3) (for exact primer positions see *Materials and Methods*). (*b*) Gel electrophoresis of reverse transcription–PCR products, which correspond to the expected sizes. *Lane 1*: DNA size marker (100 bp ladder); *lane 2*: PCR with primer pair 1/2; *lane 3*L PCR with primer pair 1/3; *lane 4*: negative control with total RNA; *lane 5*: negative control without mRNA. Moreover, a 600 base fragment was amplified, indicating an additional ankyrin-3 splice variant not isolated by reverse transcription–PCR.

with the ank-3/a anti-serum. The anti-serum should detect all ankyrin-3 isoforms containing the C-terminal part of the regulatory domain, which includes all ankyrin-3 isoforms presently known.

All living layers of the epidermis in neonatal mouse skin showed an intense ankyrin-3 staining (**Fig 9**). In the basal layer of epidermis, including the hair follicles the most intense fluorescence was observed. Ankyrin was detected in zones of cell–cell as well as of cell–matrix contact. Beside the prominent staining at the plasma membrane, a light punctate staining was seen in the cytoplasm. The stratum corneum was not stained. A similar distribution was noted in human epidermis (data not shown).

Localization of ankyrin-3 and ank-3_{93} in mouse keratinocytes For the better evaluation of the intracellular localization of ankyrin-3, we performed immunostaining of ankyrin-3 in primary mouse keratinocytes (**Fig 10**). Staining with ank-3/a revealed that ankyrin-3 was distributed almost evenly over the complete cytoplasm with a slight increase in fluorescence intensity in the nuclear perimeter (**Fig 10*a***). No significant codistribution with actin could be found (data not shown).

Staining with the ank-3/b anti-serum revealed a vesicular distribution of ank-3_{93} (**Fig 10*b***), which was not identical to the Golgi apparatus (data not shown). In addition, there was a weak but

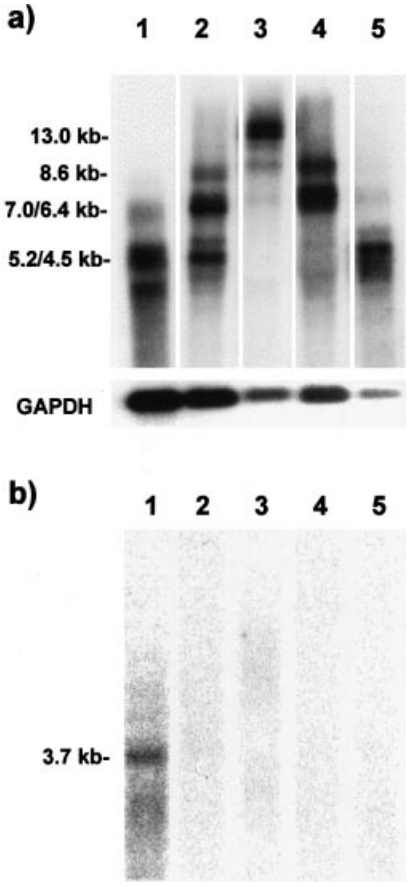

Figure 7. Ankyrin-3 is expressed in multiple splice variants in several mouse tissues. (*a*) Expression of ankyrin-3 in mouse tissues: skin (1), kidney (2), brain (3), heart (4), and liver (5). The mRNA of ankyrin-3 splice variants was analyzed by hybridization with a cDNA encoding part of the regulatory domain of ankyrin-3. The amount of RNA loaded in each lane was controlled by hybridization with mouse glyceraldehyde-3-phosphate dehydrogerase cDNA. mRNA sizes were estimated relative to 16S and 23S ribosomal RNA. The sizes of the mRNAs detected in mouse skin are denoted on the left side of the panel (b). Detection of the 3.7 kb mRNA encoding the new ankyrin splice variant ank-3_{93} exclusively in mouse skin, after rehybridization of the same northern blot with a splice variant specific riboprobe.

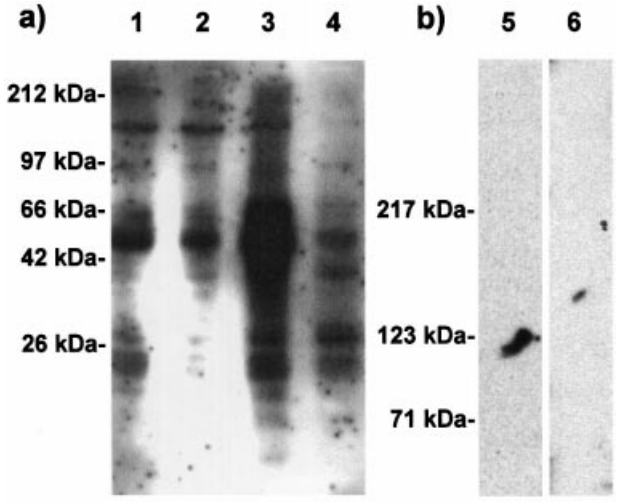

Figure 8. Multiple isoforms of ankyrin-3 can be detected in mouse skin on the protein level. (*a*) Immunoblot analysis of ankyrin-3 in mouse skin (*lane 1*), dermis (*lane 2*), epidermis (*lane 3*), and keratinocytes (*lane 4*). Total protein extracts were immunoblotted and incubated with ankyrin-3 anti-serum directed against the regulatory domain. On the left side, the protein marker sizes are denoted. (*b*) Mouse skin protein extract was incubated without (*lane 5*) and in the presence of the peptide used for immunization (*lane 6*) to test the specificity of the ank-3/b serum. In the presence of the competitor, the signal was absent. On the left side, the protein marker sizes are denoted.

distinct staining along the plasma membrane that did not colocalize with desmoplakin (data not shown). When cells were fixed briefly, a strong staining was observed in the nucleus. Upon prolonged fixation, the nuclear staining disappeared, as reported for the armadillo family (e.g., Mertens *et al*, 1996) (**Fig 10*b*** *inset*).

In conclusion, we have identified a novel skin-specific ankyrin-3 isoform, which represents the first ankyrin lacking all ANK repeats and the N-terminal part of the spectrin-binding domain. It is expressed in mouse skin together with at least two other ank-3 splice variants. Future studies will address the function of ank-3_{93}, which represents the first skin-specific ankyrin.

DISCUSSION

In order to gain an insight into the molecular organization and distribution of ankyrin-3 in epidermis, we have started its analysis in mouse skin. In this study, we describe the cDNA cloning of ankyrin-3 splice variants from mouse skin. Most importantly, we have isolated a novel skin-specific ankyrin-3 isoform, ank-3_{93}, which results from a new splice pattern that has not been described before. Using a cRNA probe and a newly generated anti-serum that specifically reacted with ank-3_{93}, we have demonstrated that ank-3_{93} is expressed exclusively in mouse skin. Here, the immunofluorescence analysis indicated a predominant localization along the basolateral membranes of the basal epidermal layer. In addition, all living strata of mouse epidermis were labeled throughout the cytoplasm. A similar pattern was observed in primary mouse keratinocytes grown in the presence of elevated Ca^{2+} ion levels. Most notably, ank-3_{93} was also localized in distinct nuclear patches, providing appropriate fixation conditions. Such a dual localization has by now been reported for a number of proteins, including members of the armadillo family (Hatzfeld, 1999). Given that ank-3_{93} lacks part of the spectrin-binding domain and all of its ANK repeats, we suggest that a dual localization would be compatible with its domain structure. Whether ank-3_{93} performs functions diferent from known ankyrins, will be analyzed in the future.

The main difference between ankyrin-3 isoforms is the presence or absence of part or all of the membrane-binding repeat domain. Whereas large ankyrin isoforms typically are located underneath the plasma membrane, small isoforms lacking the repeat domain seem to localize at other membranes inside the cell (Devarajan *et al*, 1996; Hoock *et al*, 1997). This is an important observation, as the complete sequence of ankyrin-3 splice variants is contained within the full-length ankyrin-3, which is located under the plasma membrane. The mechanism responsible for the exclusion of large isoforms from intracellular membranes is not known. Potential mechanisms could involve phosphorylation of ankyrin, association with other proteins, or maybe a specific subcellular localization during the biosynthesis of ankyrins. Our analysis of the ankyrin-3 expression in mouse skin revealed for the first time, that in skin, like in other tissues, a multitude of ankyrin splice variants exists. In this study, we report on three ankyrin-3 isoforms. Based on their domain structure and known sequence we predict that the two large isoforms, ank-3_{214} and ank-3_{190} are localized at the plasma membrane, as they contain the full-length membrane-binding domain with all ANK repeats. The third isoform, which we termed ank-3_{93}, has a unique domain structure: it lacks all ANK repeats and 75 amino acids of the N-terminal part of the spectrin-binding domain. It was shown that the N-terminal 70 amino acids of the spectrin-binding domain of ankyrin-1 are most important for the regulation of the spectrin-binding affinity and the distinctive cellular localization of ankyrin. This unique N-terminal subdomain of the spectrin-binding domain has been suggested to regulate the

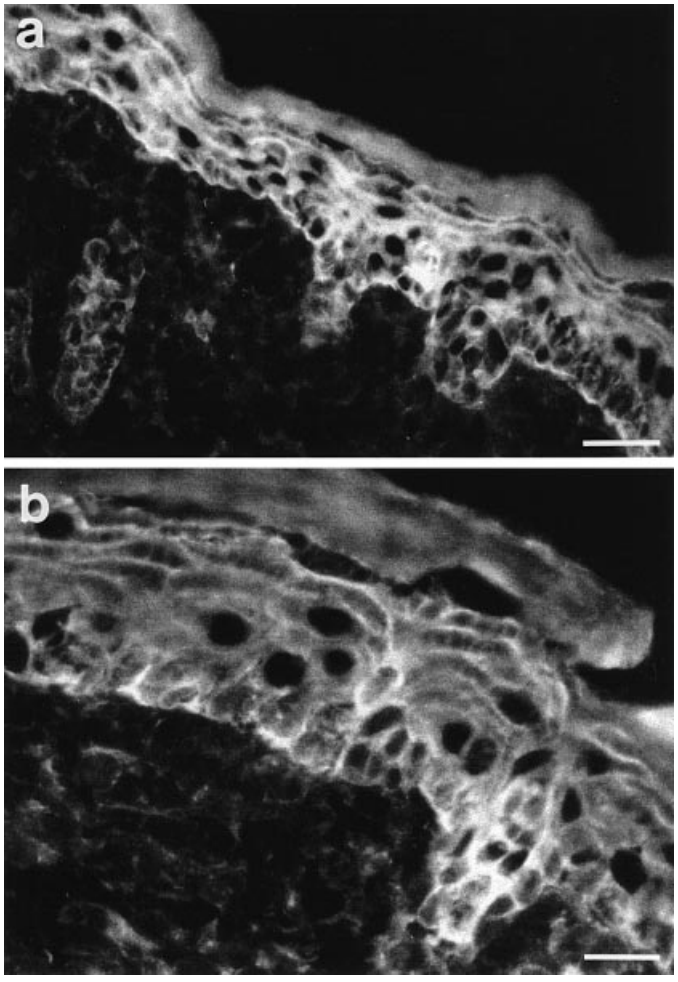

Figure 9. Ankyrin-3 is localized predominantly in all living layers of the epidermis and hair follicles of mouse skin. (*a*) Immunofluorescence analysis of neonatal mouse skin with the ank-3/a anti-serum revealed an intense membrane bound fluorescence and a diffuse but punctate cytoplasmic staining in all living layers of the epidermis, including the hair follicles; *scale bar*: 18 μm. (*b*) At higher magnification, the predominant localization in the cell-cell and cell–matrix contact zones of the basal layer become visible; *scale bar*: 11 μm.

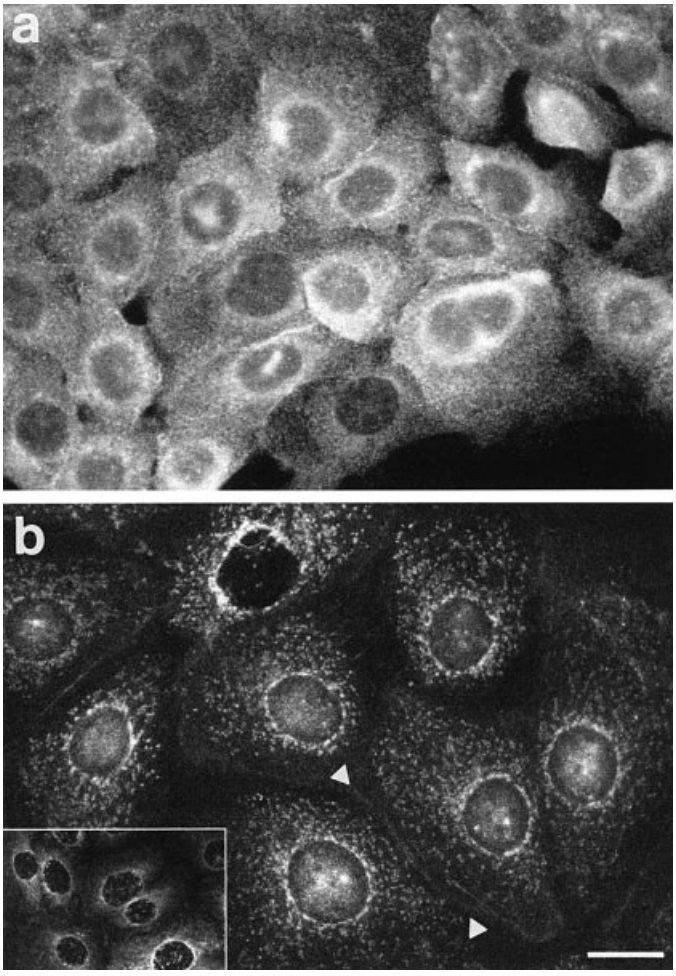

Figure 10. Ankyrin-3 localization in mouse primary keratinocytes. (*a*) Immunofluorescence analysis revealed that ankyrin-3 was distributed almost evenly over the complete cytoplasm. Note the slight increase in fluorescence intensity in the nuclear perimeter (ank-3/a anti-serum). (*b*) Ank-3_{93} exhibited a vesicular distribution in the cell (ank-3/b anti-serum) with a weak but distinct staining along the plasma membrane (*arrowheads*). After prolonged fixation, the staining in the nucleus disappeared (*inset*). *Scale bar*: 18 μm.

specific affinities of ankyrin family members to spectrin (Platt *et al*, 1993). As this domain is missing in ank-3_{93}, the spectrin affinity probably is either no longer modulated, or different ankyrin domains are required for this function. As we have shown by northern and western blot analysis, it is a skin-specific isoform of minor abundance, but the functional significance of the protein remains to be clarified. In mouse keratinocytes, we found a vesicular distribution of ank-3_{93}. Several small isoforms of ankyrin have been described to localize at the Golgi apparatus, but no colocalization could be detected in immunofluorescence staining with a Golgi marker.

Ankyrin as a binding partner for integral membrane proteins and the spectrin-based cytoskeleton is believed to be a key player in the polarization of cells and in keeping up the integrity of specialized membrane domains (Kunimoto *et al*, 1991; Otto *et al*, 1991; Kordeli *et al*, 1995). Of note, ankyrin is a binding partner for transmembrane channels and was shown to be a binding partner for the amiloride-sensitive sodium-channel in renal epithelium (Smith *et al*, 1991). Recently, it was demonstrated that amiloride-sensitive epithelial sodium channels are upregulated in epidermal development (Oda *et al*, 1999). These channels might play an important part in epidermal differentiation and skin development possibly by modulating the ion transport required for the epidermal terminal differentiation. This process might be regulated by the association to ankyrins. A development-dependent expression of ankyrin isoforms has also been observed on brain-specific ankyrin splice variants (Kunimoto *et al*, 1991; Otto *et al*, 1991). In analogy to brain, there are also numerous ankyrin isoforms in skin. In agreement with cDNA sequencing and our immunohistochemical data, membrane-bound as well as cytoplasmic ankyrins were found.

Among known ankyrin-binding partners, spectrin is the best characterized. It binds to ankyrin via the 15th repeat of β-spectrin. In normal human epidermis and in keratinocytes, α-spectrin has been found localized in the cytoplasm and associated with the plasma membrane (Mutha *et al*, 1991). Interestingly, in cutaneous tumors membrane-bound spectrin was diminished or lacked completely depending on the increase of depolarization and proliferation of tumor cells, and in some cases, the cytoplasmic spectrin staining was increased instead (Tuominen *et al*, 1996).

So far, there is no *in vivo* evidence for the function of ankyrin-3 in epidermis. Based on the results of ankyrin-2 knockout mice (Scotland *et al*, 1998) it has been postulated, that ankyrin-2 might contribute to the increased mechanical stability of brain tissue by binding to a transmembrane protein (L1), and the intracellular actin–spectrin network. This interaction, which might involve additional proteins, could represent a transcellular actin-based cytoskeleton. Such an array would be analogous to the keratin–desmosome skeleton, which builds the architectural framework for

the coherence of epithelial cells. In the light of blistering skin disorders resulting from keratin mutations it will be important to characterize further the function of ankyrin in epidermis.

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