The Calcium Sensing Receptor and Its Alternatively Spliced Form in Murine Epidermal Differentiation*  

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We have recently reported that human keratinocytes express both the full-length calcium sensing receptor (CaR) and an alternatively spliced form lacking exon 5, which were suggested to be involved in calcium induced keratinocyte differentiation. To understand further the role of these CaRs, we analyzed the structure of mouse CaRs, and investigated their role using a mouse model in which only the full-length CaR was disrupted. Our results show that both the full-length and the alternatively spliced variant lacking exon 5 encoding 77 amino acids of the extracellular domain were expressed in mouse epidermis. The deletion of the full-length CaR increased the production of the alternatively spliced form of CaR in mutant mice. The keratinocytes derived from these mutant mice did not respond to extracellular calcium, suggesting that the full-length CaR is required to mediate calcium signaling in the keratinocytes. The loss of the full-length CaR altered the morphologic appearance of the epidermis and resulted in a reduction of the mRNA and protein levels of the keratinocyte differentiation marker, loricrin. These results indicate that CaR is important in epidermal differentiation, and that the alternatively spliced form does not fully compensate for loss of the full-length CaR.

Differentiation of cultured keratinocytes is tightly controlled by the concentration of extracellular calcium (1, 2). Elevation of extracellular calcium ([Ca$^{2+}$]o) above 0.05 mM (calcium switch) inhibits proliferation and induces the onset of terminal differentiation accompanied by elevated expression of transglutaminase (3), involucrin (4, 5), and, subsequently, loricrin (6) and profilaggrin (7). Furthermore, the gradient of calcium within the epidermis, with highest levels in the stratum granulosum and its levels decrease as the cells differentiate, whereas the transcript of the spliced variant is expressed throughout the differentiation process. These molecular changes are consistent with the decrease of [Ca$^{2+}$]i and IP$_3$ response to [Ca$^{2+}$]i, during differentiation. These results suggest a role for full-length CaR in human keratinocyte differentiation that is not filled by the alternatively spliced form (11).

Here, we investigated the expression of CaR in mouse epidermis. The complete nucleotide sequence of mouse CaR cDNA was determined. The function of the CaR was analyzed using a mouse model in which the CaR gene was disrupted. To our initial surprise, mutant mice continued to express CaR. However, we then determined that the mouse epidermis, like the human keratinocyte, produce both the full-length and alternatively spliced form of CaR lacking the human equivalent of exon 5 (originally reported as exon 4 (Ref. 14) but renamed exon 5 by a recent study of the genomic structure of CaR (Ref. 15)). The disruption of the CaR in this mouse model resulted in the sole production of the alternatively spliced variant because of the fortuitous insertion of the neomycin cassette into exon 5. This mouse model provided us the opportunity to examine the differentiation of mouse epidermis in which only the alternatively spliced form was expressed. This report contains the first complete nucleotide sequence of mouse CaR cDNA, and our initial description of the consequences to the keratinocyte of deletion of the full-length but not the alternatively spliced form of the CaR. These results indicate that the full-length CaR is important for normal epidermal differentiation in vivo presumably by mediating the calcium signaling required for keratinocyte terminal differentiation.

EXPERIMENTAL PROCEDURES  

CDNA Cloning and Sequencing—A partial mouse CaR cDNA clone (mCaR-R) was isolated using RT-PCR from total RNA from the kidneys of either wild type (+/+) or mutant (−/−) mice. A set of primers flanking exon 5 of the mouse CaR was obtained from Dr. C. Ho (Harvard Medical School, Cambridge, MA): sense primer (mCaR1309F, 5′-CAA GGT CAT TGT CGT TTT CTC CAG C-3′), and antisense primer (mCaR2307R, 5′-GCA ATG CAG GAG TGG TGG TAC G-3′). The latter includes 2 mismatches to the mouse CaR sequence subsequently
found, but it was adequate for the initial experiments. The 3' clone of mouse CaR was obtained by 3' RACE. Briefly, total RNA from mouse kidney was reverse transcribed (Superscript II, Life Technologies, Inc.) using the poly(T) adaptor (5'-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT-3'). The cDNA was amplified by PCR using the gene-specific sense primer (5'-ATT AAT TCT GTC CAC AAT GG-3') and antisense adaptor primer (5'-CCA TTG TGG ACA GAC TTC CT-3') most distal to the poly(A) tail using terminal deoxynucleotidyl transferase. The cDNA was amplified by PCR using the primer set described in RT-PCR below. The PCR products were subcloned into a pCR II or pCR2.1 vector (Invitrogen). The isolated cDNA clones were sequenced on both strands using vector-specific and gene-specific primers using a Dye Terminator cycle sequencing kit (Applied Biosystems) on an Applied Biosystems 373A automated DNA sequencer. The DNA sequence was analyzed using the GCG DNA analysis package at the Computer Graphics Laboratory at the University of California, San Francisco.

FIG. 1. Nucleotide sequence and its deduced amino acid sequence of mouse CaR. The cDNA of mouse full-length CaR contains 4550 bp including an open reading frame of 3236 bp (525–3761) having a translation initiation codon assigned to the first in-frame ATG with Kozak context.

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Protein Analysis of CaR by Western Blot—

Crude plasma membranes were also prepared from HEK293 cells transfected by human cDNA of full-length or spliced variant CaR (11). Briefly, the tissues or cells were sonicated, and the membrane fractions were extracted with radioimmunoprecipitation buffer containing 1% deoxycholate, 1% Triton X-100, 0.1% SDS. Protein concentration in these membrane preparations was determined using the Pierce BCA protein assay (Pierce). The CaR proteins were analyzed by Western blot as described (11). Briefly, the membrane protein samples were electrophoresed through polyacrylamide gels and electrophototted onto nylon membranes. After blocking the membrane protein samples were electrophoresed through polyacrylamide gels and electrophototted onto nylon membranes. After blocking, the membrane protein samples were electrophoresed through polyacrylamide gels and electrophototted onto nylon membranes.
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RESULTS

Complete Nucleotide Sequence of Mouse CaR—To initiate the sequencing of mouse CaR, both the full-length and anticipated alternatively spliced form, we first generated partial cDNA clones by RT-PCR using primers spanning exon 5 (14). The RNA from the wild type (+/+) mice produced a 0.8-kilobase pair DNA of mouse CaR (mCaR-R). Mutant mice (−/−) generated a smaller DNA fragment (mCaR-S). Sequencing demonstrated that the clone mCaR-S has an in frame deletion of 231 bp of exon 5, which would cause deletion of 77 amino acids, equivalent to the naturally produced CaR spliced variant in human keratinocytes. The complete nucleotide sequence of mouse CaR was then determined using 5′ and 3′ RACE. The cDNA of mouse full-length CaR contains 4550 bp including an initiation codon assigned to the first in frame ATG with Kozak context of 1079 nucleotides (Fig. 1) (GenBank accession no. AF110178). The entire nucleotide sequence of mouse CaR consists of three major domains: a large hydrophilic extracellular domain (EC), a transmembrane (TM) domain, and an intracellular domain (Fig. 2). The mouse CaR is highly homologous to the CaR from the rat (98% amino acid identity, respectively) (18), human (95%) (19), and bovine (93%) (20), and it has less homology with puffy fish CaR (21) (83%).

The full-length mouse CaR consists of three major domains: a large hydrophilic extracellular domain (EC), a transmembrane (TM) domain, and an intracellular domain (Fig. 2). The mouse CaR is highly homologous to the CaR from the rat (98% amino acid identity, respectively) (18), human (95%) (19), and bovine (93%) (20), and it has less homology with puffy fish CaR (21) (83%) which has a much shorter intracellular domain (Fig. 2).

The position of exon 5, which is deleted in the spliced variant, is indicated as a region having 77 amino acids with 2 N-linked glycosylation sites and 10 acidic amino acids in the position of exon 5, which has a much shorter intracellular domain (Fig. 2). The region of exon 5 was highly conserved among mouse, human, and bovine as well as in rat and pig CaR.

Fig. 1—continued
species, having only 1 amino acid difference from rat and human CaR among 77 amino acids, suggesting that the mouse alternatively spliced variant of CaR would show the same loss of acute calcium signaling as the human variant (11). The mouse CaR is also homologous to metabotropic glutamate receptors, which belong to G protein-coupled receptor family 3, and showed the typical structural features conserved in this family. The hydrophobic segment in the N-terminal side of the EC domain (aa 148–176) was identified, which may contribute to the ligand binding pocket (20). The 17 conserved Cys and the 6-Cys cluster in the C-terminal part (560–585) of the extracellular domain may be involved in disulfide bonds. The 7-TM domain includes a conserved cytoplasmic loop (795–805) having a PKC site (646) between the 5th and 6th TM. The C-terminal cytoplasmic domain (217 amino acids) contains 6 potential phosphorylation sites for PKC at 794, 888, 895, 915, 993, and 1059 in addition to two sites in the TM domain at 646, 699. Potential phosphorylation sites for cAMP-dependent protein kinase were present at aa 899 and 900, suggesting that cAMP also may modulate the function of CaR. The nucleotide sequence of mouse CaR reported here is 100% identical to a partial 500-bp CaR cDNA isolated from the mouse osteoblast (GenBank accession no. AF002015) (22).

**CaR mRNA Expression in Mutant Mice**—To determine whether the expression of the alternatively spliced form of CaR would be affected by targeted disruption of the full-length CaR, we next compared CaR mRNA expression in wild type (+/+), heterozygous (+/−), and homozygous (−/−) CaR mutant mice. Both full-length and the spliced variant of CaR were detected by RT-PCR using a primer set encompassing exon 5. Both the 1007-bp full-length CaR (upper band) and the 777 bp spliced variant (lower band) were expressed in the epidermis of (+/+) and (+/−) mice (Fig. 3B, lanes 1 and 2), although the epidermis of (−/−) mice expressed only the spliced variant (B, lane 3). In contrast, kidney, which is an abundant source of CaR, produced only the full-length CaR in the wild type (+/+) mice (A, lane 1), although it expressed the spliced variant in mutant (−/−) mice (A, lane 3). The heterozygous mice (+/−) produced both forms in the kidney (A, lane 2). The intermediate band (A, lane 2) observed in the sample from (+/−) mice is a hybrid of the full-length and variant forms. The level of G3PDH transcripts showed no significant differences among the three samples of

![FIG. 2. Alignment of the amino acid sequence of mouse CaR (mCaR) with rat (rCaR), human (hCaR), bovine (bCaR), and puffy fish (fCaR). The consensus sequence among these five receptors was calculated and shown as CON on the top of the line. The same amino acid is shown by a dot (•), and missing amino acid is indicated by —. Exon 5 encoding 77 amino acids (460–537) is boxed. The seven transmembrane domains are indicated as TM1–TM7.](image-url)
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FIG. 3. CaR mRNA expression was analyzed in wild type (+/+)
(lanes 1 and 4), heterozygous (+/−) (lanes 2 and 5), and homozy- 
gous (−/−) (lanes 3 and 6) mice. The epidermis (B) and the kidney
(A) were dissected from each group of mice. The expression of CaR
(lanes 1–3) was analyzed by RT-PCR. The two amplified DNA bands are
shown by arrows. The upper band was consistent with the M of the
full-length CaR (1007 bp), and the lower band with the M of the spliced
variant lacking exon 5 (777 bp) (lanes 1–3). The same RNA was ampli-
fied by a primer set for G3PDH as a control (A and B, lanes 4–6). Two
independent PCR analysis showed the same results.

FIG. 4. CaR protein was expressed in the skin of CaR knockout
mice. The CaR protein was detected by immunostaining. The skin
section of wild type (+/+) (A) and mutant mice (−/−) (B–D) (7 days old)
were stained with a polyclonal anti-CaR antibody as described under
"Experimental Procedures." The expression of CaR was detected in the
epidermal layer as well as in the hair follicles in both wild type (+/+)
and knockout (−/−) mice. No positive staining was observed in the
(−/−) skin section incubated with pre-immune IgG (C). When the
section was treated with the antibody preabsorbed with the peptide
(215–237) against which the antibody was raised, only slight staining of
hair follicles was observed (D).

epidermis (B, lanes 4–6) and kidney (A, lanes 4–6). These results indicate that these two forms of CaR are expressed in
the mouse epidermis even in the wild type animal, and that the
alternatively spliced variant is expressed in other tissues such
as the kidney when the full-length form is deleted. Whether
expression of this mRNA leads to production of protein CaR is
the next question we addressed.

Protein CaR Expression in CaR Mutant Mice—The CaR mu-
tant mice (−/−) were originally reported not to produce CaR
protein (14). However, the mRNA data suggested that at least
in the skin and kidney, the alternatively spliced CaR protein
was likely to be produced in mutant (−/−) mice. In order to
verify this prediction, we immunostained the skin from (+/+)
and (−/−) mice using a polyclonal antibody against CaR (11).
The CaR was detected throughout the upper layers of epider-

dim of the 7-day-old wild type (+/+ ) mice (Fig. 4A). The
epithelial cells around the hair follicle also stained strongly for
CaR (A). Similar staining was observed in the skin of the CaR
mutant (−/−) mice (B). The staining with pre-immune IgG did
not show significant signal (C). When the antibody was pre-
adsorbed with excess peptide against which the antibody was
raised, the staining was markedly reduced (D). These results
show that CaR is present in the epidermis on both wild type
(+/+) and heterozygous (+/−) mice. We next analyzed CaR proteins
by Western blot. The membrane fraction from the mouse kid-
ney was prepared. The wild type (+/+) and heterozygous (+/−)
mice expressed two major bands of CaR, estimated as 140
and 160 kDa (Fig. 5, lanes 1 and 2). These two bands corre-
spond in size of the two differently glycosylated forms of human
CaR expressed in HEK293 cells transfected with cDNA for the
full-length CaR (lane 4) (11). In contrast, the homozygous mu-
tant (−/−) mice had only a ~130-kDa band (lane 3), which corre-
sponds to the single band of CaR transfected with the human
spliced variant cDNA (lane 5) (11). The size of the
~130-kDa band is consistent with the spliced variant lacking
77 amino acids (lane 3) having altered glycosylation. The bands
at ~130, ~140, and ~160 kDa were removed by pre-adsorption
of antibody (data not shown). The lower band around 100 kDa
was non-specific (lanes 1–3). The CaR protein was less readily
detected in epidermal samples using Western analysis unlike
immunolocalization. These results indicate that the full-length
CaR is produced in the wild type (+/+ ) mice, although the
spliced variant of CaR protein is still expressed in knockout
mice (−/−).

The [Ca2+], Response to [Ca2+]i in Keratinocytes from CaR
Mutant Mice—We have previously demonstrated that cultured
human keratinocytes increase their [Ca2+]i in response to

FIG. 5. Western analysis of the CaR in membrane protein pre-
parations from knockout (−/−) mice. The membrane proteins were
isolated from the kidney of mutant mice (lanes 1–3) and HEK 293 cells
transfected with human full-length CaR (lane 4) or spliced variant CaR
(lane 5). The CaR was detected using a monoclonal antibody against
CaR (ADD). The wild type (+/+ ) (lane 1) and heterozygous (+/−)
samples (lane 2) showed the ~140- and ~160-kDa bands, which corre-
spond to the glycoylated forms of CaR of human full-length CaR in
HEK293 cells (lane 4). The homogygous (−/−) mice expressed a ~130-
kDa band (lane 3), corresponding to the human spliced variant (lane 5)
expressed in HEK293 cells. The lower band was nonspecific. The spec-
ificity of the upper three bands were confirmed by pre-adsorption of
the antibody with peptide against which the antibody was raised.
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raised \([Ca^{2+}]_i\) and that this response is necessary for calcium-induced keratinocyte differentiation. To determine whether the alternatively spliced CaR can substitute for the full-length CaR, we compared the \([Ca^{2+}]_i\) response to \([Ca^{2+}]_i\) in keratinocytes from wild type and mutant mice. The keratinocytes were isolated from the mouse epidermis and cultured in low Ca\(^2+\) (0.03 mM) to prevent differentiation. The \([Ca^{2+}]_i\) response to \([Ca^{2+}]_i\) (2.0 mM) was measured using Fura-2. The keratinocytes from wild type (+/+ mice) had lower basal \([Ca^{2+}]_i\), of 57 nM but increased this \([Ca^{2+}]_i\), to 891 nM after addition of 2.0 mM \([Ca^{2+}]_i\) (Fig. 6). In contrast, keratinocytes from mutant mice (−/−) showed higher basal \([Ca^{2+}]_i\), (283 nM), and increased their \([Ca^{2+}]_i\), only slightly to 333 nM following the calcium switch (Fig. 6). These results indicate that the full-length CaR is required for normal acute \([Ca^{2+}]_i\), response to \([Ca^{2+}]_i\), and this function cannot be replaced with the alternatively spliced CaR.

Morphological Analysis of the Skin from Knockout Mice—To determine whether the loss of the full-length CaR is accompanied by altered epidermal differentiation, we analyzed the skin of the CaR mutant (−/−) mice. By 3 days after birth, the skin from mutant mice was shinier, less pigmented, and appeared thinner. This difference was less apparent after 7 days. The histopathology of 3-day-old mutant mice (−/−) showed a modest reduction in the number of nucleated epidermal cell layers, with a disordered differentiation sequence, demonstrated by abnormal polarity and flattening from the basal through suprabasal layers in comparison to wild-type epidermis (Fig. 7). In addition, both the stratum granulosum and the density of keratohyalin granules appeared to be reduced in the epidermis of mutant mice (Fig. 7). The same results were obtained in comparison with wild-type littermates and with wild-type animals from different litters. These results indicate that the CaR plays an important role in vivo during epidermal morphogenesis.

Loricin Expression in the Epidermis of CaR Mutant Mice—To examine the effect of deletion of the full-length CaR on differentiation, we next compared the expression of the terminal differentiation marker, loricin, in 3-day-old mutant (−/−) mice (Fig. 8, B, D, and F) with wild-type (+/+ mice) (A, C, and E). The histologic appearance (A and B), the loricin mRNA (C and D), and protein levels of loricin (E and F) are shown in the same skin samples. Whereas loricin mRNA and protein levels and localization in wild-type epidermis were as expected (C and E), the epidermis of the mutant (−/−) mice was altered (D and F). In wild type epidermis, loricin mRNA was found in the stratum granulosum (C), where it was very abundant. In contrast, an extremely low (almost undetectable) signal for loricin mRNA was present across the suprabasal layers of the epidermis in mutant mice (D), and most cells in the stratum granulosum did not show any accumulation of loricin message. Even those occasional cells of the stratum granulosum which had a stronger signal, stained much less intensely than granular cells in the wild-type epidermis. This expression pattern of the loricin gene was paralleled in the localization of its protein product. In wild type epidermis, loricin was localized in a sharply demarcated manner to the stratum granulosum (E). In mutant epidermis, however, a weak, diffuse immunostaining was seen (F) across the suprabasal epidermis, with lack of increased staining in the stratum granulosum. Therefore, in the absence of the full-length CaR, loricin expression and localization are markedly reduced.

These results strongly suggest that the full-length CaR is required for epidermal differentiation.

**DISCUSSION**

The identification of the CaR provided an important step toward our understanding of how calcium regulates cell function. The ability of calcium to regulate keratinocyte growth and differentiation is well established (1, 2). The discovery that the CaR, which was originally identified in the parathyroid gland and kidney, was also found in keratinocytes (10) suggested a common mechanism for calcium signaling in these different cell types. Deletions of the full-length CaR by homologous recombination in which the mutant gene contained a neomycin cassette in the mouse equivalent of exon 5 resulted in a phenotype comparable to neonatal hyperparathyroidism (14). However, when we examine the epidermis of these mice using an antibody to the CaR, we found abundant protein CaR. Since we had previously observed that human keratinocytes produce an alternatively spliced form of CaR in which same exon 5 was deleted, we asked whether the mouse epidermis did likewise, and if so whether this would explain the finding that the CaR
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To initiate this investigation, we first determined the complete nucleotide sequence of mouse CaR. The mouse CaR is highly homologous to CaR from other species including the rat (18), human (19), bovine (20), and puffy fish (21). The structural conservation of EC and TM domains were high (>90% amino acid identity) including fish CaR, suggesting the evolutionary conservation of these regions. The intracellular domain has more variation in amino acids and in length, suggesting that the signaling functions of this receptor may vary from fish to mammals.

As in human keratinocytes, the mouse epidermis makes both the full-length and the spliced variant lacking exon 5. As shown first in human keratinocytes, and now in mouse keratinocytes from CaR mutant mice, the region of exon 5 is critical to the function of CaR at least with respect to the initial response to calcium shown by IP3 production and [Ca2+]i response. The mechanism of functional abolishment by exon 5 is not well understood, although several possibilities were considered. A change in binding affinity to calcium may be caused by the deletion of 10 acidic amino acids in this region of CaR. In addition, the glycosylation of the two N-linked glycosylation sites may be critical for correct folding or transport of CaR protein to the plasma membrane, the spliced variant has an altered glycosylation pattern. The full-length CaR is expressed in normal cells as two bands: the ~160- and ~140-kDa bands. From the analysis of human CaR, we predict that the ~160-kDa band is the mature functional form of CaR, and that the ~140-kDa band may be an intermediate product of CaR because it has endoglycosidase H-sensitive high mannose type glycosylation.

The availability of a mouse model in which the full-length but not the spliced variant CaR was “knocked out” gave us the opportunity to determine the degree to which the alternatively spliced form could compensate for loss of the full-length form in terms of epidermal differentiation. Previous studies with this model (11) clearly showed loss of normal calcium homeostasis in that these animals were severely hypercalcemic and died within a few weeks of birth. The keratinocytes from the CaR mutant mice did not respond to elevated [Ca2+]i, with an increase in [Ca2+]i, clearly showing that the full-length CaR is required to mediate calcium signaling. However, we also observed that the CaR mutant keratinocytes maintained a higher basal [Ca2+]i level compared with normal cells, suggesting additional alterations in calcium regulation by CaR mutant keratinocytes. Higher resting [Ca2+]i levels were also observed in calreticulin-deficient mice, in which the [Ca2+]i, response to ATP or bradykinin was abolished (23). Therefore, a rise in [Ca2+]i, under circumstances in which normal calcium signaling is disrupted may result in increased [Ca2+]i, by way of compensation. The in vivo significance of this altered calcium signaling may be reflected in the altered differentiation pattern of the epidermis, as demonstrated by both the changes in morphology and expression of loricrin. The epidermis of the CaR mutant mice showed disordered differentiation with reduction of the number of epidermal layers, especially within the first few days of the birth. These differences were not observed after day 7. However, the differentiation marker loricrin still was decreased in mutant mice at this stage.

These alterations in calcium response, skin morphology, and loricrin expression are not likely to result secondarily from the hypercalcemia of these animals. The calcium response was measured in cultured keratinocytes grown in low (0.03 mM) calcium conditions, which should obviate differences in calcium concentrations in vivo. The changes in morphology were only observed shortly after birth, and they were normalized with time, whereas the rise in serum calcium was progressive. Loricrin expression ought to be increased by hypercalcemia, not decreased. Therefore, the observed changes are more consistent with an intrinsic defect in calcium signaling by the CaR mutant (~/-) keratinocytes than a secondary response to hypercalcemia.

In summary, we have reported the complete structure of mouse CaR and its alternatively spliced variant. Both are produced by the epidermis, but when the full-length CaR is deleted, the spliced variant is produced in other tissues that normally express only the full-length CaR. Keratinocytes lacking the full-length CaR do not respond to calcium normally, indicating that the full-length CaR is required for normal calcium signaling. The skin lacking the full-length CaR showed thinner epidermis with disordered differentiation, and decreased loricrin expression. These results indicate that CaR is required for normal epidermal differentiation in vivo, presumably by mediating the calcium signaling required for normal keratinocyte differentiation.

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