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Alternatively spliced forms of the cGMP-gated channel in human keratinocytes

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Abstract Alternatively spliced forms of the α subunit of the cGMP-gated channel have been cloned from human keratinocytes. One form encodes a complete channel which is almost identical to the rod photoreceptor. A second spliced variant would encode a protein missing a portion of the intracellular hydrophilic domain and the putative first transmembrane domain. Both complete and spliced variants of the channel also were found in epidermis. The expression of the complete form of the channel was induced by levels of extracellular calcium which promote keratinocyte differentiation. The cGMP-gated channel may play an important role in calcium induced keratinocyte differentiation by mediating Ca^{2+} entry.

Key words: Ion channel; cGMP; Cloning; Keratinocyte differentiation; Alternative splicing

1. Introduction

The proliferation and differentiation of cultured epidermal keratinocytes are regulated by extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) [1,2]. Elevation of $[Ca^{2+}]_o$ (calcium switch) inhibits proliferation and induces aspects of terminal differentiation such as cell stratification and cornification. Several studies [3–8] have demonstrated that both an initial rise of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and a subsequent Ca^{2+} influx are required for keratinocyte differentiation. Finally, the basal level of $[Ca^{2+}]_i$ substantially increases as these cells differentiate [6,7]. These results suggest that ion channels may have an important role in keratinocyte differentiation by conducting Ca^{2+} influx. A non-selective cation channel (NSCC) which was permeable to and activated by Ca^{2+} was described in human keratinocytes [9,10]. Channel blockage inhibited calcium induced keratinocyte differentiation [11]. A Ca^{2+} permeable channel gated by nicotine has also been detected in keratinocytes [12]. Arsenault et al. isolated a partial cDNA clone encoding the N-terminal portion of a putative cyclic nucleotide-gated channel (CNG1) from a human keratinocyte cDNA library [13]. The nucleotide sequence encoded by this clone was 98% identical to the α subunit of the cGMP-gated channel originally described in retinal rods [14,15]. Although this channel family was originally thought to be restricted sensory cells such as rods (CNG1), cones (CNG3) and olfactory cells (CNG2), they are now recognized to be widely distributed in non-sensory tissues (see review [16–18]). As these channels are permeable to divalent cations, especially Ca^{2+} [19,20], they have been implicated in the rise of intracellular Ca^{2+} by mediating calcium entry [21,22].

Raised extracellular calcium, which induces keratinocyte differentiation, also increases the level of nitric oxide (NO) [23] and inducible nitric oxide synthase mRNA [24] in these cells. Since guanylate cyclase is stimulated through NO regulated pathway, raised extracellar Ca^{2+} also may mediate an increase of intracellular cGMP level.

In the current study we report that mRNA encoding a full-length cGMP-gated channel, which could conduct Ca^{2+}, is present in keratinocytes. Alternatively spliced variants lacking the N-terminal hydrophilic domain and/or first putative transmembrane domain were also cloned from human keratinocytes. The expression of these two forms of the channel is differentially regulated by calcium induced differentiation. These results suggest that the channels might play an important role in mediating transmembrane Ca^{2+} entry during calcium induced keratinocyte differentiation.

2. Materials and methods

2.1. Cell culture

Epidermis was isolated from newborn human foreskin. SCC12F2 and SCC12B2 (derived from human facial skin SCC) were gifts from H. Green. Primary keratinocytes, epidermoid carcinoma A-431, immortalized HACAT cells were cultured as described [25]. Briefly, cells were grown in Keratinocyte Growth Medium (KGM, Clonics) with 0.07 mM Ca^{2+} and switched to the same medium containing 1.2 mM Ca^{2+} to stimulate keratinocyte differentiation.

2.2. Northern hybridization

The total RNA was isolated from keratinocytes by a guanidinium thiocyanate-phenol-chloroform extraction method using RNAzol B (Tel-test Inc. TX) [26]. Poly(A) RNA was purified using oligo (dT) cellulose column chromatography. The poly(A) RNA (5 μg each lane) was denatured in 2.2 M formaldehyde and electrophoresed on a 0.7% agarose gel. The RNA was directly transblotted onto GeneScreen membrane (DuPont) and probed with a ^{32}P-labeled 0.75-kbp *Pst*I/*Bam*HI fragment of the partial cDNA clone (F8) for a cGMP channel previously isolated from the human keratinocyte cell line (COLO-16) [13]. The membranes were washed under stringent conditions (0.1×SSC, 55°C). Following autoradiography and washing, the blot was re-probed with a labeled glyceraldehyde-3-phosphate dehydrogenase (G3PDH, Clontech) cDNA as a control to estimate RNA loading. The message size was estimated by comparison with molecular weight markers (RNA ladder, 0.24–9.5-kbp BRL).

2.3. cDNA cloning and sequencing

mRNA isolated from confluent differentiated keratinocytes cultured

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in KGM medium containing 1.2 mM Ca^{2+} was reverse transcribed into total cDNA (Superscript II, BRL) using a poly T primer [27]. Since the partial cGMP channel previously isolated from keratinocytes appeared to be essentially identical to the retinal rod cGMP-gated channel, primers were designed to span the complete open reading frame of the channel. These primers would thus also be capable of amplifying any alternatively spliced variants which contain the exons encoding the extreme 5′ and 3′ regions but which were missing internal exons. These primers were 5′ sense primer (−60 to −41, 5′-CAC CAG TGA CGT CCA CTT CT-3′) and 3′ antisense primer (5′-TGA GGC ATG TCC CTG TTA AT-3′). Following first and second strand synthesis, PCR amplification was performed using a mixed Pwo DNA polymerase (Expand long template PCR system, Boehringer) for 30 cycles. The PCR product was analyzed by electrophoresis on a 1% agarose gel and PCR products were subcloned into the pCR II vector (Invitrogen). Colonies were screened with ^{32}P-labeled oligonucleotide probes designed against an internal sequence from the rod channel from cGMP-binding domain sequence (5′-TGT GGT GGC AGA TGA TGG AGT CAC TCA GTT TGT GGT A-3′). Positive clones were sequenced on both strands by using vector specific primers and 10 gene specific primers using a Dye termination cycle sequencing kit (ABI) on an Applied Biosystems 373A automated DNA sequencer. For cDNA clone hkGMPc-2,3, only the 5′ end of the cDNA was sequenced. The DNA sequences were analyzed using the GCG8 DNA analysis package at the Computer Graphics Laboratory of the University of California, San Francisco.

2.4. RT-PCR analysis

cGMP-gated channel isoform expression in keratinocytes was determined by RT-PCR using primers which spanned the missing exons (exons 6,7,8). Total RNA was isolated from cultured keratinocytes or from normal epidermis which was separated from neonatal foreskin by dispase treatment. cDNA pools prepared as described above were amplified by PCR using the same sense primer (from 1st exon) used for cloning the channel and an anti-sense primer designed from the 10th exon (693–674, 5′-CCT TTA CCA GCA GTC CTT GT-3′). Each cDNA was also amplified using a pair of primers for human G3PDH (Clontech) as a control for cDNA loading in the PCR assay. Amplification was carried out using Taq polymerase PLUS (Stratagene) for 25 cycles. The PCR products were analyzed by electrophoresis on a 3% agarose gel. Molecular weights of the bands were estimated using ϕX174/*Hae*III fragments (BRL).

3. Results

3.1. The cGMP-gated channel is expressed in keratinocytes

A 339-bp partial cDNA fragment of a putative cGMP-gated channel which was previously isolated from COLO-16 cells [13] was used to probe keratinocyte mRNA by Northern hybridization. A 4.6-kbp transcript was detected in normal differentiated keratinocytes (Fig. 1, lane 4) and in human epidermal carcinoma cells A-431 (lane 3). In contrast, no transcripts were detected in squamous cell carcinoma lines SCC12F2 and SCC12B2 (lanes 1 and 2) or the immortalized HACAT keratinocyte cell line (data not shown). The size of the message was larger than the 3.2-kbp and 3.5-kbp transcripts reported in human retinal rods. These results showed that transcripts with high homology to the retinal rod cGMP-gated channel are expressed in normal human keratinocytes, but with a different size than that reported in retinal rods.

3.2. Molecular cloning of the keratinocyte cGMP-gated channel

RT-PCR amplification of total keratinocyte RNA using primers spanning the complete open reading frame of the cGMP-gated channel resulted in an approximately 2-kbp DNA product. Cloning and sequencing of the PCR products yielded a cDNA clone (hkGMPc-1) which encoded a cGMP-gated cation channel with high sequence homology to the human rod channel [14] (Fig. 2). The only major difference was in the 5′ the non-coding sequence in which the keratinocyte channel did not contain an *Alu* sequence which was previously assigned as exon 2 in the rod channel [14]. Within the coding region, the keratinocyte channel sequence differed at 5 nucleotides which resulted in one amino acid change. The conversion of D118N has been reported to occur as a benign polymorphism in 15% of the population [28]. In addition to the cDNA clone encoding the full-length cGMP channel, three alternatively spliced transcripts (hkGMPc-2, -3, -4) were obtained. All of these clones contained in-frame deletions, and they could be conceptually translated to yield different isoforms of the channel protein (Fig. 3). The cDNA clone hkGMPc-2 lacked the putative first transmembrane domain (S1). A second spliced variant, hkGMPc-3, had a deletion of the N-terminal half of the Lys and Glu-rich hydrophilic domain (K/E domain) and S1. The last variant, hkGMPc-4, lacked the whole hydrophilic domain and S1.

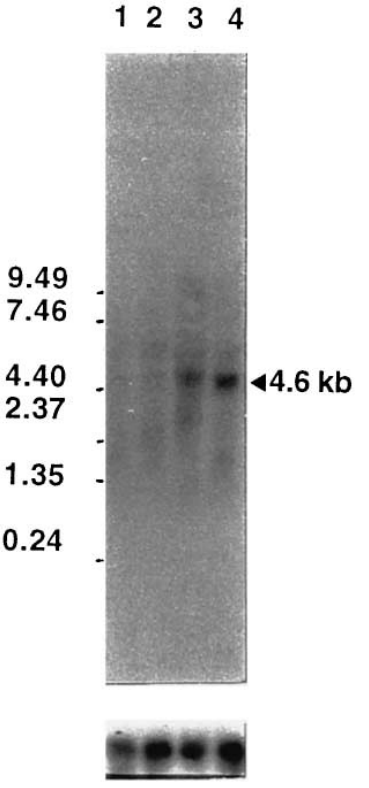
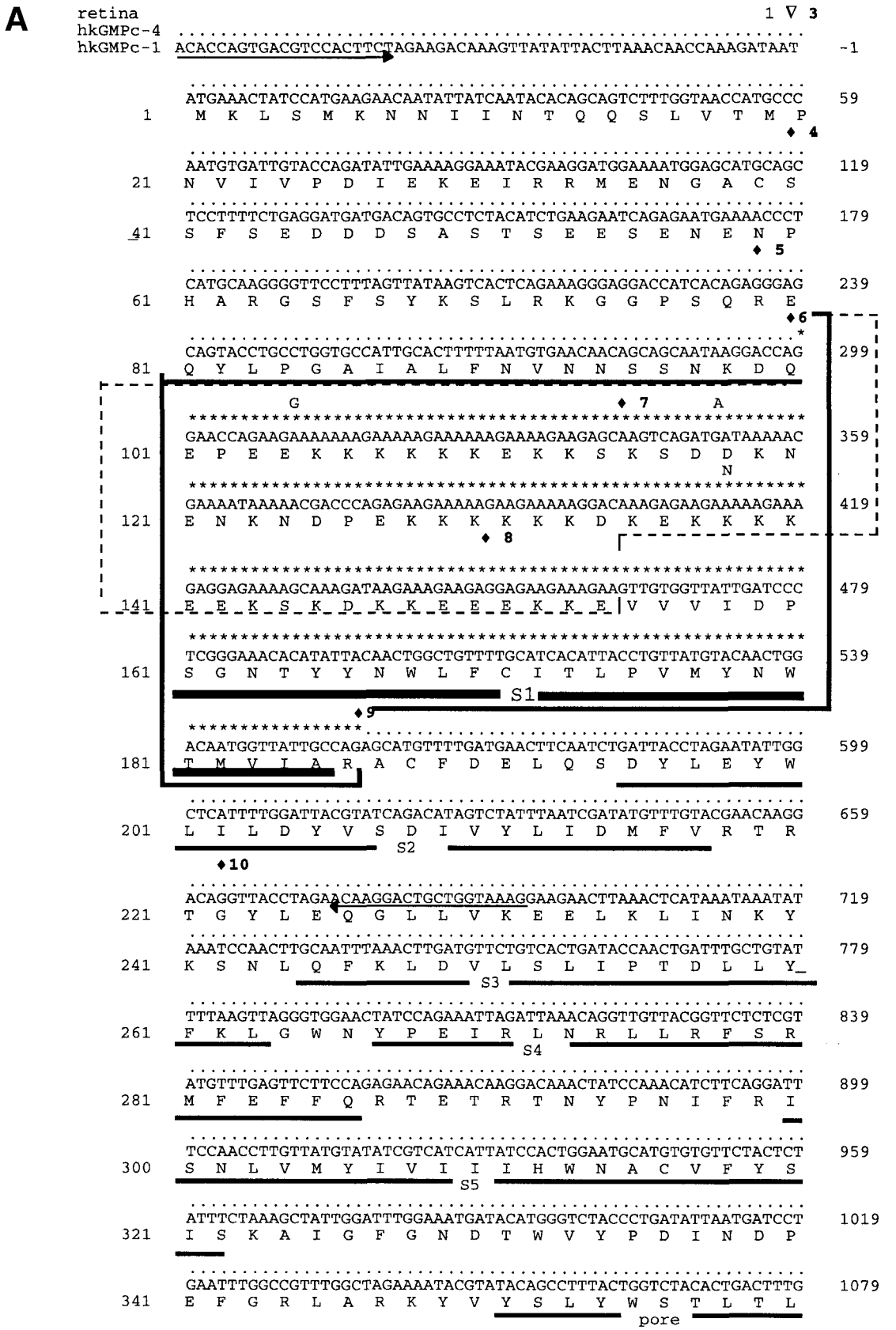


Fig. 1. Northern hybridization of cGMP-gated channel transcripts in human keratinocytes. Poly(A) RNA (5 μg) was electrophoresed, transblotted, and probed with a ^{32}P-labeled partial channel clone (F8, *Pst*I-*Bam*HI fragment, 339 bp) [13]. A control probe (G3PDH) was used to normalize the amount of RNA as shown in the bottom of the blot. The membrane was washed under stringent conditions (0.1×SSC, 55°C). A 4.6-kbp message was detected in normal differentiated keratinocytes (lane 4), and epidermoid carcinoma A-431 (lane 3), but not from squamous carcinoma SCC12F2 or SCC12B2 lines (lanes 1 and 2).

3.3. The cGMP-gated channels are regulated in keratinocyte differentiation

The expression of different forms of the cGMP-gated channel was analyzed by RT-PCR, using primer sets that would produce amplified bands of differing sizes to distinguish the presence of different spliced variants. Undifferentiated kera-



tinocytes were grown in 0.07 mM Ca^{2+} until 70% confluent, then switched to 1.2 mM Ca^{2+}, a concentration that promotes cell differentiation. Two PCR product bands were detected: a 753-bp upper band which corresponds to the calculated MW of the complete isoform, and a 495-bp lower band which represents one of the alternatively spliced forms (hkGMPch-4)

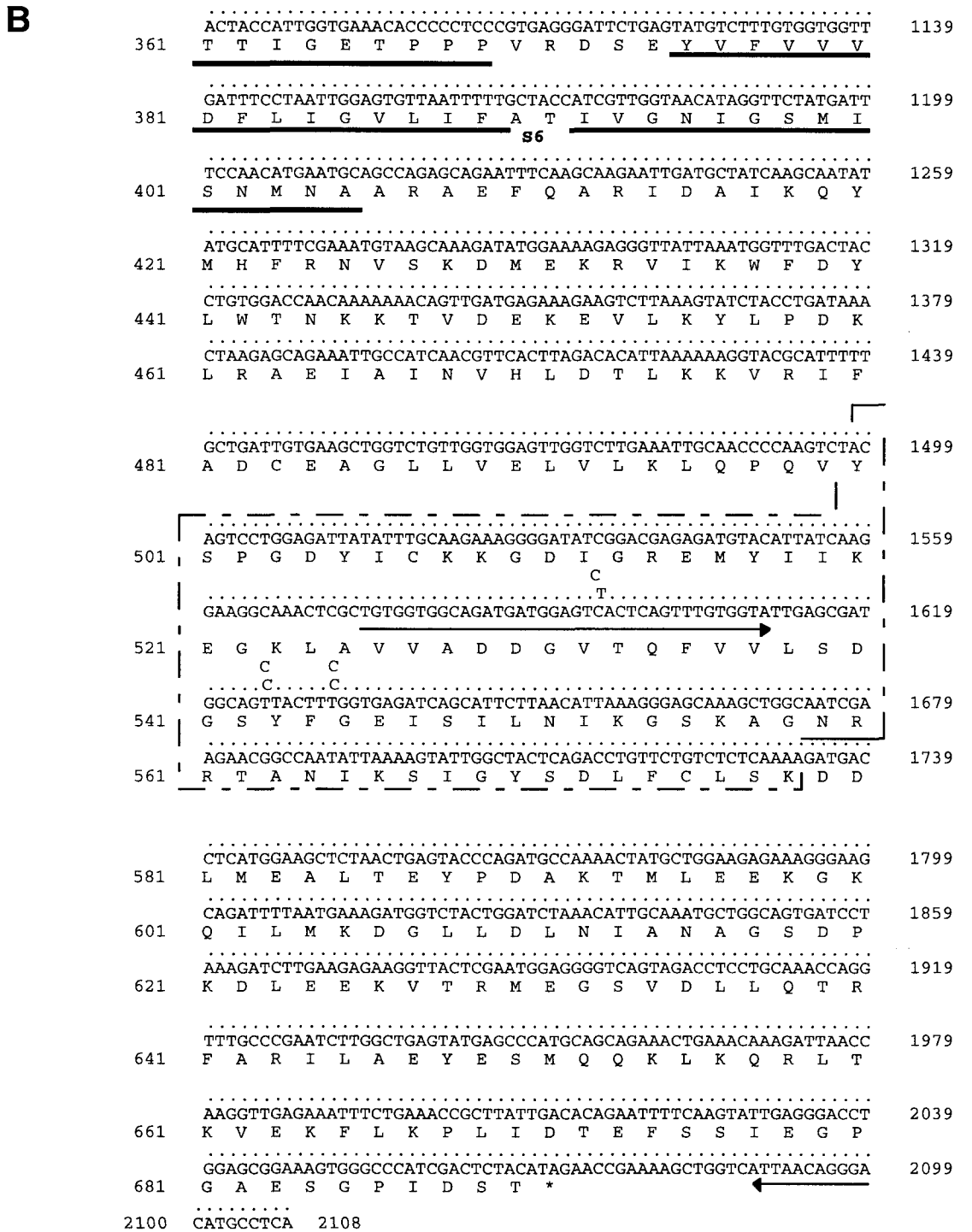


Fig. 2. Aligned nucleotide sequences of the keratinocyte cGMP-gated channel and its alternatively spliced isoform. The nucleotide sequences of a cDNA clone (hkGMPc-1) which encodes the complete form of the channel (middle row) and its deduced amino acid sequence (bottom row) are shown. The putative domain structure is shown by the boxed (----) region for the N-terminal Lys-rich hydrophilic domain, boxed (—— – ——) region for the C-terminal cGMP-binding domain, and bold underlined regions for the six transmembrane domains (S1–S6) and pore region (———), respectively. The number of exon and exon/intron boundaries are shown by ♦. The nucleotide sequence of one of the alternatively spliced channel transcripts (hkGMPch-4) is aligned above the sequence of the complete form. The shared nucleotides are shown by dots (·) and different nucleotides are listed. This spliced variant lacks three exons (6–8) (nucleotide 298–546) shown as boxed by bold lines, and missing nucleotides are shown by asterisks (*). This region includes the intracellular Lys and Glu-rich hydrophilic (K/E) domain (---- boxes) and the first transmembrane domain (—— S1 ——) shown by bold underline. Sense and antisense primers, used to clone the channel from keratinocytes, are shown by arrows at the 5′ and 3′ ends of the sequence. The sequence of the human rod channel [14] is also aligned on the top. The major difference between the keratinocyte and rod channels are in the 5′ non-coding sequence. The keratinocyte channel does not contain an *Alu* sequence which is inserted at the position of ∇ and is assigned as exon 2 in the rod channel. Within the coding region, the keratinocyte channel (hkGMPc-1) differs in 4 nucleotides which result in one amino acid change at 118 which is shown under the amino acid sequence. Oligonucleotide probes used for screening colonies are shown as arrows in the cGMP-binding domain.

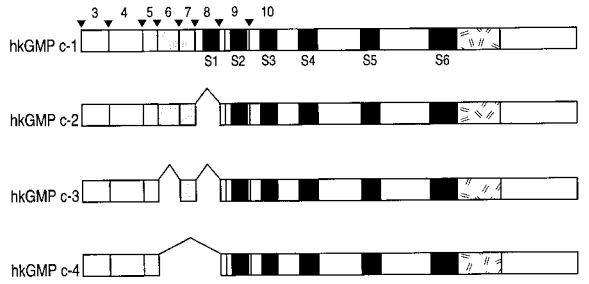


Fig. 3. Alternatively spliced forms and missing domains of cGMP-gated channels in keratinocytes. In addition to the complete isoform, three alternatively spliced transcripts were cloned. All three splice variants contain in-frame deletions, such that they can be translated to yield different isoforms of the cGMP-gated channel. These forms differ from the complete form by lacking portions of either the N-terminal hydrophilic (K/E) domain and/or the putative first transmembrane domain (S1). The putative domains and exons are shown schematically. The numbers of the exons and exon/intron boundaries are shown by arrows on the complete form of the channel (hkGMPc-1, top). The K/E domain is shown by the shadowed regions. Six transmembrane domains are shown as S1–S6 (black boxes), and the cGMP-binding domain is shown as patterned boxes. One of the alternatively spliced forms, hkGMPc-2, (second row) which lacks only the 8th exon encoding the S1 domain appears to be a minor transcript in epidermis. Another form, hkGMPc-3, (third row) which lacks the 6th and 8th exons corresponding to the N-terminal half of K/E domain and the S1 domain, was not detected in RT-PCR analysis of keratinocyte mRNA. The last isoform, which is expressed in keratinocytes, hkGMPc-4, lacks three exons (6th, 7th and 8th) encoding both the entire K/E region and the S1 domain.

lacking both the N-terminal domain (K/E domain) and the first transmembrane domain (S1) encoded by exons 6, 7, and 8 (Fig. 4). The expression of the complete form of the channel (753 bp) was increased after keratinocytes were induced to differentiate by 1.2 mM Ca^{2+} (Fig. 4a; after 8 h, lane 3; after 24 h, lane 4). PCR bands representing the same two channel isoforms also were detected in RNA isolated from the epidermal layer of newborn human foreskin (Fig. 4b, lane 2) as well as cultured differentiated keratinocytes maintained with 1.2 mM Ca^{2+} for 3 days (Fig. 4b, lane 1). The spliced variant lacking only S1 (hkGMP-2) also was detected below the upper band as a minor component (646 bp) in epidermis as well as cultured differentiated keratinocytes (Fig. 4b, lane 2). These results suggest that the same alternative splicing events are involved in keratinocyte differentiation in vivo. The other alternatively spliced variant (hkGMPc-3) which was isolated by cDNA cloning, was not detected in abundance in the RT-PCR analysis, suggesting that it is a very rare splicing event.

4. Discussion

We have shown by molecular cloning and RT-PCR analysis that transcripts encoding the α subunit of the cGMP-gated channel and a spliced variant lacking the hydrophilic (K/E) and first transmembrane domains (S1) are present in keratinocytes. We speculate that the alternatively spliced variant might play a role in regulating the channel function by deletion of S1 and K/E domains. In other tissues, alternatively spliced forms of cyclic nucleotide-gated (CNG) channels have been briefly described [14,22,29]. These include channel isoforms missing either the S1 domain [14], the S2 domain [22], or the K/E domain [29]. Our major variant (hkGMPc-4) which was detected in keratinocytes lacked both the S1 and

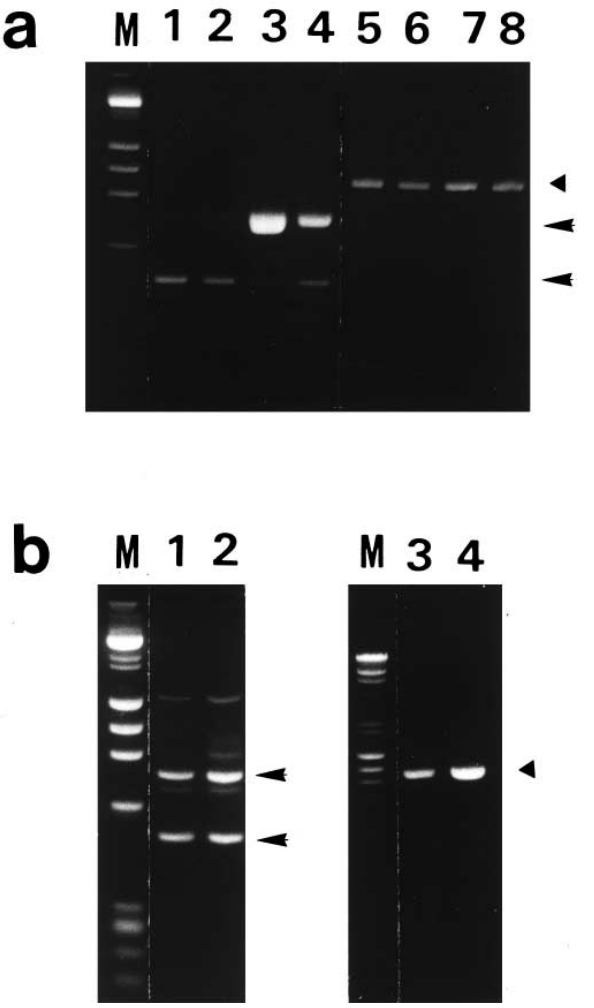


Fig. 4. RT-PCR analysis of channel expression. Primers spanning the missing region (exons 6, 7 and 8), which was identified in the alternatively spliced cDNA clones isolated from cultured keratinocytes, were used to analyze expression of splice variants. Each alternatively spliced transcript would be detected as a different size PCR product. (a) Human keratinocytes were grown in 0.07 mM Ca^{2+} until 70% confluent, then switched to 1.2 mM Ca^{2+} to promote keratinocyte differentiation. RNA was isolated before and at different time points after 1.2 mM calcium was added (4–24 h), with the 24 hour sample taken from cultures which had reached confluence. RNA from each sample was reverse transcribed and amplified by PCR. The PCR products from keratinocytes grown in 1.2 mM calcium for 0 h (lane 1), 4 h (lane 2), 8 h (lane 3) and 24 h (lane 4) were analyzed on a 3% agarose gel. (b) Epidermis excised from human foreskin (b, lane 2) and differentiated keratinocytes cultured with 1.2 mM Ca^{2+} for 3 days (b, lane 1) were analyzed in same way. Two major DNA bands were amplified as shown by arrows (←). The upper band was consistent with the calculated molecular weight (753 bp) for the complete form of the channel (hkGMPc-1). The lower band (495 bp) was consistent with one of the alternatively spliced forms (hkGMPc-4) which is lacking exons 6, 7, and 8. The other alternatively spliced version of the human keratinocyte cGMP-gated channel, hkGMPc-2 was detected as a minor transcript (646 bp) below the upper band. The third variant, hkGMPc-3, which would have produced bands of 604 bp, was not detected. These same cDNA samples were analyzed by PCR using a set of primers specific for human G3PDH as a control. Approximately the same amount of a 1-kbp DNA band for G3PDH (shown by closed triangle) were detected in lanes of calcium induced keratinocytes (a; lanes 5, 0 h; 6, 4 h; 7, 8 h; 8, 24 h), epidermis (b, lane 4) and differentiated keratinocytes (b, lane 3) demonstrating that equal amount of RNA were used in the analysis of channel expression.

K/E domains. A minor variant (hkGMPc-2) which lacks only the S1 domain was identical to a variant previously described in human retinal rods [14]. This form was reported to be non-functional [14]. The deletion of the K/E domain in the cone channel (CNG3) in the pineal gland increased cGMP sensitivity by two-fold [22]. The N-terminal hydrophilic domain of the HERG K^+ channel, which is evolutionarily the most closely related to the CNG channel within the channel superfamily, has been reported to be involved in the assembly of the channel [30–32]. The deletion of the S1 domain from the voltage-gated K^+ channel disturbed the assembly of channels and abolished its function [33]. Since cGMP-gated channels are thought to form tetramers containing α and β subunits in retinal rods [17], the alternatively spliced form of the channel, which lacks the S1 and K/E domains, might regulate channel function by either changing its oligomeric structure and/or altering cGMP sensitivity.

We also have demonstrated that the expression of these channels are differentially regulated during calcium induced differentiation. The transcript encoding the functional full-length cGMP-gated channel was increased in calcium induced keratinocyte differentiation. This observation suggests that this form of the channel might play a role during keratinocyte differentiation to maintain $[Ca^{2+}]_i$ levels through transmembrane calcium entry. Recently a calcium receptor (CaR) which recognizes elevated $[Ca^{2+}]_o$, was cloned from human keratinocytes [34]. This G-protein coupled membrane receptor was expressed in undifferentiated keratinocytes. This receptor would increase $[Ca^{2+}]_i$ through the release of intracellular calcium stores by increasing the metabolism of inositol triphosphate. It would be interesting to investigate the relative importance of the cGMP-gated channel versus the CaR in regulation of $[Ca^{2+}]_i$ levels during keratinocyte differentiation.

Further functional studies of the isoforms of cGMP-gated channel could elucidate a regulatory mechanism of Ca^{2+}entry during calcium induced keratinocyte differentiation.

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