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Basis for Intracellular Retention of a Human Mutant of the Retinal Rod Channel α Subunit

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Abstract. A mutant of the α subunit of the retinal rod cyclic GMP-gated channel, [Arg654(1-bp del)], corresponding to a truncated αR654Dstop subunit, was previously described in patients with retinitis pigmentosa: when expressed in HEK-293 cells, this mutated α subunit was retained inside the cell, but had normal channel activity in one case where it reached the plasma membrane, indicating that the mechanism of targeting is altered by the mutation, but not the function of the channel. The corresponding mutants of the bovine rod channel (αR656D stop), and of the closely related olfactory neuron channel (αR632D-stop) α subunits were expressed in *Xenopus* oocytes and their activity was analyzed by patch-clamp. Like their human homologue, these two channels have no activity, and we show that their GFP fusion proteins are accumulated into intracellular compartments. The truncation alone or the R/D mutation alone do not prevent or modify channel activity, indicating that neither the R656 residue nor the C-terminal domain downstream of R656 is necessary for homomeric channel targeting and function. Several mutations of R656 and of the preceding residues in the R656Dstop mutant disclose that the motif responsible for the absence of channel activity is an endoplasmic reticulum retention signal (KXKXXstop) in which the nature of the residues in positions −1 and −4 is determinant.

Key words: Cyclic GMP-gated channel — Retinitis pigmentosa — Endoplasmic reticulum — Patch clamp — Confocal microscopy

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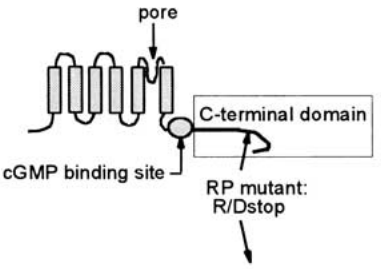
Abbreviations: CNG: cyclic nucleotide-gated; RP: Retinitis Pigmentosa; GFP: green fluorescent protein; EC_{50}: ligand concentration producing half-maximal excitation; ER: endoplasmic reticulum

Introduction

The retinal rod cyclic GMP-gated channel belongs to the family of cyclic nucleotide-gated (CNG) channels involved in sensory transduction, which are activated by direct binding of the cyclic nucleotide to a site situated in the cytoplasmic C-terminal region. The rod channels, situated in the rod outer segment plasma membrane, are responsible for the entry of cations (mainly sodium, but also calcium) into the cell in the dark. Upon light-induced activation of the phototransduction cascade, the concentration of cGMP is reduced, which results in closure of the channels and hyperpolarization of the cell. Mutations in various proteins of this cascade lead to hereditary diseases known as "retinitis pigmentosa" (RP), which are characterized by progressive photoreceptor degeneration.

CNG channels are tetramers composed of at least two types of subunits (*review*: Zagotta & Siegelbaum, 1996): α subunits, which can form channels when expressed alone, and β subunits, which cannot. A mutation in the C-terminal domain of the human α subunit of the retinal rod cGMP-gated channel downstream of the cGMP binding site ([Arg654(l-bp del)], which produces the R654D mutation and truncation of the subsequent last 32 residues), was found to be associated with RP in homozygous individuals (Dryja et al., 1995). Upon transfection into HEK-293 cells of the mutated cDNA, channels are expressed in the cytoplasm instead of the plasma membrane; in one patch, which contained a single R654Dstop channel, the expressed channel had the characteristics of α*wt* channels (EC_{50} for cGMP and open probability), indicating that the reason for intracellular retention is not improper folding or assembly of the channel subunits (which would result in impaired function), but rather mistargeting of a functional protein (Dryja et al., 1995). We have searched for the molecular basis of the retention of this mutant in an attempt to un-

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(1) IANAGSDPKDLEEKVTRMEGSVDLLQTRFARILAEYESMQQKLKQRLTKVEKFLKPLIDTEFSSIEGPWSESGPIDST--
(2) IANAGSDPKDLEEKVTRMESSVDLLQTRFARILAEYESMQQKLKQRLTKVEKFLKPLIDTEFSAIEGSGTESGPTDSTQD
(3) EVAASMEV-DVQEKLEQLETNMDTLYTRFARLLAEYTGAQQKLKQRITVLETKMK-QNNEDDSLSDGMNSPEPPAEKP--
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Figure 1. Alignment of the C-terminal domains of the α subunits of three cyclic nucleotide-gated channels: the human and bovine rod channel and the bovine olfactory neuron channel. (1) human rod: 609–686; (2) bovine rod: 611–690; (3) bovine olfactory neuron : 588–664. The aligned domains start immediately downstream of the nucleotide binding site. The residue R654 (human rod), which is mutated into a D in the RP mutant R656Dstop, and the corresponding R656 (bovine rod) and R632 (bovine olfactory neuron) are boxed. A schematic representation of CNG channel secondary structure is drawn above the alignment.

derstand the mechanism of the rod channel targeting to the plasma membrane.

The R654 residue is situated 46 residues downstream of the C-terminal end of the nucleotide binding site, and is conserved in the α subunits of the rod channel and of other members of the CNG channel family of different species. Fig. 1 shows the alignment of the C-terminal ends of the human and bovine rod and of the closely related bovine olfactory α subunits (Kaupp et al., 1989; Pittler et al., 1992; Dhallan et al., 1990; Ludwig et al., 1990). This domain is almost identical in the human and bovine rod α subunits (91.2% identity in the sequence shown), and a sequence of 26 residues encompassing R654 is fairly well conserved between the human rod and the bovine olfactory a subunits (73.1% identity), suggesting that it might play an important role. The wrong targeting of the human R654Dstop mutant could indicate that the mutation and/or the truncation alters or removes α plasma membrane-targeting signal. Anterograde signals required for surface expression of membrane proteins have been previously described (Schulein et al., 1998; Sharma et al., 1999): in particular, two residues in the C-terminal domain of the SUR1 subunit of K_{ATP} channels (L1566 and F1574) are necessary for channel transit to the cell surface (Sharma et al., 1999). Interestingly, a leucine and a phenylalanine residue (L662, F670), also separated by 7 residues, which are conserved in the bovine rod but not in the bovine olfactory neuron α subunit, are missing in the truncated α subunit of the human R654Dstop rod channel. Alternatively, since the last five residues of the R654Dstop mutant constitute an endoplasmic reticulum (ER) dilysine retention sequence of the KXKXX type (*review*: Teasdale & Jackson, 1996), another possibility is that this sequence induces ER targeting.

Mutants of the α subunits of the bovine rod and of the bovine olfactory neuron channels were constructed to investigate the reason for the mistargeting of the human rod RP mutant. The human rod α subunit is highly homologous to the bovine rod α subunit, which is almost the same protein, with 90.9% identity for the complete sequence; it is also homologous, to a lesser extent, to the bovine olfactory neuron α subunits, with 59. 1% identity.

Materials and Methods

Channel Expression

The bovine rod (Kaupp et al., 1989) and olfactory α subunits (Ludwig et al., 1990) were a gift of Prof. U.B. Kaupp. Mutations were constructed by PCR and verified by sequencing. The [S65T]GFP cDNA (Clontech) was fused in-frame to the N-terminal ATG codon of the rod or olfactory α cDNA after removal of the stop codon; a Kozak consensus sequence (Kozak, 1984) was engineered upstream of the GFP ATG codon, and the constructions were inserted into the high expression vector pGemHe downstream of the untranslated sequence of the *Xenopus* β-globin gene (Liman, Tytgat & Hess, 1992). The rod β subunit previously described (Pagès et al., 2000) was used. Capped mRNAs were synthesized in vitro from linearized plasmids in the presence of RNAcap structure analogs (New England Biolabs), and injected into *Xenopus* oocytes (25 ng/oocyte).

Patch-Clamp Recording of Excised Inside-Out Patches

The solution in the pipette and in the perfusion medium was : 100 mM KCl, 10 mM EGTA/KOH, 10 mM HEPES/KOH, pH 7.2. The cytoplasmic face of the excised patch was superfused by solutions containing variable cGMP concentrations using an RSC100 rapid solution changer (Bio-Logic, Claix, France). The saturating cGMP concentration was 500 μM. Currents induced by voltage steps (500 msec, ± 80 mV) were recorded with an RK-400 patch amplifier (Bio-Logic), low-pass filtered at 300 Hz and digitized at 1 kHz (each record averaged 3 times) using pCLAMP 6.0 (Axon Instruments, Union City, CA). The series resistance was compensated for (resulting value <1 MΩ). Dose-response curves were obtained by plotting the current at +80 mV as a function of nucleotide con-

centration after subtraction of the leak current. Data were fitted to the Hill equation $I / I_{max} = 1/(1+(EC_{50}/X)^{n_H})$, with EC_{50} = ligand concentration that gives half maximal effect, n_H = Hill number, and X = ligand concentration, using Microcal Origin software.

Fluorescence Microscopy

Oocytes were fixed 7 days after mRNA injection (1 hour immersion in 4% paraformaldehyde followed by 1 hour in 10% and 1 hour in 20% sucrose solutions), embedded in Tissue-Tek OCT compound (Sakura, Zoeterwoude, NL), and frozen. Frozen blocks were cut to obtain ~50-μm slices. Slides were counterstained for 10 seconds in 0.01% Evans blue, a nonspecific protein dye with red fluorescence, in order to mask nonspecific green fluorescence. Localization of GFP fusion protein was analyzed by confocal laser scanning microscopy (TCS-SP2, Leica). [S65T]GFP fluorescence was excited at 488 nm using an argon laser; GFP emission was collected between 505 nm and 530 nm. The red fluorescence was collected between 580 nm and 660 nm. The green and red images were superimposed.

Results

Role of the Truncation and of the R/D Mutation for the Activity of the Bovine Rod Homologue of the Human RP α Subunit Mutant

Two truncated mutants of the bovine rod α subunit were constructed and analyzed for cGMP-induced currents: αR656stop (truncated after residue R656) and αR656Dstop (truncated at position 656 with the additional R656D mutation), the latter corresponding to the human αR654Dstop RP mutant described by Dryja et al. (1995). Examples of current records are shown in Fig. 2, and the results are displayed in Table 1: αR656stop gives rise to large cGMP-induced currents similar to control wild-type α channels, and the EC_{50} for cGMP obtained from dose-response curves are also similar. On the contrary, as previously described for the human αR654Dstop mutant expressed in HEK-293 cells, the αR656Dstop mutant gives rise to very small or non-measurable currents. Similar results are obtained with the mutant of the olfactory channel α subunit (α_{olf}R632Dstop) truncated at the corresponding place downstream of the binding site (Fig. 1 and Table 1).

The R656D mutation in a non-truncated rod α channel with a complete C-terminal domain does not prevent channel activity, and the EC_{50} of this mutant channel for cGMP is similar to that of the wild type channel (Table 1).

Since in the eye the rod channel is a hetero-oligomer consisting of α and β subunits, we also investigated whether co-expressing the β*wt* subunit, which does not form functional channels when expressed alone, is able to rescue the mutated α subunit. As shown in Table 1, co-expression of the β*wt* subunit with the αR656Dstop subunit does not restore channel activity.

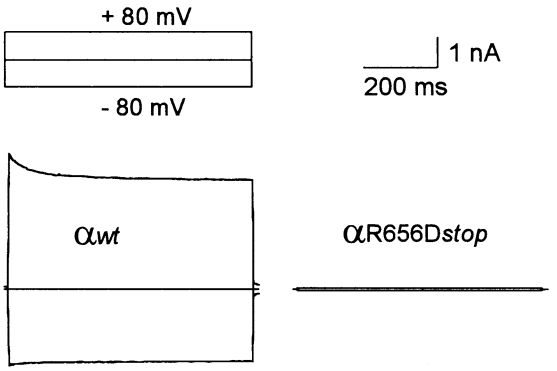

Figure 2. Records of cGMP-induced currents obtained with one patch from an oocyte expressing the rod α*wt* channels and one patch from an oocyte expressing the rod αR656Dstop channels. cGMP concentration: 500 μM. The voltage protocol is shown above the α*wt* currents. The amplitude of the current at +80 mV was 3.65 nA for α*wt* and 41 pA for αR656Dstop, i.e., about 2700 and 30 channels, respectively, taking the value of 1.35 pA given in Pagès et al., (2000) for the unitary current.

Localization of Truncation Mutants in the Oocyte

Although the human RP-mutant α subunit was previously shown to be retained inside the cell, no indication is available concerning its localization and its fate within the cell. In order to get more information on the intracellular localization of the mutated channels in the oocyte, we constructed GFP fusion proteins, in which GFP was fused to the N-terminal end of the bovine rod and olfactory neuron α subunits (wild type and with the RP mutation). The control GFP $-\alpha_{rod}wt$, had very little activity, suggesting that it was either inactive or badly targeted to the plasma membrane. On the other hand, the N-terminal GFP fusion protein of the olfactory α subunit, GFP- $\alpha_{olf}wt$, gave rise to cGMP-induced currents of similar amplitude and characteristics (EC_{50} for cGMP: 2.2 ± 0.6 μM) as those observed with the α_{olf}wt channel (EC_{50} for cGMP: 2.2 ± 0.5 μM), and could therefore be used as a control for the localization of wild-type channels in the oocyte by fluorescence microscopy.

Confocal micrographs of oocytes expressing the GFP-$\alpha_{olf}wt$ and the truncated mutant GFP-α_{olf}R632Dstop channels are shown in Figure 3. Bright green fluorescence was observed in the plasma membrane of ~30% of the oocytes expressing GFP-$\alpha_{olf}wt$ channels. In oocytes expressing the truncated mutant, no clearly delimited green fluorescence was observed in the plasma membrane, although sometimes, faint green fluorescence appeared localized in small vesicles adjacent to the plasma membrane. Green fluorescence was observed in the cytoplasm for both wild-type and R632Dstop channels, but presented clearly distinct characteristics. In the case of

Table 1. cGMP-induced currents in patches from oocytes expressing wild-type or mutated rod α, rod (α + β), and olfactory neuron α channels

Mutant	Current Mean ± SE (nA)	Control	Current Mean ± SE (nA)
Rod		Rod	
αR656Dstop	0.01 ± 0.01 (18)	α*wt*	3.9 ± 0.2 (10)
αR656stop	3.5 ± 0.9 (5)		
αR656D	6.9 ± 2.9 (7)		
Rod		Rod	
αR656Dstop + β*wt*	0.04 ± 0.02 (25)	α*wt* + β*wt*	3.4 ± 0.9 (6)
Olfactory		Olfactory	
αR656Dstop	0 (4)	α*wt*	2.1 ± 0.1 (6)

For each channel type, the wild-type mRNA was injected the same day and in the same lots of oocytes as the mutant mRNAs. The number of experiments is indicated in parentheses.

The amplitude of cGMP-induced currents varies widely from one experiment to another and from one oocyte to another, and has no absolute meaning (the standard error, SE, gives an idea of this variation); the important result is that with the R/Dstop mutants, the currents were small or null in all tested oocytes, between 2 and 10 days after injection of the mRNAs. With the wild-type channels and the 2 other mutants (αR656stop and αR656D), large currents were observed from the second or third day after mRNA injection, and remained large whenever tested (up to 7 to 10 days according to the experiments).

The EC_{50} for cGMP were similar for the rod α*wt* (27 ± 2 μM), αR656D (27.3 ± 4 μM), αR656stop (26.4 ± 4 μM).

R632Dstop mutant, the fluorescence was highly concentrated in numerous dense vesicular-like compartments. In oocytes expressing the wild-type channel, the green cytoplasmic fluorescence appeared as fine structures or sparse, faintly labeled spots. Qualitatively similar results were observed in three different series of experiments. Thus, the reason for the absence of activity of olfactory neuron R632Dstop channels is retention inside the cell, as previously reported by Dryja et al. (1995) for the human RP mutant rod channel. The particular fluorescence pattern of GFP-α_{olf}R632Dstop mutant is moreover suggestive of an accumulation in some sort of intracellular compartment. GFP-αR656Dstop mutants (bovine rod channel) similarly show absence of fluorescence in the plasma membrane and accumulation of intense green fluorescence in intracellular vesicles (*not shown*).

Mutations of the Potential Endoplasmic Reticulum Retention Signal

The fact that the last 5 residues of the rod mutants (human: αR654Dstop, and bovine: αR656Dstop) and of the corresponding olfactory neuron mutant (αR632Dstop) constitute a known C-terminus ER retention sequence (KXKXXstop) for type I and type III membrane proteins (Teasdale & Jackson, 1996), in which the C-terminal domain is cytoplasmic, suggests that the reason for the wrong targeting of these mutants might be the accidental creation of an ER retention sequence. It is, however, puzzling that the rod αR656stop mutant, which has the same potential ER retention sequence, but terminates with an R, is normally expressed in the plasma membrane. As shown in Table 2, replacing the D656 residue in the rod αR656Dstop mutant by K, N and E did not prevent expression in the plasma membrane either, indicating a crucial role for D656 in the retention process.

Several other mutants were constructed in order to test the ER retention sequence hypothesis (Table 2). An ApaI site (GGG CCC) was introduced in the cDNA at the place corresponding to M649Q650 in order to facilitate the mutation of residues from Q651 to Q655. Like the simple R656Dstop mutant, this triple-truncated mutant (M649G/Q650P/R656Dstop) did not give rise to cGMP-induced currents, indicating that retention is independent of the M649Q650 residues. The strategy was to swap the GPQKLKQD656stop terminal sequence of the truncated mutant by the last 8 residues 683–690 of the α*wt* subunit (GPTDSTQD690stop), which possesses a unique ApaI site at position G683P684, and vice-versa.

The truncated mutant terminating with the last 8 residues of the α*wt* subunit, GPTDSTQD656stop, had normal currents, suggesting a potential role of the reduced QKLKQDstop sequence. We then reintroduced K654 by mutation of the GPTDSTQD656stop mutant, and then K654 + K652. However, as would have been expected if retention was due to the KXKXX terminal sequence, reintroducing both K654 and K652 in the GPTDSTQD656stop mutant did not provoke retention of the channel: the mutant terminating with GPT*K*S*K*QD, like those terminating with GPTDSTQD and GPTDS*K*QD, had normal currents (Table 2). In contrast, almost no currents were obtained for the mutant with the additional S653L mutation (terminating with GPT*KLK*QD656).

The symmetric α*wt* mutants terminating with the last 6 residues of the R656Dstop mutant (GPQKLKQD690stop) or with the mutated sequence GPTKLKQD690stop had no or almost no activity

Table 2. cGMP-induced currents from truncated α656stop and complete α*wt* (690stop) homomeric bovine rod channels with mutations in their C-terminal sequence

Mutant	Current Mean ± SE (nA)		Current in control α*wt* Mean ± SE (nA)	
656stop:				
MQQKLKQR656stop	3. 5 ± 0.9	(5)		
MQQKLKQ[D]656stop	**0.01 ± 0.01**	**(18)**	**3.9 ± 0.2**	**(10)**
MQQKLKQ[K]656stop	2.2 ± 0.9	(8)		
MQQKLKQ[N]656stop	1.2 ± 0.3	(5)		
MQQKLKQ[E]656stop	3.7 ± 0.9	(9)		
[GP]QKLKQ[D]656stop	**0**	**(14)**	**2.4 ± 0.6**	**(6)**
[GPTDST]Q[D]J656stop	1.8 ± 0.6	(8)		
[GPTDS]KQ[D]656stop	3.2	(1)		
[GPT]K[S]KQ[D]656stop	4.6 ± 2.8	(2)		
[GPT]JKLKQ[D]656stop	**0.08 ± 0.04**	**(30)**	**3.3 ± 0.9**	**(8)**
690stop:				
GPTDSTQD690 (wt)	7.2 ± 6.7	(7)		
GP[QKLK]QD690	**0**	**(5)**	**1.8 ± 0.5**	**(8)**
GPT[K]S[K]QD690	1.3 ± 0.46	(10)		
GPT[KLK]QD690	**0.12 ± 0.04**	**(25)**	**2.4 ± 0.6**	**(6)**

The C-terminal domain (649–690) of the bovine rod α*wt* subunit (see Fig. 1) is: MQQKLKQ[R]LTKVEKFLK-PLIDTEFSAIEGSGTES*GP*TDSTQD*stop*. "656stop" mutants are α subunits truncated after residue 656, and "690stop" mutants are complete α subunits. Mutated residues are boxed. Mutants that produce no or almost no currents are marked in bold letters. For each of these mutants, the control α*wt* mRNA was injected the same day and in the same lot of oocytes. The number of experiments is indicated in parentheses.

either, while the GPTKSKQD690stop mutant gave rise to normal currents (Table 2). Results in Table 2 therefore show that the KLKQDstop terminus sequence is necessary for suppressing the activity; however, the ER retention sequence KXKXXstop is not sufficient for retention of the αR656Dstop channels in the cytoplasm, since the nature of at least 2 other residues in positions (−4) and (−1) (L653/687 and D656/690) also has a major importance. It can be noted that the 2 mutants terminating with *KLKQD*, in which the residue at position (−5) is T instead of Q, gave rise to very slightly increased currents, suggesting that the residue in position (−5) may also play a role. A potential role of Q655/689 (position −2) was not tested.

Discussion

The results indicate that both the R656D mutation and the truncation are necessary for retention of homomeric rod α channels, and that neither the R/D mutation nor the truncation alone modifies the channel activity. The C-terminus of the rod α subunit downstream of R656 (bovine channel) does not, therefore, contain any plasma membrane-targeting domain, and is not important for homomeric channel assembly and function. It must be noted, however, that, in the rod, the channels are heteromers (*see* below), and that they are specifically targeted to the outer segment plasma membrane, with a much lower channel density in the inner segment plasma membrane (Matthews & Watanabe, 1988), and exclusion from the disc membrane. The study of the underlying sorting mechanisms would require the use of transgenic animals.

In agreement with previous results showing that the human rod R654Dstop mutant is mostly retained in the cytoplasm (Dryja et al., 1995), confocal images (Fig. 3) indicate that the reason for the absence of channel activity of the olfactory neuron R632Dstop mutant is that it is retained inside the cell rather than being expressed in an inactive form at the plasma membrane. The micrographs show, moreover, that the mutated channel is not degraded, but accumulated into intracellular compartments. In addition, these results indicate that the effect of the R/Dstop mutation is neither specific to the host cell (*Xenopus* oocytes in our experiments, HEK-cells in the experiments of Dryja et al., 1995), nor to the rod channel: the human rod and the bovine olfactory neuron α subunits, which are closely related, but present only 59.1% identity (compared to 90.9% identity for the human and bovine rod α subunits), are both prevented to reach the plasma membrane, suggesting that the basis for retention could be the C-terminal KXKXX retention motif.

The results of the mutagenesis study show that the KLKQDstop terminus sequence in the bovine rod αR656Dstop truncated mutant is necessary for the absence of activity of homomeric channels; moreover, the same sequence introduced in place of the last five residues of the complete α subunit has the same effect. Of all the mutated rod α subunits tested (Table 2),

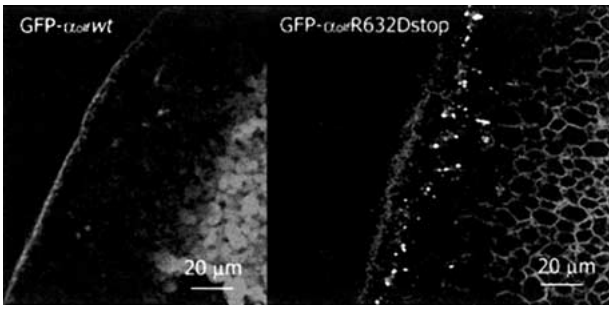

Figure 3. Confocal micrographs of *Xenopus* oocytes injected with mRNAs for GFP fusion proteins of the olfactory channel α subunit (GFP-$\alpha_{olf}wt$ and GFP-α_{olf}R632Dstop). A single confocal scan is shown for each oocyte. In the central part of the oocytes, the cytoplasm is filled with closely packed yolk platelets (5–15 μm diameter, right side of each image), the fluorescence of which is shifted from green/yellow to red by Evans Blue staining. The empty appearance of yolk platelets in the oocyte expressing the mutated channel is not typical. In the micrograph of the oocyte expressing the wild-type channel, green fluorescence is observed in the plasma membrane, a line of small vesicles situated immediately underneath the plasma membrane, and a few larger spots in the peripheral zone in which no yolk platelets are present. In the micrograph of the oocyte expressing the mutant channel, green fluorescence is observed in small vesicles underneath the plasma membrane, and mainly in large intensely labeled vesicles in the peripheral zone. In control oocytes, which were not injected with GFP-fusion protein mRNA, only the red fluorescence due to Evans Blue staining was observed.

only the equivalent of the human RP mutant and the 4 other mutants terminating with the same KLKQDstop sequence (GPTKLKQD656stop, GPQKLKQD656stop, GPTKLKQD690stop and GPQKLKQB69Qstop) have no channel activity, and modification of a single residue in this sequence (−1 or −4) is sufficient to restore normal expression of functional channels at the plasma membrane. The reason for the absence of activity of the equivalent human RP mutant R654Dstop was previously shown to be retention inside the cell (Dryja et al., 1995), which was not due to misfolding since in one case, where a single channel was expressed at the plasma membrane, this mutant had the characteristics of wild-type channels. We moreover observe that the GFP-αR656Dstop fusion protein is accumulated in intracellular compartments like its olfactory neuron homologue.

The cytoplasmic KXKXX terminal motif of membrane proteins has been extensively studied, and was previously shown, from a site-directed mutagenesis study, to be necessary and sufficient for ER retention (Jackson, Nillson & Peterson 1990), provided that the distance between the transmembrane domain and the dilysine motif is not shorter than 5 residues, or that the dilysine motif is not preceded by an amphipathic helix (Teasdale & Jackson, 1996). We also searched whether known proteins with a C-terminal KXKXX motif may localize to a different target. A ScanProsite (ExPASy) search for the KXKXXstop pattern in human, mouse and *Arabidopsis* entries of Swiss-Prot data bank (chosen as three well represented species) reveals that almost all of the membrane proteins with a cytoplasmic KXKXXstop C-terminal sequence localize to the ER. In human entries, 27 proteins out of the 109 hits are identified as membrane proteins; among these, 24 are type I or III known ER membrane proteins, one is a probable ER protein, one is localized in Golgi vesicles, and one is a subunit of a heteromeric sodium channel (in which the retention motif could be masked by association with another subunit, as reported for the K_{ATP} channel subunit Kir6.2 (Zerangue et al., 1999)). In mouse entries, 6 proteins of the 31 hits are membrane proteins, all of which are identified as ER resident; in *Arabidopsis* entries, 5 proteins of the 13 hits are membrane proteins, all of which are also identified as ER resident. Altogether, this suggests that the R/Dstop mutants of the three cyclic nucleotide-gated channels (human and bovine rod, bovine olfactory neuron) as well as the complete bovine rod α subunit with engineered KLKQDstop C-terminus are likely to be retained in the ER. Note also that ER-retention induced by the dilysine motif has been described as a nonsaturable process (Jackson et al., 1990), which agrees well with the high GFP-fluorescence intensity of the compartments observed with the olfactory channel R632Dstop mutant (Fig. 3). Although a few channels manage to reach the plasma membrane in our experiments, it should be emphasized that the currents in the oocytes expressing the RP mutant channels are of the order of 1% or less of the currents in the control oocytes.

Examination of the C-terminal sequences of known ER membrane proteins terminating by the KXKXX motif (Teasdale & Jackson, 1996, and our ScanProsite search) does not reveal any precise requirement for the residues in the last (−1) and the −4 positions; in fact, none of these proteins terminates with KLKQD. In our experiments, however, normal targeting and activity is observed when D656 is exchanged for R, N, K or E, or when L653 is exchanged for S. The complete α subunit, in which two lysines have been introduced in position −3 and −5 ($\underline{KS}\underline{K}QD_{690}$), is also normally targeted to the plasma membrane, in contrast to the complete α subunit terminating with $\underline{KLK}$QD, which has no channel activity. It is possible that the requirements observed in the present work may depend on the sequence and structure of the preceding domain, or of another domain that would interact with the KLKQD motif. However, identical results are observed when the KLKQDstop (no activity) or KSKQDstop (normal targeting and activity) sequence is situated at residues 652–656 or at residues 686–690. Moreover, secondary-structure predictions suggest α-helical arrangement for residues 633–653 versus loop arrangement for residues 667–690 (Fig.4). Alternatively, the im-

αwt C-terminal domain (residues 616-690):
SDPKDLEEKVTRMESSVDLLQTRFARILAEYESMQQKLKQRLTKVEKFLKPLIDTEFSAIEGSGTESGPTDSTQD
LLL..HHHHHHHHHHHHHHHHHHHHHHHH..HHHHHHHHHHHHHHHH..LLLL..LLLLLLLLLLLLLLLLL

αR656stop (616-656):
SDPKDLEEKVTRMESSVDLLQTRFARILAEYESMQQKLKQR
LLLHHHHHHHHHHH...HHHHHHHHHHHHHHHHHHHHH.LL

αR656Dstop (616-656):
SDPKDLEEKVTRMESSVDLLQTRFARILAEYESMQQKLKQD
LLLHHHHHHHHHHH...HHHHHHHHHHHHHHHHHHHHHLLL

αQKLKQD690stop (616-690):
SDPKDLEEKVTRMESSVDLLQTRFARILAEYESMQQKLKQRLTKVEKFLKPLIDTEFSAIEGSGTESGPQKLKQD
LLL..HHHHHHHHHHHHHHHHHHHHHHHH..HHHHHHHHHHHHHHHH..LLLLLLLLLLLLLLLLLLLLLLL

Figure 4. Secondary structure prediction for the C-terminal domain of the bovine rod α subunit, wild-type and mutated. The secondary structure was predicted by the PredictProtein server (Burkhard Rost, CUBIC, Columbia Univ, New York, NY) (Rost & Sander, 1993). Only the "Subset (SUB sec)" is indicated: a subset of the prediction, for all residues with an expected average accuracy > 82%. A period means that no prediction is made for this residue, as the reliability is: Rel < 5 (the probabilities are scaled to the interval 0–9). *H* = helix, *E* = extended (sheet), *L* = loop. The complete α subunit has 690 residues. Note that replacing R by D in the truncated α656stop mutant, or TDSTQD by QKLKQD in the complete α subunit, does not significantly modify the secondary-structure prediction.

portance of X residues may depend on specific receptors of the species or tissue in which the protein is expressed (in the present experimental *Xenopus laevis* oocytes). The ScanProsite search for the KXKXXstop pattern in *X. laevis* entries of Swiss-Prot and TrEMBL found 14 sequences, none of which was identified as a membrane (or ER resident) protein. ER resident membrane proteins terminating with the closely related dilysine KKXX have been shown to bind to the COPI coatomer, involved in retrograde and perhaps anterograde transport between ER and the Golgi complex, although the COPI subunit(s) responsible for binding is still under debate (Gomez et al., 2000). The ScanProsite search for the KKXXstop pattern *X. laevis* entries found 22 matches, of which a single one is a membrane protein, which indeed localizes in the ER (3-hydroxy-3-methylglutaryl-coenzyme A reductase). This protein terminates with KKAA. The site of KXKXX motif binding has not as yet been studied, and could be distinct from that of KKXX motif binding. The number of known resident ER membrane proteins from *X. laevis* does not, therefore, allow any speculation on the importance of the X residues for retention in the ER.

It can be noted that CNG channels are tetramers, with probable dimerization at the level of the cyclic nucleotide binding site (*review*: Richards & Gordon, 2000). Therefore, in the assembled homomeric channel, when the monomer terminates with a retention motif, 4 such motifs lie in close proximity (perhaps arranged as 2 dimers). This could perhaps participate in the mechanism of retention: tetramerization of the COPI vesicles protein p23, which binds COPI through a C-terminus motif structurally similar to the KKXX motif, was shown to induce the polymerization of coatomer, required for the budding reaction (Reinhard et al., 1999).

Our results suggest that mutagenesis of channel proteins normally targeted to the plasma membrane, which can be detected with the highly sensitive patch-clamp technique, allowing quantification of functional protein expression at the plasma membrane, could prove a useful tool for the study of retention signals and their receptors.

Finally, the fact that coexpression of β*wt* subunits with the αR656Dstop mutant does not restore channel activity suggests that in the heteromeric assembly, no domain in the β subunit is able to mask the accidental retention sequence in the mutant α subunit. However, it is also possible that the heteromeric αR656Dstop/βwt channels are not functional for a different reason: the absence of the C-terminal domain in the α subunit may expose a retention sequence in the β subunit, or impair proper assembly of the heteromeric channel. This question will be addressed elsewhere.

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