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Genetics

Mutations in the gene encoding the α subunit of the rod cGMP-gated channel in autosomal recessive retinitis pigmentosa

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ABSTRACT **Mutations in the genes encoding two proteins of the retinal rod phototransduction cascade, opsin and the β subunit of rod cGMP phosphodiesterase, cause retinitis pigmentosa (RP) in some families. Here we report defects in a third member of this biochemical pathway in still other patients with this disease. We screened 94 unrelated patients with autosomal dominant RP and 173 unrelated patients with autosomal recessive RP for mutations in the gene encoding the α subunit of the rod cGMP-gated cation channel. Five mutant sequences cosegregated with disease among four unrelated families with autosomal recessive RP. Two of these were nonsense mutations early in the reading frame (Glu76End and Lys139End) and one was a deletion encompassing most if not all of the transcriptional unit; these three alleles would not be expected to encode a functional channel. The remaining two mutations were a missense mutation (Ser316Phe) and a frameshift [Arg654(1-bp del)] mutation truncating the last 32 aa in the C terminus. The latter two mutations were expressed *in vitro* and found to encode proteins that were predominantly retained inside the cell instead of being targeted to the plasma membrane. We conclude that the absence or paucity of functional cGMP-gated cation channels in the plasma membrane is deleterious to rod photoreceptors and is an uncommon cause of RP.**

Retinitis pigmentosa (RP) is a genetically heterogeneous set of diseases in which affected individuals develop progressive degeneration of the rod and cone photoreceptors. Patients typically experience night blindness by age 20 followed by progressive loss of peripheral visual field and later central visual field that leads to blindness usually in middle age. Oral vitamin A supplementation slows the course of the disease in most cases (1). Dominant, recessive, X chromosome-linked, and digenic patterns of inheritance are exemplified by families with RP, and even among the families with the same inheritance pattern, there is nonallelic heterogeneity. More than 15 genes have been implicated by linkage studies, five of which have been identified to date. Two of these genes encode proteins known to function in the phototransduction pathway [namely, rhodopsin (2) and the β subunit of rod cGMP-phosphodiesterase (3)], two are photoreceptor-specific proteins of unknown function [peripherin/*RDS* (4–6) and *ROM1* (6)], and one is an unconventional myosin (7).

Here we report the analysis of the gene encoding the α subunit of the rod cGMP-gated cation channel, which is the protein involved in the last stage of the phototransduction pathway (for review, see ref. 8). The rod cGMP-gated cation channel is a heterooligomer composed of two homologous subunits (α and β), each with cytoplasmic N and C termini, six putative transmembrane domains, and a pore region (9–11). The genomic structure and sequence of the 10 exons encoding the α subunit have been determined (12, 13), allowing one to screen this sequence for defects in patients. This α subunit can also be expressed *in vitro* and its channel activity can be quantified (9, 13).

MATERIALS AND METHODS

Ascertainment of Patients. Patients were categorized by the inheritance pattern of the disease as described (14). A set of 96 unrelated individuals without known photoreceptor degeneration were recruited as normal controls. After informed consent, blood samples were collected from each patient and normal control, and leukocyte DNA was purified.

Screening for Mutations. The single-strand conformation polymorphism technique was used to screen for point mutations or other small-scale sequence changes (15). The PCR was carried out in 96-well microtiter plates with the following sets of primers (sense/antisense): exon 3, TTCTCCTCAGATATGAAACT/TAAATTAAAGAACTGTAGCT; exon 4, CTGCAGTGGTTGAAAACATT/GCACCAAGGGATGGATCATA; exon 5, TATTGTGATTTCACTGCATG/GATTCAGATATATTCCTACA; exons 6 and 7, AAATAAGAGTGGAAGACTGT/GGAAAATCATCCCTGCATCT; exon 8, TTCTTGTATCTTGGTAACTA/TGAACTTGGAAACTAGAAAT; exon 9, TTTATTTATACAAGTGTTTC/AAATATTCAAAACTGAACAT; exon 10a, AAGGAGAAACACTGAATTGA/CGTAACAACCTGTTTAATCT; exon 10b, GTTAGGGTGGAACTATCCAG/CCTATGTTACCAACGATGGT; exon 10c, CTTTGTGGTGGTTGATTTCC/TTGCAATTTCAAGACCAACT; exon 10d, TTTGCTGATTGTGAAGCTGG/GCCAAGATTCGGGCAAACCT; exon 10e, CAGTAGACCTCCTGCAAACC/CATGTCCCTGTTAATGACCA. Variant bands were evaluated by direct genomic sequencing of the corresponding PCR-amplified DNA segments (16). Sequence variations expected to affect protein sequence or expression were evaluated by the single-strand conformation polymorphism technique for cosegregation with the disease in the relatives of the respective probands.

In selected cases, Southern blot analysis was used to screen for gene deletions or rearrangements with radiolabeled cDNA fragments (probe pCNCG) derived from the human α subunit of the cGMP-gated channel gene (13).

Expression of the Ser316Phe and Arg654(1-bp del) Alleles *in Vitro*. The cDNA encoding the α subunit of the human cGMP-gated cation channel was inserted into the polylinker site of pCIS (17). *In vitro* mutagenesis of the wild-type channel cDNA to produce the Ser316Phe and Arg654(1-bp del) mutations identified in patients was carried out with standard procedures with single-stranded DNA and oligonucleotides.

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Abbreviation: RP, retinitis pigmentosa.

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For expression of the proteins, cultured human embryonic kidney cells (HEK-293) were transfected with a mixture of expression plasmid (10 μg), carrier DNA (pBluescript, 10 μg), and simian virus 40 tumor antigen expression plasmid (RSV-TAg, 1 μg) by the calcium phosphate method (18).

Electrical Recordings. At 48–72 hr after transfection, patch-clamp recordings were made from excised inside-out membrane patches of transfected HEK-293 cells at room temperature. The patch pipette contained "0-Ca^{2+}" solution (140 mM NaCl/5 mM KCl/1 mM Na_2-EGTA/10 mM Hepes·NaOH, pH 7.6). The bath perfusate before establishment of a membrane seal was Ringer's solution (140 mM NaCl/5 mM KCl/10 mM Hepes·NaOH/2 mM $CaCl_2$/1 mM $MgCl_2$, pH 7.6). Before excision of the patch, the bath perfusate was switched to "0-Ca^{2+}" solution. Appropriate concentrations of cGMP were added to the bath solution.

Immunocytochemistry. Transfected cells were harvested from a culture dish, centrifuged, and, after resuspension, pipetted onto a poly(D-lysine)-coated microscope slide. The cells were fixed overnight in 4% (wt/vol) paraformaldehyde in 100 mM sodium phosphate (pH 7.3) at 4°C and then washed in phosphate-buffered saline (PBS). Triton X-100 (0.1%) was present in all incubation and wash buffers. The cells were incubated overnight with the monoclonal antibody PMc 2G11 (19) (1:50 dilution in PBS) at 4°C. After two washes in PBS, the cells were incubated with the secondary antibody (fluorescein-conjugated goat anti-mouse immunoglobulin, Cappel, 1:50 dilution) in PBS for 2 hr at room temperature, then washed, and sealed with coverslips. The cells were viewed on a confocal laser scanning microscope at 1-μm-thick optical sections. The gain and blackness levels were set so that in each optical section, white in the pseudo-color image corresponded to the maximum signal intensity.

RESULTS

Sequence Variants Discovered. A total of 94 unrelated patients with autosomal dominant RP and 173 unrelated patients with autosomal recessive RP were evaluated for mutations in the gene encoding the α subunit of the cGMP-gated channel. The entire coding sequence and a variable amount of intron sequence flanking the exons were screened. Among the patients with dominant RP were 16 known to have pathogenic mutations in the rhodopsin or peripherin/*RDS*

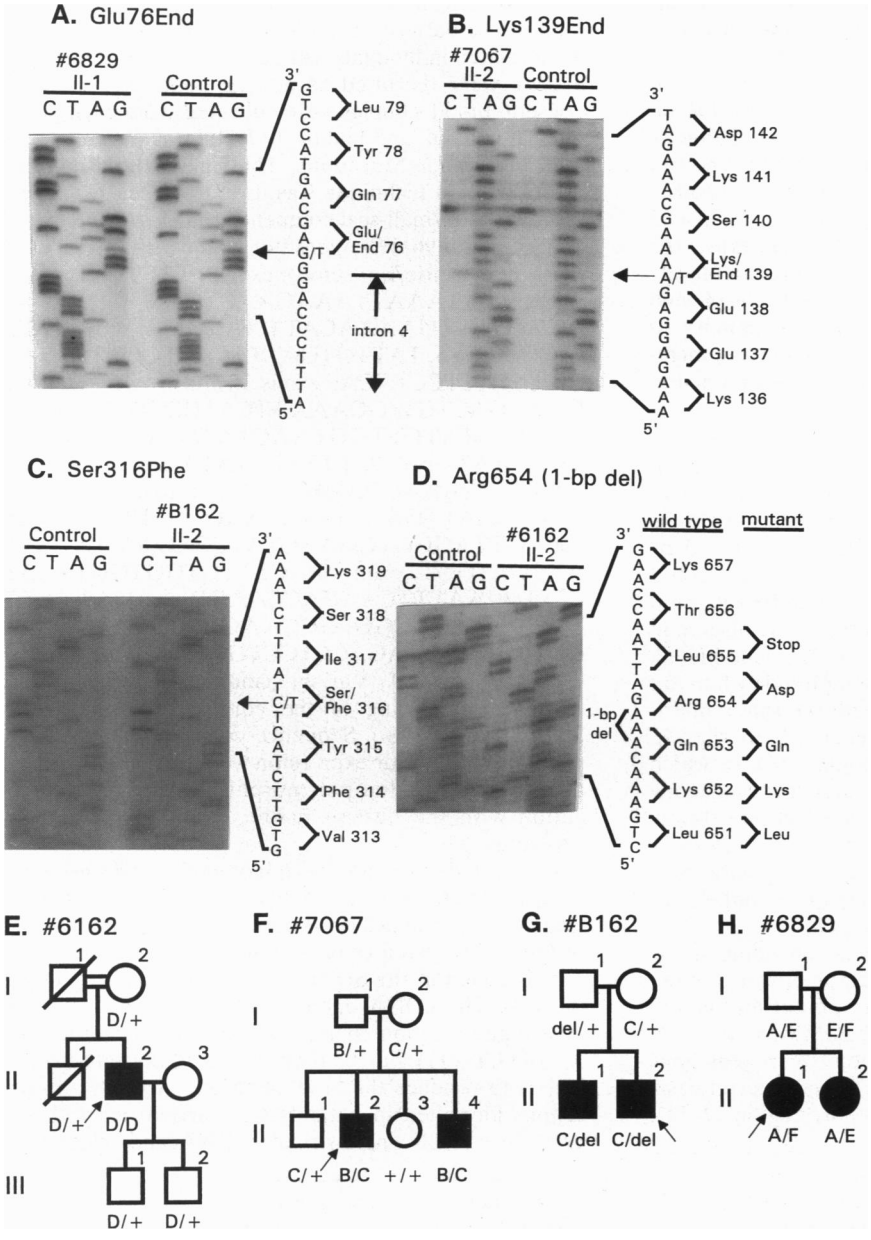

FIG. 1. Sequences of mutations in the gene encoding the α subunit of the channel protein and their cosegregation with recessive RP. (*A–D*) Results of directly sequencing PCR-amplified DNA from patients with the mutations Glu76End, Lys139End, Ser316Phe, and Arg654 (1-bp del), respectively. In *A–D*, the sequence from an individual without the respective mutation is also shown as a normal control. (*C*) The mutation Ser316Phe appears monoallelic because individual II-2 has a deletion of the other allele. (*E–H*) Transmission of these alleles in the families that segregate them. The alleles are designated as follows: A, Glu76End; B, Lys139End; C, Ser316Phe; D, Arg654 (1-bp del); del, deletion of most or all of the transcriptional unit; E, Asp at codon 114; F, Asn at codon 114. In family 6162 (*E*), members I-1 and I-2 are first cousins. An arrow marks the proband in each pedigree.

Table 1. Allele frequencies of nonpathogenic variants

Variation	No. of respective alleles: Normal controls	Autosomal dominant RP	Autosomal recessive RP
Arg28Gln (CGA/CAA)	ND	185/3	339/7
−29 bp exon 4 (A/T)	149/43	ND	126/40
−26 bp exon 6 (C/T)	147/45	150/38	277/69
Asp114Asn (GAT/AAT)*	167/25	155/33	308/38
Glu-100 (GAA/GAG)*	186/6	184/4	339/7
Intron 8 poly(A) repeat	ND	ND	ND
Thr-469 (ACA/ACG)	ND	188/0	345/1
Phe-540 (TTC/TTT)	ND	ND	329/17

ND, allele frequency not determined.
*Published sequence is the less common allele (18).

genes, and among the patients with recessive RP were 6 known to have pathogenic mutations in the rhodopsin, *ROM1, RDS*, or the rod β-phosphodiesterase genes. We identified two categories of deviations from the published sequence (13). The first group of anomalies were deemed polymorphisms or rare variants unrelated to disease because (*i*) they were predicted not to alter the sequence of the encoded protein, (*ii*) they did not cosegregate with RP in families carrying them, or (*iii*) they were found with approximately equal frequency among patients with dominant disease, recessive disease, or no disease (i.e., unaffected individuals who served as controls). In this category of presumably nonpathogenic sequence variations were three silent changes in the coding region (affecting codons Glu-100, Thr-469, and Phe-540), two missense changes (Arg28Gln and Asp114Asn), two single-base variations in introns (A vs. T 29 bp upstream of exon 4 and C vs. T 26 bp upstream of exon 6), and a variation in the length of a poly(A) repeat in intron 8 (see Table 1 for allele frequencies).

The second group of anomalies all altered the sequence or expression of the encoded protein and were ultimately interpreted as pathogenic mutations. Of the five mutations in this group, three clearly were null alleles: two nonsense mutations occurring early in the reading frame (Glu76End and Lys139End, Fig. 1*A* and *B*) and a deletion of most or all of the transcriptional unit that was indicated by the reduced intensity of hybridizing bands by Southern blot analysis (data not illustrated). Although the remaining two mutations altered the sequence of the encoded protein, it was not obvious from inspection whether they would interfere with the protein's function. These were a missense mutation (Ser316Phe, Fig. 1*C*) in the fifth putative transmembrane domain and a frameshift mutation [Arg654(1-bp del), Fig. 1*D*] near the end of the reading frame that leads to the alteration of aa 654 and truncation of the C-terminal 32 aa residues.

Cosegregation Analysis. Four families with autosomal recessive RP each segregated one or two of the five mutations. In three of the families, the affected individuals were either homozygous for a mutation [Arg 654(1-bp del) in family 6162, Fig. 1*E*] or were compound heterozygotes (Lys139End and Ser316Phe in family 7067, Fig. 1*F*; Ser316Phe and gene deletion in family B162, Fig. 1*G*). Unaffected relatives either had no mutation or were heterozygote carriers for only one mutation. Based on the cosegregation of these mutations with the disease and the results from the expression of the Ser316Phe and Arg654(1-bp del) mutations *in vitro* (see below), we concluded that these mutations are the cause of RP in these three families.

In the fourth family with recessive RP (family 6829, Fig. 1*H*), the nonsense mutation Glu76End was present heterozygously in the two affected sisters and in their unaffected father. No mutation was detected in the maternally derived allele in the two affected sisters. In fact, analysis with the polymorphism Asp114Asn showed that they received different alleles at this locus from their mother (Fig. 1*H*). Because of the absence of a pathogenic mutation in the maternally derived alleles in these two sisters, it is possible that there is a pathogenic mutation in a gene encoding another subunit of the channel protein or some other protein that interacts with the α subunit and that the combination of the two defects is the cause of RP. This explanation would be reminiscent of the digenic inheritance that has been previously discovered among some families with RP in which affected members are double heterozygotes for mutations in the unlinked peripherin/*RDS* and *ROM1* genes (6). An alternative possibility is that this mutation is not the cause of RP in this family.

One individual with autosomal dominant RP carried the Ser316Phe mutation heterozygously. Five available affected members of the corresponding family (6003) had been previously found to carry heterozygously the rhodopsin missense mutation Pro347Leu (20), whereas the channel protein mutation was present only in two affected siblings and their unaffected mother. There was no clear difference in phenotype between the relatives who carried only the rhodopsin mutation

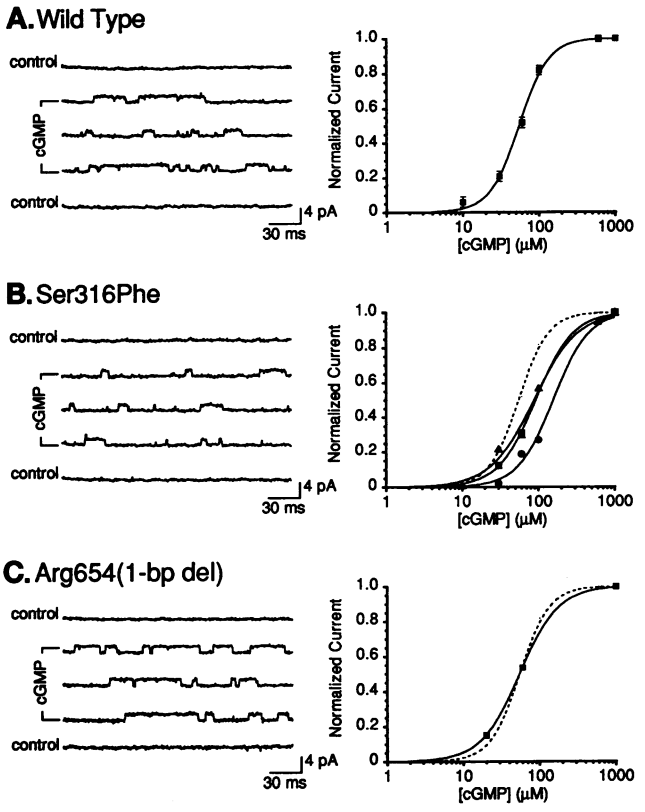

FIG. 2. Electrophysiological data from excised patches of plasma membrane from HEK-293 cells transfected with cDNAs encoding the wild-type channel protein and the Ser316Phe and Arg654(1-bp del) mutants. (*Left*) Sample electrical records from responsive patches in the absence and presence of cGMP for the three cases. cGMP concentration was 10 μM in *A*, 10 μM in *B*, and 60 μM in *C*. Patches in *A* and *B* had more than one channel, while patch in *C* had a single channel. Membrane potential was held at +60 mV. (*Right*) Dose–response relation between normalized mean current and cGMP concentration for the three channel proteins. Averaged data are shown for the wild type, and individual experiments are shown for the mutants. Curves are least-squares fits to the data based on the Hill equation, $j = [cG]^n/([cG]^n + K_{1/2}^n)$, where j is normalized current, [cG] is cGMP concentration, $K_{1/2}$ is the half-activating cGMP concentration, and n is the Hill coefficient. $K_{1/2}$ and n values, respectively, are 55 μM and 2.3 in *A*; 93 μM and 1.8 (■), 88 μM and 1.5 (▲), and 157 μM and 1.9 (●) in *B*, and 55 μM and 1.7 in *C*. The dashed curves in *B* and *C* are identical to the solid curve in *A*.

and the siblings who carried both the rhodopsin and the channel protein mutations. We concluded that the rhodopsin mutation was the cause of the siblings' retinitis pigmentosa and that in addition they carried the recessive channel protein mutation Ser316Phe by chance and without obvious effect.

Expression of the Ser316Phe and Arg654(1-bp del) Alleles *in Vitro*. To check for any functional defects in the Ser316Phe and Arg654(1-bp del) mutant proteins that might support their role in RP, we introduced these mutations into a rod channel cDNA sequence and transiently expressed the mutant proteins in cultured HEK-293 cells. Inside-out patches of plasma membrane were excised from transfected cells and tested for sensitivity to bath-applied cGMP. With either mutant, the frequency of detecting functional cGMP-activated channels was extremely low. With the Ser316Phe point mutation, only 3 out of a total of 85 patches tested responded to cGMP. Two of these responsive patches apparently had a single cGMP-gated channel, while the third had perhaps 7 channels, as judged from the size of the macroscopic current at a saturating cGMP concentration. In parallel transfections with the wild-type cDNA, 15 out of 28 tested patches were responsive to cGMP; the induced current was also generally much larger, indicating the presence of a much greater number of channels (between 8 and 470). With the Arg654(1-bp del) mutation, only 1 out of 83 patches responded to cGMP, and it again had a single channel; in contrast, 17 out of 37 patches in parallel experiments with the wild-type channel responded to cGMP with much larger currents (equivalent to between 13 and 170 channels per patch). Despite the low frequency of detected mutant channels in the plasma membrane, these channels nonetheless did not show significant deviations from the normal channels in either the open-channel current or the dependence on cGMP concentration. In Fig. 2 *Left*, single-channel openings of the wild-type and the two mutant channels are compared. In all three cases, the single-channel current at +60 mV was ≈1.8 pA. The open times also appeared to be comparable, though this parameter has not been examined closely. In Fig. 2 *Right*, the dose–response relations between current activation and cGMP concentration are plotted. There is good agreement in this relation between wild-type channel and the Arg654(1-bp del) mutant. The deviation in this relation between wild-type and the Ser316Phe mutant also may not be significant, because the sample size for the mutant is too small; $K_{1/2}$ values for the wild-type channel have been observed to be as large as 100 μM (10, 13). The possibility that the cGMP-gated channels on the plasma membrane of cells transfected with the mutant cDNAs represented endogenous channels on HEK-293 cells seems unlikely because previous work using mock-transfected cells (18) or cells transfected with the channel β-subunit cDNA alone (10) has not detected these open-channel events.

Table 2. Summary of immunofluorescence experiments

	Wild type	Ser316Phe	Arg654 (1-bp del)
No. of transfections	2	2	3
Total no. of cells examined	200	200	300
No. of cells with staining only of cell circumference	152 (76)	14 (7)	27 (9)
No. of cells with staining only of cell interior	0	1 (0.5)	215 (72)
No. of cells with staining of both circumference and cell interior*	48 (24)	185 (92.5)	58 (19)

Values in parentheses are percents.

*In this category, the cells expressing the wild-type sequence generally showed stronger staining of the circumference than the cell interior, whereas cells expressing either mutant sequence showed stronger staining of the cell interior.

To examine whether the paucity of expressed mutant channels on the plasma membrane of HEK-293 cells is due to poor expression of the proteins or their failure to reach the surface membrane, we stained the transfected cells with the monoclonal antibody PMc 2G11 (19), which recognizes an epitope on the C-terminal segment; this epitope is still present in the Ser316Phe and Arg654(1-bp del) proteins. Confocal microscopic images of the stained cells clearly show the wild-type protein located almost exclusively on the surface membrane of the HEK-293 cells (Fig. 3*A*), whereas the mutant proteins appear to be predominantly trapped inside the cell (Fig. 3 *B* and *C*). Collected data leading to a similar conclusion are presented in Table 2. Mock-transfected cells were also examined, and they did not show any staining (data not shown). Thus, the mutant proteins were expressed, and the rare presence of functional channels as assayed electrophysiologically appeared to be due to their failure to reach the plasma membrane.

DISCUSSION

We have presented evidence that mutations in the gene encoding the α subunit of the cGMP-gated channel protein are

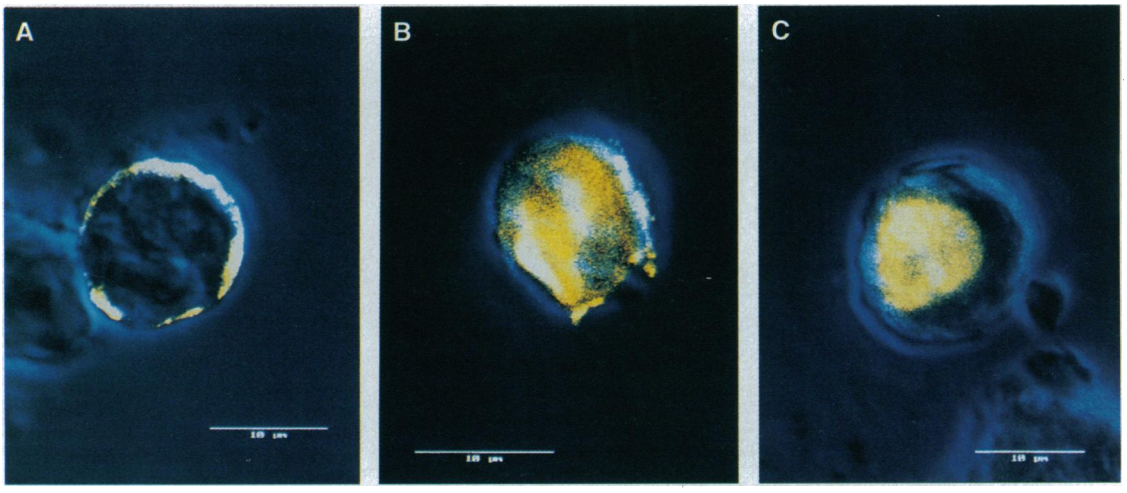

FIG. 3. Confocal images of immunofluorescence staining from the expressed wild-type and mutant channels in HEK-293 cells, with the antibody PMc 2G11. In each case, the optical section is horizontal and is roughly through the middle of the stained cell, with the yellow/white color indicating the location of the protein. (Bars: 10 μm.) (*A*) Wild type. (*B*) Ser316Phe mutant. (*C*) Arg654(1-bp del) mutant.

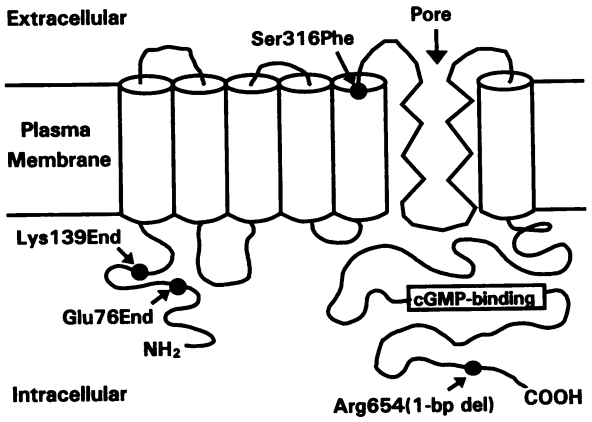

FIG. 4. Diagram showing the putative folding pattern of the channel α subunit in the membrane (11). The locations of the pore and the cGMP-binding domain are indicated. Arrows point to the first residue altered by four of the mutations described in this paper. The fifth mutation is a deletion of the entire gene (not illustrated).

a cause of autosomal recessive RP. Three of the mutant alleles (Glu76End, Lys139End, and a deletion of the gene) are null either because they would encode a protein without the transmembrane domains and the pore forming region (Fig. 4) or because they would encode no protein at all. When expressed *in vitro*, the remaining two alleles [Ser316Phe and Arg654(1-bp del)] encoded channels that, while functional, mostly failed to reach the plasma membrane. Based on these observations, it is likely that the pathogenesis of photoreceptor degeneration due to these mutations is a consequence of the paucity or lack of cGMP-gated channels on the plasma membrane of the rod outer segments. An alternative explanation, at least for the Ser316Phe and Arg654(1-bp del) mutant proteins, would be that these mutant proteins cause disease by accumulating inside the rod inner segment and by interfering with protein processing or the general metabolism of the photoreceptors, as thought to be the case for some rhodopsin mutants causing autosomal dominant RP (21, 22). However, this latter explanation does not seem likely in view of the recessive nature of these channel mutations. The molecular basis for the intracellular retention of the Ser316Phe and Arg654(1-bp del) mutant proteins remains to be identified.

In normal rod photoreceptors, wild-type channels close in the light due to a reduction in cytoplasmic cGMP. In patients with RP due to these recessive null mutations, the rod photoreceptors would functionally mimic wild-type photoreceptors exposed to constant bright light because there would be few or no open cGMP-gated channels. It remains undetermined whether this predicted perturbation in the physiology of photoreceptors is actually the cause of their degeneration.

Mutations associated with the α subunit of the cGMP channel were found to be responsible for only 3 or 4 out of 173 unrelated cases of recessive RP and none of 94 cases of dominant RP. Since about half of all cases of RP are due to autosomal recessive mutations, the gene encoding the α subunit of the channel protein accounts for only $\approx$1% of cases of this disease. If one includes both dominant and recessive mutations at the six RP loci so far identified, one locus (rhodopsin) accounts for $\approx$10% of all cases (20) and the remaining five loci together (peripherin/*RDS*, *ROM1*, the β subunit of cGMP-phosphodiesterase, myosin VIIA, and the α subunit of the cGMP-gated channel) account for another $\approx$10% of cases (refs. 3, 6, and 23 and unpublished observations). The nonallelic heterogeneity in RP is highlighted by the recognition that $\approx$80% of cases are still without an identified cause. Since three of the six identified genes code for proteins in the phototransduction cascade, it is likely that genes encoding other members of this pathway will be found to be the cause of some of the remaining cases.

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