

The destabilization of human GCAP1 by a proline to leucine mutation might cause cone-rod dystrophy

Richard J. Newbold¹, Evelyne C. Deery¹, Caroline E. Walker¹, Susan E. Wilkie², Narayanaswamy Srinivasan³, David M. Hunt², Shomi S. Bhattacharya² and Martin J. Warren^{1,+}

¹School of Biological Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK, ²Department of Molecular Genetics, Institute of Ophthalmology, 11–43 Bath Street, London EC1V 9EL, UK and ³Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

Received 18 August 2000; Revised and Accepted 8 November 2000

Guanylate cyclase activating protein-1 (GCAP1) is required for activation of retinal guanylate cyclase-1 (RetGC1), which is essential for recovery of photoreceptor cells to the dark state. In this paper, experimentally derived observations are reported that help in explaining why a proline→leucine mutation at position 50 of human GCAP1 results in cone-rod dystrophy in a family carrying this mutation. The primary amino acid sequence of wild-type GCAP1 was mutated using site-directed mutagenesis to give a leucine at position 50. In addition, serine replaced a glutamic acid residue at position 6 to promote N-terminal myristoylation, yielding the construct GCAP1 E6S/P50L. The enzyme was over-expressed in *Escherichia coli* cells, isolated and purified before being used in assays with RetGC1, characterized by circular dichroism (CD) spectroscopy, and investigated for protease resistance and thermal stability. Assays of cyclic guanosine monophosphate (cGMP) synthesis from guanosine triphosphate by RetGC1 in the presence of E6S/P50L showed that E6S/P50L could activate RetGC1 and displayed similar calcium sensitivity to wild-type GCAP1. In addition, E6S/P50L and wild-type GCAP1 possess similar CD spectra. However, there was a marked increase in the susceptibility to protease degradation and also a reduction in the thermal stability of E6S/P50L as observed by both the cGMP assay and CD spectroscopy. It is therefore suggested that although GCAP1 E6S/P50L has a similar activity and calcium dependency profile to the wild-type GCAP1, its lower stability could reduce its cellular concentration, which would in turn alter [Ca²⁺] and result in death of cells.

INTRODUCTION

Within inherited retinopathies, cone and cone-rod dystrophies occur with a frequency of ~1 in 10 000 of the population. Cone

dystrophies are characterized by a loss of central vision with retention of peripheral sight. In cone-rod dystrophy, some loss of peripheral vision is observed although it may occur to a lesser degree and at a later stage. One of the seven loci so far identified with autosomal dominant cone and cone-rod dystrophy is 6p21, where mutations in *GUCA1A* [guanylate cyclase activating protein-1 (GCAP1)] are associated with the disorders. The role of GCAP1 in the visual transduction system is outlined below.

The absorption of light by photoreceptor cells in the eye results in a brief voltage pulse, as a consequence of closing cation channels in the plasma membrane. The cation channels close due to a decrease in cyclic guanosine monophosphate (cGMP) produced by activation of an enzyme cascade, which hydrolyses the cGMP after absorption of light. Recovery from this light stimulation requires the re-synthesis of cGMP, which is initiated by the light-induced drop in cellular [Ca²⁺]. The reduction in [Ca²⁺] occurs due to the closure of the cGMP-gated cation channels that allow the influx of intracellular calcium (1,2). The enzyme that produces cGMP in photoreceptor cells is retinal guanylate cyclase-1 (GUCY2D or RetGC1) (3). RetGC1 synthesizes cGMP, in the cell, only when stimulated by GCAP1. Thus, GCAP1 is a key component in the restoration of the dark state of photoreceptor cells (4).

GCAP1 is a member of the calcium-binding EF-hand protein family that includes calmodulin and recoverin (5). In contrast to the behaviour of other EF-hand proteins, which are stimulated by high calcium levels, GCAP1 acts as an activator only at low levels of Ca²⁺, typically when [Ca²⁺] drops below ~300 nM (6). GCAP1 has four EF-hand motifs but only three of these are functional and able to bind calcium. A crystal structure of recoverin in the Ca-free form has been elucidated (7), as have nuclear magnetic resonance (NMR) structures of recoverin in the Ca-free and Ca-bound states (8,9) and a recent NMR structure of GCAP2 (10). Currently, recoverin provides the only model for the conformational changes that EF-hand proteins undergo on calcium binding. The NMR structures of myristoylated recoverin in the Ca-free and Ca-bound states reveal considerable changes in the tertiary structure on calcium binding, including a dramatic change in the position of the myristoyl group. The myristoyl group in GCAP1 is believed to help in targeting the

⁺To whom correspondence should be addressed. Tel: +44 207 882 7718; Fax: +44 208 983 0973; Email: m.j.warren@qmw.ac.uk

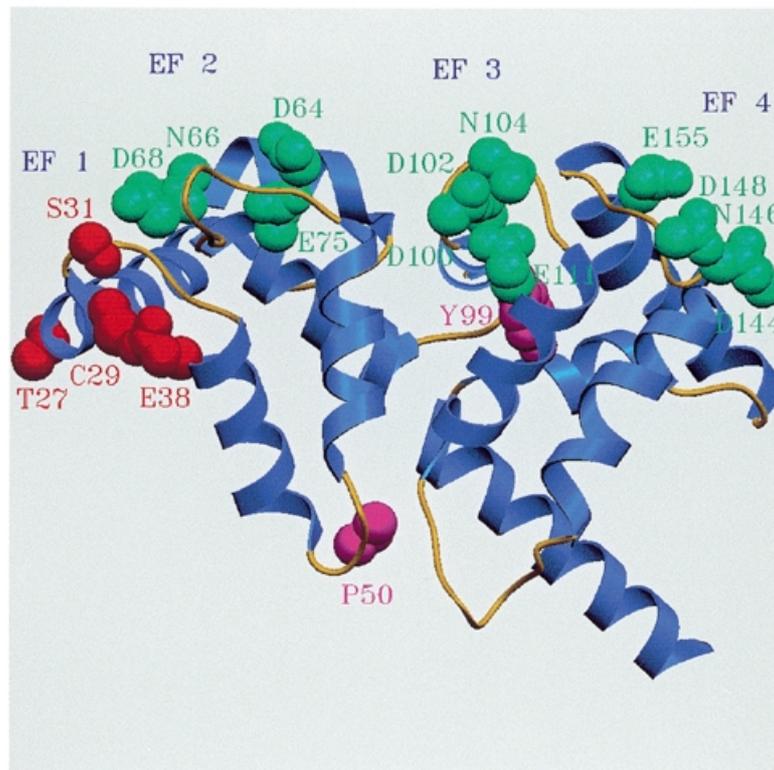


Figure 1. Model structure of GCAP1 showing the overall fold of the model. The helices are rendered in blue, with loops shown in orange. The key residues determining the calcium-binding ability of an EF-hand are shown in the space-filling model and are labelled. The EF-hand 1 is shown in red as it is predicted to be incapable of binding calcium, and the calcium-binding EF-hands 2–4 are shown in green. Amino acid residues 50 and 99, those which correspond to the detected mutations in GCAP1, are coloured pink. The figure was produced using SETOR (26).

protein to the membrane and its absence has been observed to prevent GCAP1 activation of RetGC1 (11). A pictorial representation of a model of GCAP1 (unmyristoylated for clarity) is shown in Figure 1, and highlights the positions of the EF-hands and the mutations discussed in this paper.

Two separate mutations in GUCA1A (GCAP1) have been reported. The first mutation, Y99C, causes decreased visual acuity and loss of colour vision after the age of 20, followed by progressive central atrophy. Electrophysiological testing confirmed the general loss of cone function with preservation of rod function. The second mutation, P50L, is associated with a marked variability in phenotype, ranging from minimal abnormalities of macular function to cone–rod dystrophy. For instance, one patient became symptomatic only in her sixth decade with mild photophobia yet her son, in his third decade, had rapid involuntary movement of the eyes, moderate photophobia and reduction of his central vision.

A biochemical analysis of the effect of the Y99C mutation has previously been reported and these studies revealed the mutant GCAP1 to be a constitutively active protein (12,13). Thus, even at high physiological levels of Ca^{2+} , the Y99C variant is still able to activate RetGC1, resulting in altered cGMP levels. The observed phenotype must be due to an association between the elevated levels of cGMP and cone cell death. In this paper, the functional properties of the P50L mutation are investigated in order to determine whether it

causes a similar biochemical response or whether another mechanism needs to be invoked to explain its pathology.

RESULTS

A modified version of human GCAP1, containing a change at codon 6 such that serine was encoded rather than glutamate, was cloned into pET3a and expressed in *Escherichia coli* cells. The change at codon 6 does not affect the activity of the GCAP1 (14) but permits more efficient myristoylation of the protein in *E. coli* (15,16), which is essential for the functioning of GCAP1. In this study, the E6S variant is referred to as wild-type protein, with the E6S/P50L variant called *P50L* and the E6S/Y99C variant called *Y99C*. Two mutant variants of this E6S protein were also made using site-directed mutagenesis, Y99C and P50L, which correspond to mutations associated with cone and cone–rod dystrophies. The integrity of the constructs used in this study was confirmed by sequencing of the relevant plasmid inserts. The pET3a vectors, encoding the aforementioned GCAP1 variants (wild-type, P50L and Y99C) were transformed into *E. coli* together with pBBlyS to allow concerted expression of GCAP1 and *N*-myristoyltransferase.

When expressed in *E. coli*, in the presence of myristic acid, the proteins were easily visualized when crude cell extracts were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were purified as described in Materials and Methods by differential ammonium

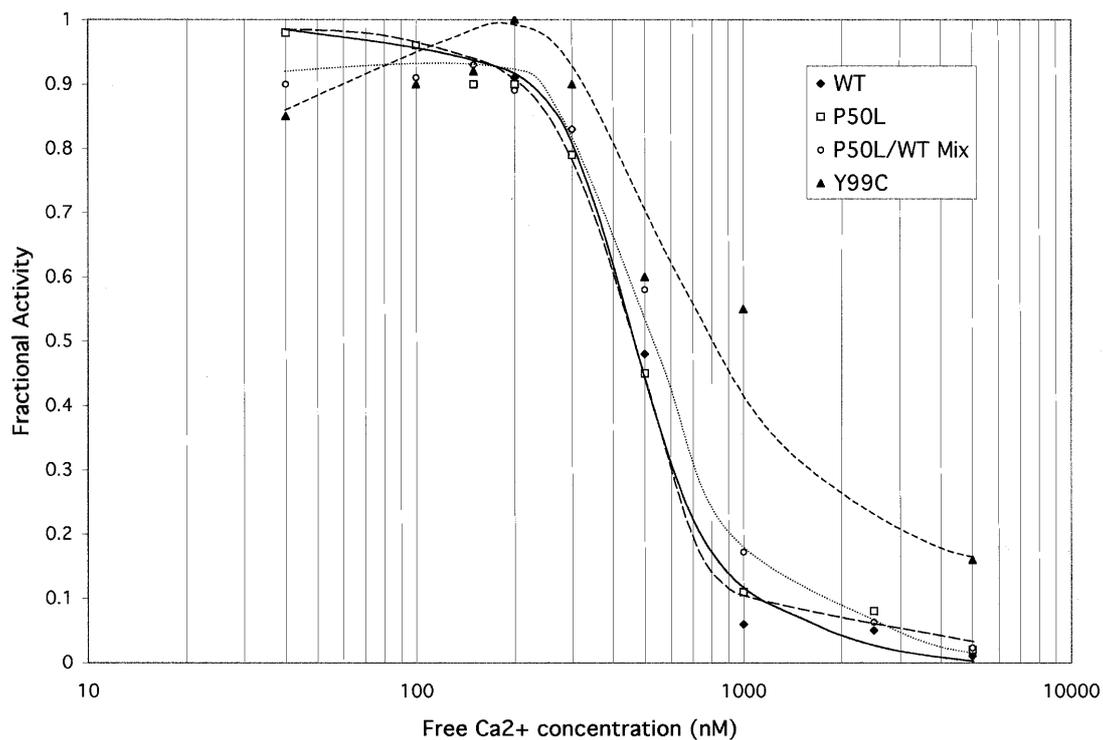


Figure 2. Ca^{2+} sensitivity of wild-type and P50L GCAP1 in the RetGC1-stimulation assay. Stimulation of RetGC1 activity by GCAP1s at varying calcium concentrations, showing that the P50L mutant possesses similar calcium sensitivity to the wild-type protein and that the Y99C mutant shows shifted calcium sensitivity to the wild-type protein, leading to constitutive activation at physiological $[\text{Ca}^{2+}]$.

sulphate fractionation, gel filtration and ion exchange chromatography. Analysis of fractions from the S300 column indicated that the majority of GCAP1 is present as a high molecular mass aggregated form, eluting with the void volume. This aggregation is probably a consequence of the expression of human GCAP1 in a prokaryotic host rather than of the presence of the myristoyl group, because samples prepared without the myristoyl group also showed similar aggregatory properties and elution characteristics from the S300 gel filtration column (unpublished data). This aggregated form of GCAP1 was found to have little activity and was therefore discarded.

The fractions containing less aggregated GCAP1 were pooled and further purified by anion exchange chromatography. Subsequent analytical gel filtration studies revealed that the less aggregated GCAP1 fraction comprised monomeric protein (data not shown). The two-column purification was sufficient to yield a sample of >90% purity, which was found to be highly active in RetGC1 assays. Protein yields were routinely 2–3 mg/l of culture for the wild-type, Y99C and P50L forms of the protein. The molecular masses of the protein samples were checked using MALDI-TOF mass spectrometry, and the major peaks confirmed the presence of the myristoyl group on full-length protein constructs (data not shown).

Protein activity

In this study human RetGC1, expressed in hk293 cells, was used in conjunction with bacterially expressed human GCAP1. The RetGC1/GCAP1 assay therefore represents a homologous

human system, as opposed to heterologous bovine/human systems, which have been described previously.

The activity of RetGC1 is stimulated by GCAP1 at low concentrations of calcium. In the absence of GCAP1, RetGC1 is also stimulated by Mn^{2+} /Triton X-100, which has been used to quantitate general RetGC1 catalytic activity (17). In assays with RetGC1 it was found that both the wild-type and mutant forms of GCAP1-stimulated guanylate cyclase (GC) activity by at least 10-fold and showed similar levels of stimulation to that observed for manganese stimulation of fresh RetGC1 preparations. This demonstrates that the bacterial expression of human GCAP1 produces a soluble, monomeric and active protein, which is effective in this system. In addition, the production of protein in relatively high yields provides the opportunity for concerted structural studies on the human protein, including circular dichroism (CD) spectroscopy, without the necessity of refolding protein from inclusion bodies.

The sensitivity of the wild-type and P50L proteins to Ca^{2+} was assayed over a $[\text{Ca}^{2+}]$ range of 40–5000 nM. The profiles, as shown in Figure 2, show no significant differences between the wild-type and P50L forms of GCAP1, in contrast to the results obtained with Y99C, which show a shift of ~20–200 nM with respect to the profile observed for the wild-type (18,13) (Fig. 2). Mixing wild-type GCAP1 with the P50L form yielded a similar profile to that observed for the wild-type sample alone (Fig. 2). The P50L mutation would therefore not appear to affect the ability of the GCAP to respond to physiological concentrations of Ca^{2+} .

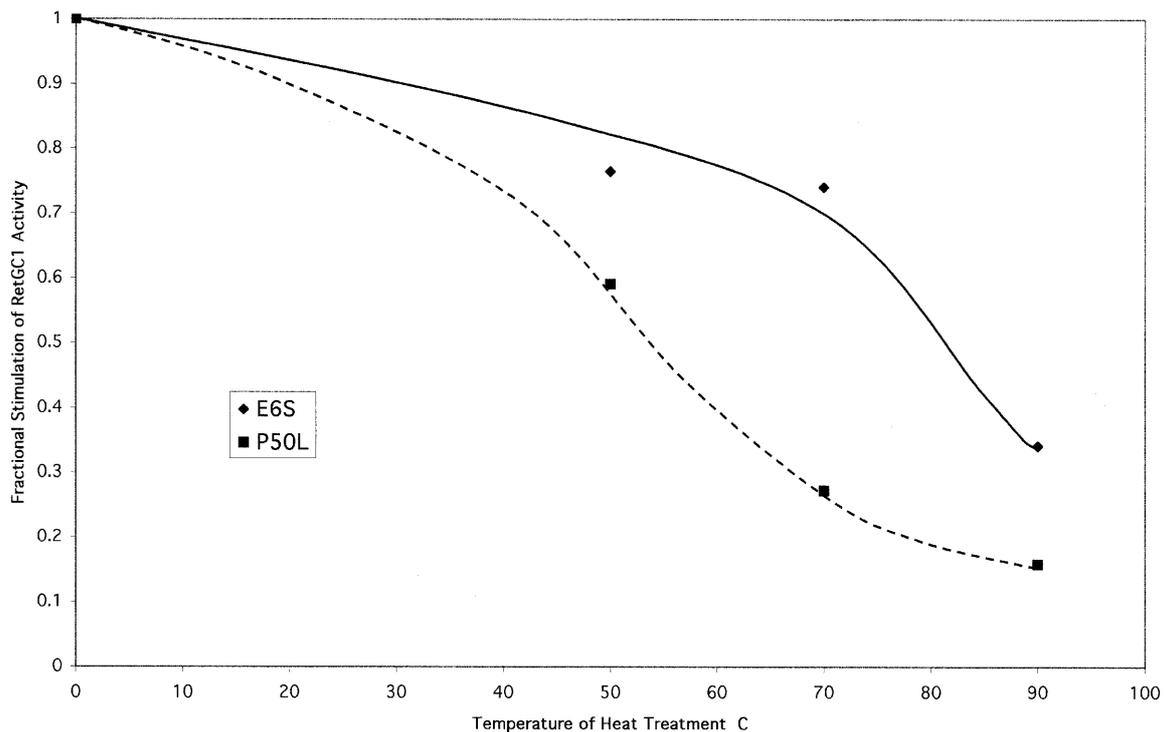


Figure 3. Temperature sensitivity of activation of RetGC1 by wild-type GCAP1 and P50L. Reduction of stimulation of RetGC1 activity by GCAP1s at elevated temperatures, showing the decreased thermal stability of the P50L mutant compared with the wild-type protein.

Thermal stability

The thermal stability of the wild-type and P50L forms of GCAP1 was investigated by heating the GCAP samples to a range of different temperatures (37, 50, 70 or 90°C) prior to their addition to the GC assay mixture. The results of this procedure, shown in Figure 3, demonstrate that the P50L variant is dramatically more thermally labile than wild-type GCAP1. Indeed, they indicate that the P50L activity is half its maximum value at ~55°C, which is ~25°C lower than that observed for the wild-type protein.

Protease susceptibility

Visualization of the effect of trypsinization of GCAP1 by SDS-PAGE showed that the P50L substitution increases the protease susceptibility of GCAP1 (Fig. 4). Addition of Ca^{2+} decreases the susceptibility to trypsin activity of both forms of GCAP1 (Fig. 4). These calcium protection results show a similar trend to those shown previously (18), where trypsin-derived fragments of GCAP1 at low $[\text{Ca}^{2+}]$ were smaller than those produced under high $[\text{Ca}^{2+}]$. In contrast to the protease susceptibility of the P50L mutant, the Y99C shows matching protease resistance to the wild-type protein, with a similar quantity of residual trypsin-resistant core remaining after prolonged incubation with the protease.

Protein structure

The far-UV CD spectra of the two mutant forms of GCAP1 are indistinguishable from that of the wild-type protein, are typical

of a well-folded globular protein with a high helical content (Fig. 5) and are consistent with the model shown in Figure 1. The lack of differences between the predicted secondary structural contents of the three forms of GCAP1 indicates that the mutations probably do not cause gross conformational changes to the native fold.

However, on heating the P50L and wild-type samples, differences in the far-UV spectra were observed, as shown in Figure 5, with a greater decrease in the helical content of P50L compared with that observed for the wild-type protein at the same temperature. This clearly suggests that the P50L variant is more susceptible to thermal denaturation than either the wild-type or Y99C versions of the protein, agreeing with the greater reduction in activity for P50L following heat treatment.

DISCUSSION

Cone dystrophies are characterized by the loss of cone cells, especially in the macula, and result in a loss of central vision (19). Previously, it was reported that patients with a Y99C mutation in GCAP1 had a phenotype that showed reduced visual acuity and loss of colour vision prior to visual loss. However, even at later stages of the disease, the peripheral visual field was preserved, indicating that the rod cells are largely unaffected (12). Patients with a P50L mutation, as reported by Downes *et al.* (20), show marked differences in phenotype compared with those who carry a Y99C mutation. A key difference is the range of symptoms observed for patients with a P50L mutation, even in the same family, in contrast to the highly distinctive Y99C phenotype, which shows relatively little variation in expression. The P50L mutation has produced

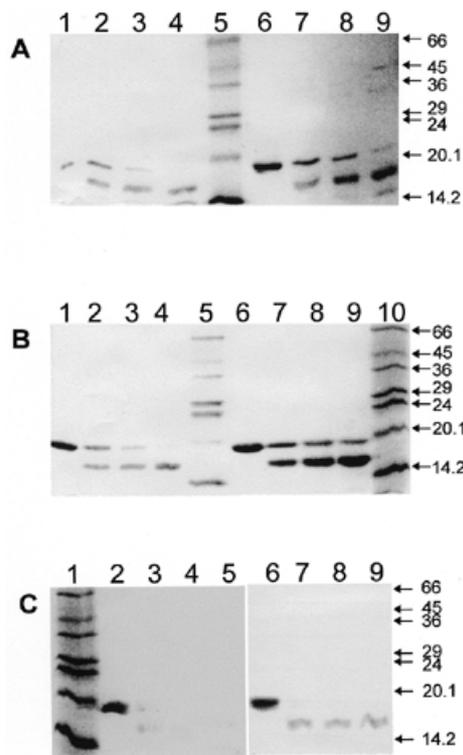


Figure 4. SDS-PAGE analysis of trypsinized GCAP1 samples in the absence and presence of calcium. Coomassie Blue-stained 17.5% polyacrylamide gels reveal the progressive trypsinization of wild-type, Y99C and P50L samples in the presence (1 mM) and absence of calcium with time. (A) Wild-type. Lane 1 (no calcium), T = 1 min; lane 2 (no calcium), T = 15 min; lane 3 (no calcium), T = 30 min; lane 4 (no calcium), T = 60 min; lane 5, Dalton VII markers (66, 45, 36, 29, 24, 20.1 and 14.2 kDa); lane 6 (1 mM Ca²⁺), T = 1 min; lane 7 (1 mM Ca²⁺), T = 15 min; lane 8 (1 mM Ca²⁺), T = 30 min; lane 9 (1 mM Ca²⁺), T = 60 min. (B) Y99C. Lane 1 (no calcium), T = 1 min; lane 2 (no calcium), T = 15 min; lane 3 (no calcium), T = 30 min; lane 4 (no calcium), T = 60 min; lane 5, Dalton VII markers (66, 45, 36, 29, 24, 20.1 and 14.2 kDa); lane 6 (1 mM Ca²⁺), T = 1 min; lane 7 (1 mM Ca²⁺), T = 15 min; lane 8 (1 mM Ca²⁺), T = 30 min; lane 9 (1 mM Ca²⁺), T = 60 min; lane 10, Dalton VII markers (66, 45, 36, 29, 24, 20.1 and 14.2 kDa). (C) P50L. Lane 1, Dalton VII markers (66, 45, 36, 29, 24, 20.1 and 14.2 kDa); lane 2 (no calcium), T = 1 min; lane 3 (no calcium), T = 15 min; lane 4 (no calcium), T = 30 min; lane 5 (no calcium), T = 60 min; lane 6 (1 mM Ca²⁺), T = 1 min; lane 7 (1 mM Ca²⁺), T = 15 min; lane 8 (1 mM Ca²⁺), T = 30 min; lane 9 (1 mM Ca²⁺), T = 60 min.

similar cone dystrophy to Y99C, a mild dystrophy with low macular involvement and a moderately severe cone-rod dystrophy (20). In terms of the mutation effect at the molecular level, the P50L mutation does not produce a protein with altered calcium sensitivity, which is in marked contrast to the Y99C mutation. The activities of the P50L mutant and the wild-type protein were also indistinguishable, suggesting that activation of RetGC1 by the P50L form of GCAP1 is not affected by this mutation. In addition, structural studies of wild-type GCAP1 and the P50L and Y99C mutant forms of GCAP1 show almost identical CD spectra in the far-UV region, suggesting that there are no significant differences between them in terms of secondary structure.

However, there are marked differences in the stability of the wild-type and P50L variants. The protease susceptibility studies showed that the P50L form is more readily digested by

trypsin. In addition, thermal stability studies of the activation of RetGC1 by GCAP1 show a dramatic decrease in the thermal stability of the P50L form, with a transition midpoint temperature (T_m) ~25°C lower than that observed for the wild-type protein. In addition, the CD spectra show a bigger decrease in helical content at elevated temperatures for the P50L mutant form, also indicating a less stable structure. The dependence of the disease on the stability of the protein may, therefore, help in explaining the broad phenotypic description of the P50L form of cone-rod dystrophy, since it is likely to vary with the genetic make-up of the individual.

The identification of P50L as the mutation responsible for autosomal dominant cone-rod dystrophy (20) was initially unexpected, given that residue's probable position within GCAP1. Homology modelling of the Y99C mutation of GCAP1 onto the crystal structure of recoverin (12), a 23 kDa Ca²⁺-binding protein which inhibits phosphorylation of rhodopsin at high Ca²⁺ concentrations (21), showed that the mutation is likely to affect the local conformation around the EF₃-hand, with probable consequences with regard to that motif's ability to bind Ca²⁺. Indeed, the inability to bind Ca²⁺ explains why the Y99C variant remains constitutively active. In contrast, the proline residue at position 50 in GCAP1 should be present on an external loop in the Ca-free form, which is not spatially close to any of the calcium-binding motifs. However, the Ca²⁺-bound form of recoverin undergoes a huge conformational change, as observed by comparison with the Ca-free structure (8,9). The proline in recoverin, equivalent to proline 50 of GCAP1, moves from a peripheral loop position to a key central role within the molecule, forming potential Van der Waals contacts between two helices, which form the boundary between the two halves of the recoverin molecule (8).

Alignment of published GCAP1 sequences also shows that the proline residue is conserved in species as diverse as human and leopard frog, suggesting that the identity of this residue is important. This conservation is in marked contrast to other residues in close proximity to the proline (Fig. 6). Both the homology modelling of recoverin and the conservation of the proline at position 50 throughout GCAP1 suggest that this residue may play a structural role within the protein, which may not affect the activity of the molecule but may affect its long-term stability within the cell. Proline is well known as a helix breaker and is commonly found in turns (as it is here in wild-type GCAP1), hence its replacement by leucine, which is the third most stabilizing residue within an α -helix (22), would be expected to yield considerable destabilizing conformational changes to the native GCAP1 fold.

Therefore, we propose that, although mutant GCAP1 P50L has a similar activity and calcium dependency profile to those of the wild-type GCAP1, its lower stability could reduce its cellular concentration, which would in turn alter the [Ca²⁺] within the photoreceptor cell. Imbalance of [Ca²⁺] is believed to disrupt the membrane potential of the mitochondrial outer membrane, leading to release of cytochrome *c*, with subsequent caspase activation and apoptosis (23). Although reduced levels of GCAP1 would decrease the Ca²⁺ content, any disruption to the fine balance of cellular ion levels could set in motion a series of events resulting in the observed cone-rod dystrophy phenotype.

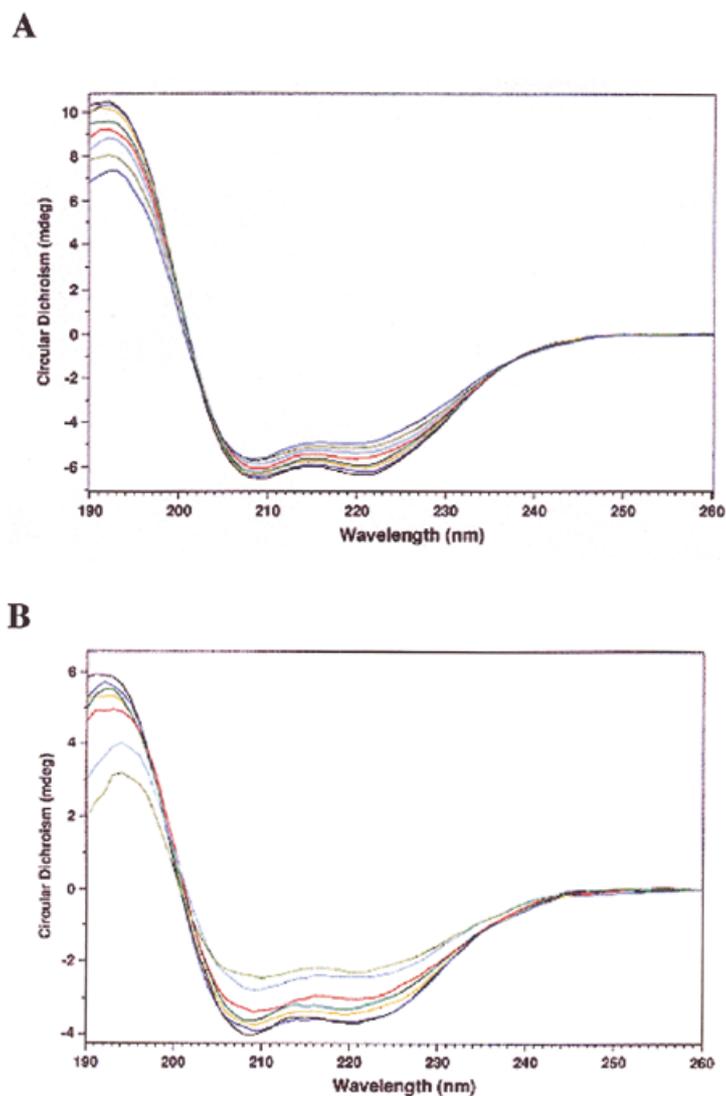


Figure 5. Far-UV CD spectra of wild-type and P50L. (A) Thermal denaturation of wild-type GCAP1 followed by measurement of its far-UV CD spectrum over a range of temperatures (20, 30, 40, 50, 60, 70, 80 and 90°C). (B) Thermal denaturation of P50L GCAP1 followed by measurement of its far-UV CD spectrum over a range of temperatures (30, 40, 50, 60, 70, 80 and 90°C).

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich unless stated otherwise.

Cloning and mutagenesis of GCAP1

GCAP1 was cloned into the M13-based plasmid M13mp18 and mutagenesis was performed with the Sculptor *in vitro* mutagenesis system (Amersham Pharmacia Biotech) using the following primers: E6S mutagenesis primer, 5'-CAC TGA CTT TCC CGA CAT CAC GTT GC-3'; P50L mutagenesis primer, 5'-GCT GGC CGA CAG GCT CAG GTT C-3' and Y99C mutagenesis primer, 5'-CCG TTG CCA TCT ACA TCA CAG AGC TTG AAG TAC CAG C-3'.

The mutagenesis products were subcloned into pET3a (Novagen) and the sequences were verified on an ABI373 automated sequencer. The wild-type, Y99C and P50L forms of GCAP1 cloned into pET3a were expressed in

BL21(DE3)(pBBlysS), where pBBlysS was cloned from pBB131 (the generous gift of Prof. J. Gordon, Washington University School of Medicine, St Louis, MO). pBB131 contains the *Saccharomyces cerevisiae* myristoyl-coA: protein *N*-myristoyltransferase gene and has been used to aid the efficient myristoylation of proteins expressed in bacterial hosts (15). This was necessary because the presence of the myristoyl group on GCAP1 has been shown to be necessary for the stimulation of RetGC1 by GCAP1 (11). The lysozyme gene, isolated by digesting pLysS (Novagen) with *Bam*HI, was cloned into pBB131. This new pET3a construct showed enhanced stability because the synthesis of the T7 RNA polymerase is then fully repressed whilst enabling the co-expression of the target protein and the *N*-myristoyl transferase.

Expression and purification of GCAP1

Cultures of *E. coli* (1 l) containing the plasmids encoding wild-type, P50L or Y99C GCAP1 were grown at 37°C in luria broth

	50	Accession Number
Human	FFGLK NLS P SASQ YVEQMF E	P43080
Cow	FFGLK NLS P WASQ YVEQMF E	P46065
Mouse	FFGLK NLS P SASQ YVEQMF E	P43081
Chicken	FFGLK NLS P SANK YVEQMF E	P79880
Frog	FFGLK NLS P ASNQ YVEQMF E	O73761

Figure 6. Line-up of GCAP1 amino acid sequences in the area close to residue 50. Conserved residues are highlighted with a dark grey background. Conservative substitutions are shown with a lighter grey tone. The mutated proline residue, Pro50, is highlighted in bold.

(LB) media, with vigorous shaking, to an OD_{600 nm} of 0.4–0.6 and induced with isopropyl- β -D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM in the presence of 1 mM myristic acid. The cultures were allowed to incubate for a further 2 h before harvesting by centrifugation. The cells were resuspended in 33 mM Tris-HCl, 20% sucrose, pH 7.4 and frozen at -20°C until required. After thawing, lysozyme (3 $\mu\text{l/ml}$ of a 5 mg/ml stock) and EDTA (to a final concentration of 1 mM) were added to the cell suspension and this was left to incubate for 30 min at room temperature on a rocking platform. After centrifugation at 10 000 g for 15 min, the supernatant (periplasmic fraction) was removed. The pellet was resuspended in ice-cold 10 mM Tris-HCl, 10 mM β -mercaptoethanol, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 1 mM EDTA pH 8.0 using a hand-held homogenizer. This suspension was centrifuged at 10 000 g for 10 min and the supernatant (cytoplasmic fraction) removed. The membranous pellet fraction was resuspended in 10 mM Tris-HCl, 0.2 M NaCl, 10 mM β -mercaptoethanol, 1 mM PMSF, 1 mM EDTA pH 8.0, again using a homogenizer. The cell suspension was then sonicated with a Sonics Vibra Cell Ultrasonic Processor, equipped with a 10 mm diameter probe (four times for 1 min at 30 W on ice) and centrifuged at 20 000 g for 30 min. The supernatant was decanted and retained and the pellet was discarded.

The supernatant was made 25% w/v with respect to ammonium sulphate and the solution clarified by centrifugation (10 000 g for 15 min). The pellet was removed and the supernatant made 50% w/v with respect to ammonium sulphate and the solution centrifuged again (10 000 g for 15 min). The resultant pellet from this cut was resuspended in 5 ml of buffer A (10 mM Tris-HCl, 0.2 M NaCl, 10 mM β -mercaptoethanol, pH 8.0). The sample was filtered through a 0.2 μm filter to remove particulate matter and applied to a column of Sephacryl S300 (Amersham Pharmacia Biotech) (2.6×70 cm), which had been pre-equilibrated in buffer A. The column was run at 1 ml/min and those fractions containing high levels of monomeric GCAP1, identified by SDS-PAGE, were pooled and dialysed against buffer B (10 mM Tris-HCl, 10 mM β -mercaptoethanol, pH 8.0).

The sample was applied to a column of DEAE-Sephadex (2.6×40 cm), which had been previously equilibrated in buffer B, and was eluted by application of a linear gradient of buffer B against buffer B + 1 M NaCl. Fractions were analysed by SDS-PAGE and those where GCAP1 constituted at least 90% of the total protein content were pooled and dialysed against 10 mM MOPS, 10 mM β -mercaptoethanol, pH 7.5 and concentrated to 0.5–2 mg/ml for use in the guanylate cyclase

assay. Alternatively, samples were dialysed against 10 mM sodium phosphate, 2 mM dithiothreitol (DTT), pH 7.5, for use in CD experiments.

RetGC1 preparation

Human RetGC1 cloned into the pRC-CMV vector (Invitrogen) (a generous gift from Prof. J.B. Hurley, University of Washington, Seattle, WA) was transfected into 293T human kidney cells (ECACC) with LipofectAmine (Gibco). Following 48 h transient expression, cells were harvested, washed in phosphate-buffered saline and resuspended in homogenization buffer (10 mM MOPS pH 7.3, 5 mM β -mercaptoethanol, 20 $\mu\text{g/ml}$ leupeptin and 1 mM PMSF). Cells were lysed by passing through a fine gauge needle five times, centrifuged to pellet debris and the membrane-associated RetGC1 was collected by 0.25 M salt precipitation and centrifugation (13 000 g for 30 min at 4°C). Membrane preparations typically yielded 2–3 $\mu\text{g}/\mu\text{l}$ RetGC1 and were stored frozen in small aliquots at -80°C until required.

GC assays

GC assays were undertaken using a modified form of the method of Laura *et al.* (24). Assays were performed in 20 μl reaction volumes containing 5 μg of RetGC1 containing membrane preparation and 1 μg of GCAP1 protein in GC buffer (100 mM KCl, 50 mM MOPS, 7 mM β -mercaptoethanol, 8 mM NaCl, 10 mM MgCl_2 and $\text{Ca}^{2+}/\text{EGTA}$, pH 7.3) with 0.4 mM ATP. The reaction was started by the addition of substrate solution [1 mM GTP, 5 mM cGMP, 0.05 μCi [α - ^{32}P]GTP and 0.00125 μCi [8^3H]cGMP (Amersham Pharmacia Biotech)]. $\text{Ca}^{2+}/\text{EGTA}$ buffers were prepared by the method of Tsien and Pozzan (25) calculated from dissociation constants of EGTA for Ca^{2+} and Mg^{2+} at pH 7.30 and 37°C . After 30 min incubation at 37°C , the reactions were stopped by heating to 100°C for 2 min and the proteins pelleted by centrifugation (10 000 g for 10 min). The reaction was analysed (6 μl) by thin layer chromatography using $\text{NH}_2\text{F}_{254}\text{S}$ anion exchange plates (BDH-Merck) with a solvent mixture of 7% 2 M LiCl, 30% EtOH, 63% H_2O . Spots corresponding to cGMP were visualized under a short wave illuminator, excised and eluted in 0.5 ml of 2 M LiCl, on an orbital shaker for 10 min. Two millilitres of H_2O and 3 ml of Lumagel Safe scintillation cocktail (Lumac) were added and the tubes mixed by inversion. Both ^3H and ^{32}P were counted in a Packard Tri-Carb 2200CA Liquid Scintillation Analyzer to determine the ability of wild-type and mutant GCAP1 to activate RetGC1.

CD spectra

CD spectra were obtained on APP π^* , Aviv 62DS and Aviv 202SF CD Spectrometers using pathlengths of 1 mm over a wavelength range of 180–300 nm. Temperature control in the cells was achieved using computer controlled circulating water baths, a manual controlled water bath and Peltier cell holders, respectively. Thermal equilibrium was ensured before measurements at each temperature increment were recorded. Dynode voltages were assessed over the first and last scans and kept below 500 V.

Protease treatment of GCAPs

GCAP1 samples were treated with L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, at a molar ratio of 1000:1, at room temperature. Aliquots were removed at 1, 15, 30, 60 and 90 min and the reaction was stopped by placing the sample in a boiling water bath for 5 min. Samples were resuspended in SDS-PAGE loading buffer and applied to 17.5% polyacrylamide gels run on a BioRad Mini-Protean II gel rig or Pharmacia Phast-Gel 8–25% gradient gels. Gels were stained with Coomassie Blue stain and the degree of degradation was assessed by eye. Samples were digested in the absence of calcium and in buffer supplemented with 1 mM CaCl₂.

Alternatively, for assessment of the effect of trypsinization on ability to activate RetGC1, GCAP1 samples were treated with trypsin (100:1) and the digestion terminated by the addition of the serine protease inhibitor PMSF. Assay of RetGC1 activity was then carried out as described above.

Pre-assay heat treatment of GCAPs

GCAP1 samples were heat treated for 5 min at 50, 70 or 90°C before being used in the assay as normal. Control samples were retained at ~0°C during the 5 min incubation rather than being heat-treated. The long-term thermal stability of GCAP1 samples was also analysed by pre-incubation at 37, 50 or 70°C for time periods of 15, 30 or 60 min.

ACKNOWLEDGEMENTS

We would like to thank Prof. J. Gordon (Department of Molecular Biology and Pharmacology, Washington University, St Louis, MO) for the generous gift of the pBB131 plasmid, Prof. J.B. Hurley (Department of Biochemistry, University of Washington, Seattle, WA) for the RetGC1 vector, Dr Dieter Becker (Applied Photophysics) for the CD spectra on the APP π^* CD spectrometer, Dr Bonnie Wallace (Birkbeck College, London, UK) for help with acquiring CD spectra on the Aviv 62DS CD Spectrometer, Dr Ronan O'Brien (University College, London, UK) for assistance in acquiring CD spectra on the Aviv 202SF CD spectrometer and the School of Pharmacy, University of London, Mass Spectrometry Service for running the GCAP1 samples on their MALDI-TOF mass spectrometer. R.J.N., E.C.R., C.E.W. and S.E.W. are supported by the Wellcome Trust (Project Grant no. 055252). N.S. is supported by a Senior Research Fellowship from the Wellcome Trust.

REFERENCES

1. Stryer, L. (1991) Visual excitation and recovery. *J. Biol. Chem.*, **266**, 10711–10714.
2. Kaupp, U.B. and Koch, K.-W. (1992) Role of cGMP and Ca²⁺ in vertebrate photoreceptor excitation and adaptation. *Annu. Rev. Physiol.*, **54**, 153–175.
3. Semple-Rowland, S.L., Lee, N.R., van Hooser, J.P., Palczewski, K. and Baehr, W. (1998) A null mutation in the photoreceptor guanylate cyclase gene causes the retinal degeneration chicken phenotype. *Proc. Natl Acad. Sci. USA*, **95**, 1271–1276.
4. Gorczyca, W.A., Polans, A.S., Surgecheva, I.G., Subbaraya, I., Baehr, W. and Palczewski, K. (1995) Guanylate cyclase activating protein, a calcium sensitive regulator of phototransduction. *J. Biol. Chem.*, **270**, 22029–22036.
5. Ikura, M. (1996) Calcium binding and conformational response in EF-hand proteins. *Trends Biol. Sci.*, **1**, 14–17.
6. Lowe, D.G., Dizhoor, A.M., Liu, K., Gu, Q., Spencer, M., Laura, R., Lu, L. and Hurley, J.B. (1995) Cloning and expression of a second photoreceptor-specific membrane retina guanylyl cyclase (RetGC), RetGC-2. *Proc. Natl Acad. Sci. USA*, **92**, 5535–5539.
7. Flaherty, K.M., Zozulya, S., Stryer, L. and Mackay, D.B. (1993) Three dimensional structure of recoverin, a calcium sensor in vision. *Cell*, **75**, 709–716.
8. Tanaka, T., Ames, J.B., Harvey, T.S., Stryer, L. and Ikura, M. (1995) Sequestration of the membrane targeting myristoyl group of recoverin in the calcium-free state. *Nature*, **376**, 444–447.
9. Ames, J.B., Ishima, R., Tanaka, T., Gordon, J.I., Stryer, L. and Ikura, M. (1997) Molecular mechanics of calcium-myristoyl switches. *Nature*, **389**, 198–202.
10. Ames, J.B., Dizhoor, A.M., Ikura, M., Palczewski, K. and Stryer, L. (1999) Three-dimensional structure of guanylate cyclase activating protein-2, a calcium sensitive modulator of photoreceptor guanylyl cyclases. *J. Biol. Chem.*, **274**, 19329–19337.
11. Frins, S., Bonigk, W., Muller, F., Kellner, R., and Koch, K.-W. (1996) Functional characterization of a guanylyl cyclase-activating protein from vertebrate rods. *J. Biol. Chem.*, **271**, 8022–8027.
12. Payne, A.M., Downes, S.M., Beasant, D.A.R., Taylor, R., Holder, G.E., Warren, M.J., Bird, A.C. and Bhattacharya, S.S. (1998) A mutation in guanylate cyclase activator 1A (GUCA1A) in an autosomal dominant cone dystrophy pedigree mapping to a new locus on chromosome 6p21.1. *Hum. Mol. Genet.*, **7**, 273–277.
13. Sokal, I., Li, N., Surgucheva, I., Warren, M.J., Payne, A.M., Bhattacharya, S.S., Baehr, W., Palczewski, K. (1998) GCAP1(Y99C) mutant is constitutively active in autosomal dominant cone dystrophy. *Mol. Cell*, **2**, 129–133.
14. Dizhoor, A.M., Boikov, S.G. and Olshevskaya, E.V. (1998) Constitutive activation of photoreceptor guanylate cyclase by Y99C mutant of GCAP-1. *J. Biol. Chem.*, **273**, 17311–17314.
15. Duronio, R.J., Rudnick, D.A., Johnson, R.L., Linder, M.E. and Gordon, J.I. (1990) Reconstitution of protein N-myristoylation in *Escherichia coli*. *Methods*, **1**, 253–263.
16. Rocque, W.J., McWherter, C.A., Wood D.C. and Gordon, J.I. (1993) A comparative analysis of the kinetic mechanism and peptide substrate specificity of human and *Saccharomyces cerevisiae* myristoyl-CoA: protein N-myristoyltransferase. *J. Biol. Chem.*, **268**, 9964–9971.
17. Potter, L.R. and Garbers, D.L. (1992) Dephosphorylation of the guanylyl cyclase—a receptor causes desensitization. *J. Biol. Chem.*, **267**, 14531–14534.
18. Rudincka-Nawrot, M., Surgucheva, I., Hulmes, J.D., Haeseleer, F., Sokal, I., Crabb, J.W., Baehr, W. and Palczewski, K. (1998) Changes in biological activity and folding of guanylate cyclase-activating protein 1 as a function of calcium. *Biochemistry*, **37**, 248–257.
19. Weleber, R.G. and Eisner, A. (1988) Cone degeneration (Bull's eye dystrophies) and colour vision defects. In Newsome, D.A. (ed.), *Retinal Dystrophies and Degenerations*. Raven Press, New York, NY, pp. 233–256.
20. Downes, S.M., Holder, G.E., Fitzke, F.W., Payne A.M., Warren, M.J., Bhattacharya, S.S. and Bird, A.C. (2000) Autosomal dominant cone and cone-rod dystrophy with mutations in the GUCA1A gene encoding guanylate cyclase activating protein-1. *Arch. Ophthalmol.*, in press.
21. Kawamura, S. (1999) Calcium-dependent regulation of rhodopsin phosphorylation. *Novartis Found. Symp.*, **224**, 208–218.
22. Fersht, A.R. (1999) Protein stability. In *Structure and Mechanism in Protein Science*. W.H. Freeman, New York, NY, pp. 508–539.
23. Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, **281**, 1309–1312.
24. Laura, R.P., Dizhoor, A.M. and Hurley, J.B. (1996) The membrane guanylyl cyclase, retinal guanylyl cyclase-1, is activated through its intracellular domain. *J. Biol. Chem.*, **271**, 11646–11651.
25. Tsien, R. and Pozzan, T. (1989) Measurement of cytosolic free Ca²⁺ with quin2. *Methods Enzymol.*, **172**, 230–262.
26. Evans, S.V. (1993) SETOR: hardware-lighted three-dimensional solid model representations of macromolecules. *J. Mol. Graph.*, **11**, 134–138.