Identification of the cyclic-nucleotide-binding domain as a conserved determinant of ion-channel cell-surface localization

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Summary

Mutations of a putative cyclic-nucleotide-binding domain (CNBD) can disrupt the function of the hyperpolarization-activated cyclic-nucleotide-gated channel (HCN2) and the human ether-a-go-go-related gene potassium channel (HERG). Loss of function caused by C-terminal truncation, which includes all or part of the CNBD in HCN and HERG, has been related to abnormal channel trafficking. Similar defects have been reported for several of the missense mutations of HERG associated with long QT syndrome type 2 (LQT2). Thus, we postulate that normal processing of these channels depends upon the presence of the CNBD. Here, we show that removal of the entire CNBD prevents Golgi transit, surface localization and function of HERG channel tetramers. This is also true when any of the structural motifs of the CNBD is deleted, suggesting that deletion of any highly conserved region along the entire length of the CNBD can disrupt channel trafficking. Furthermore, we demonstrate that defective trafficking is a consequence of all LQT2 mutations in the CNBD, including two mutations not previously assessed and two others for which there are conflicting results in the literature. The trafficking sensitivity of the CNBD might be of general significance for other ion channels because complete deletion of the CNBD or mutations at highly conserved residues within the CNBD of the related ERG3 channel and HCN2 also prevent Golgi transit. These results broadly implicate the CNBD in ion-channel trafficking that accounts for the commonly observed loss of function associated with CNBD mutants and provides a rationale for distinct genetic disorders.

Key words: Cyclic-nucleotide-binding domain, Hyperpolarization-activated cyclic-nucleotide-modulated channel, Human ether-a-go-go-related gene, Long QT syndrome

Introduction

The cyclic-nucleotide-binding domain (CNBD) exists in diverse ion-channel families including the ether-a-go-go (EAG) family (Warmke and Ganetzy, 1994), the hyperpolarization-activated cyclic-nucleotide-modulated (HCN) family (Robinson and Siegelbaum, 2003), the cyclic-nucleotide-gated (CNG) family (Zagotta and Siegelbaum, 1996) and some plant channels (Anderson et al., 1992; Sentenca et al., 1992). In general, ion channels that harbor a CNBD are directly modulated by cAMP and/or cGMP, independent of channel phosphorylation (for reviews, see Zagotta and Siegelbaum, 1996; Robinson and Siegelbaum, 2003). Previous studies have reported that mutations within the CNBD, or C-terminal truncations that encompass the CNBD, disrupt ion-channel function (Marten and Hoshi, 1997; Scott et al., 2000; Shapiro and Zagotta, 2000; Matulef and Zagotta, 2002; Mazzolini et al., 2002; Stieber et al., 2003). Although some mutations might prevent the binding of endogenous cyclic nucleotides, many of these mutations are located outside the nucleotide-binding pocket, suggesting that they cause dysfunction through some other mechanism. Recently, Proenza et al. (Proenza et al., 2002a; Proenza et al., 2002b) have provided experimental evidence for and discuss the idea that mutations in the CNBD disrupt the trafficking of HCN2 to the plasma membrane.

The human ether-a-go-go-related gene HERG encodes a CNBD-containing potassium channel involved in the repolarization of cardiac action potential (Sanguinetti et al., 1995). Abnormal HERG function is a mechanism for one form of the long QT syndrome, LQT2, a cardiac disorder associated with ventricular arrhythmias and sudden death (Curran et al., 1995; Sanguinetti et al., 1995). Loss of HERG function has been reported for LQT2 missense mutations (Satler et al., 1996) or truncations (Kupershmidt et al., 1998; Aydar and Palmer, 2001) in the C-terminus of HERG. Zhou et al. (Zhou et al., 1998) provide evidence that the LQT2 mutant V822M lacks functional channel expression because of improper trafficking. By contrast, Cui et al. (Cui et al., 2001) reported that V822M and another LQT2 mutant that fall in the C-terminus of HERG (R823W) exhibit cell-surface localization. However, Ficker et al. (Ficker et al., 2002) provide evidence that the C-terminal LQT2 mutants R823W and F805C are misprocessed and improperly trafficked. This is consistent with reports by Ficker et al. (Ficker et al., 2000) and Akhavan et al. (Akhaven et al., 2003) that LQT2 mutants R752W and N861I, both of which are within the C-terminus of HERG, are trafficking defective. These results and those of Ficker et al.
The generation of Myc- and hemagglutinin (HA)-tagged HERG has been previously described (Akhavan et al., 2003). All quantifications are averages ± standard deviation. A Student’s t-test was used for statistical analysis. Significance was indicated at *P < 0.05, **P < 0.01 or ***P < 0.001.

Cell biology and biochemistry

All biochemical experiments were performed in HEK-293T cells unless otherwise indicated. HEK, PC-12, M2, COS-7 and HL-1 mouse cardiac-muscle cells were maintained as previously described (Akhavan et al., 2003). Transfections were performed using Lipofectamine (Invitrogen). Proteasomal inhibitors and tunicamycin treatments were performed 1 day after transfection. Cells in culture were exposed to dimethylsulfoxide (100 μM), lactacystin (50 μM), ALLN (N-acetyl-leucinal-leucinal-norleucinal; 100 μM), MG132 (GBZ-Leu-Leu-Leucinol; 50 μM) or tunicamycin (5 μg ml−1) for 6-8 hours and cell lysates were analysed by immunoblotting. For Fig. 2A, cells were lysed in 2× sample buffer and, in Fig. 5A, cell lysates were subjected to high-speed centrifugation (150,000 g, 45 minutes). In all other cases, cell lysates were prepared using mild detergent conditions and insoluble material was sedimented at 16,000 g for 30 minutes (Akhavan et al., 2003). Membranes were probed with anti-HA antibodies unless otherwise indicated. Double blots were performed by simultaneous incubation of membranes with two antibodies. In all experiments, immunoblots shown on the same membrane were subject to identical conditions and all treatments were performed using master mix solutions.

Electrophysiological recordings were performed in M2 cells, which are easily amenable to patch clamp analysis. Briefly, cells were cotransfected with surface antigen CD8 to allow the selection of transfected cells with immunomagnetic Dynabeads precoated with a monoclonal anti-CD8 antibody (Dynal Biotech, Brown Deer, WI). Voltage-clamp protocols were imposed from a holding potential of −80 mV with a 4 second depolarizing pulse to potentials between −60 mV and +50 mV in increments of 10 mV.

For immunolocalization, HEK293 cells transfected on glass cover slips coated with poly-L-lysine (Sigma) were fixed in paraformaldehyde and permeabilized using 0.1% Triton X-100. specificity antibody binding was blocked with 10% goat serum followed by incubation with monoclonal anti-HA antibodies for 1 hour. The coverslips were then washed and incubated with Oregon-Green/Cy3-conjugated secondary antibody for 45 minutes. After extensive washing with PBS, the coverslips were mounted onto glass slides using ImmunolFloures Mounting Medium (ICN Biomedicals). Images were analysed using a Bio-Rad Microradiance confocal laser-scanning microscope mounted on a Zeiss 200M inverted microscope.

Proteinase K (PK) experiments were performed 1 day after transfection on 100% confluent cells. After PK treatment for 20-30 minutes at 37°C, cells were lifted as a sheet and placed in PK-blocking solution containing 10 mM HEPES, 25 mM EDTA, 20 mM Pefablock SC (Roche). In all cases, PK quantification was performed relative to the CASK (calcium/calmodulin-dependent serine protein kinase) signal.

For pulse-chase experiments, cells were labeled with 200 μCi ml−1 [35S]methionine/cysteine (Perkin-Elmer Life Sciences) for 1 hour and chased with Dulbecco’s modified Eagle’s medium containing 2 mM unlabeled methionine and cysteine. Equal amounts of protein were immunoprecipitated with anti-HA antibody, subjected to SDS-PAGE and visualized by autoradiography.

Succrose-gradient analysis was performed on cell lysates subjected to high-speed centrifugation (150,000 g, 45 minutes). The supernatants were layered on top of a 5-40% continuous sucrose-density gradient. Molecular-weight protein standards (alcohol dehydrogenase and thyroglobulin, 150 kDa and 669 kDa, respectively) were layered on a separate 5-40% sucrose gradient. Samples were centrifuged for 16 hours at 220,000 g (4°C) and fractions were collected and analysed as previously described (Manganas et al., 2001; Akhavan et al., 2003). Densitometric analysis was used for quantification of western blots as previously described (Akhavan et al., 2003). All quantifications are based on four to 11 independent experiments. The means and standard errors were calculated, and the statistical significance of the observed differences was determined using the Student’s t-test.

Sequence alignment analysis was performed using free software...
provided by the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw/).

**Results**

Disruption of CNBD underlies mistrafficking of C-terminal HERG mutants

In order to determine which LQT2 missense mutations fall within the putative CNBD, we first aligned the CNBD of HERG with those of HCN2, CAP, ERG3 and the plant KAT1 channel (Fig. 1). According to this alignment, the putative CNBD of HERG extends from residue 750 to residue 870 and encompasses seven identified C-terminal HERG LQT2 missense mutations, including R752W, F805C, F805S, S818L, V822M, R823W and N861I (Fig. 1).

If processing of HERG is sensitive to mutations throughout the entire CNBD then it might be expected that all CNBD missense mutants would fail to produce functional channels as a result of defective trafficking to the cell surface. Although this was first proposed for HERGV822M (Zhou et al., 1998), a more recent report claims that HERGV822M is normally processed and trafficked to the surface membrane (Cui et al., 2003). We therefore examined all LQT2 mutations of HERG, beginning with a detailed reevaluation of V822M. To evaluate the maturation of HERG, we used a western blot to identify a 135 kDa immature core glycosylated endoplasmic reticulum (ER) form (Fig. 2A, lower band) and a 155-kDa mature Golgi-processed EndoH-insensitive form (Fig. 2A, upper band) (Zhou et al., 1998). Previously, it has been shown that the 155 kDa fraction is sensitive to PK applied extracellularly, indicating that this band represents the species localized to the plasma membrane (Zhou et al., 1998; Akhavan et al., 2003). Our results show a single band of 135 kDa for HERGV822M, similar to that found by Zhou et al. (Zhou et al., 1998), indicating abnormal processing (Fig. 2A). The degradation of wild-type HERG (HERGwt) and HERGV822M do not seem to follow the proteasome pathway, because both are relatively insensitive to proteasomal inhibitors. We further confirm the results of Zhou et al. (Zhou et al., 1998) by showing that the single band produced in V822M is insensitive to PK treatment; the PK efficacy on CASK (an intracellular protein) and N-cadherin (N-Cad; a cell-surface protein) served as negative and positive controls, respectively (Fig. 2B). The absence of surface localization of the HERGV822M mutant was corroborated using the patch-clamp technique, which showed that the HERGV822M mutant generates currents that are similar in magnitude and kinetics to endogenous currents (Fig. 2C, Fig. 3C), indicating a lack of functional channels. To illustrate further that residue V822 of HERG is important for channel trafficking, a non-conservative substitution (V822P) was introduced (Bordo and Argos, 1991). As shown in Fig. 2A, the HERGV822P mutant has an exacerbated trafficking-deficient phenotype with significantly decreased levels of channel production (Fig. 2A, lanes 2,6,10). Moreover, HERGV822P was sensitive to a range of proteasome inhibitors that cause a significant accumulation of a 58 kDa species (Fig. 2A, lanes 10-13). One possibility is that HERGV822P is first degraded by a non-proteasomal protease to generate an N-terminal fragment of 58 kDa that is subsequently cleared by the proteasomal complex. These findings suggest that the properties of the amino acid at residue 822 can substantially modulate the production level and degradation pathway of HERG channels.

We next tested the consequence of other known LQT2 missense mutations that fall within the putative CNBD. We found that all of these mutants generate only the immature species (Fig. 3A, lanes 2,3,5,7), which is of particular significance because this represents the first demonstration that HERG750S and HERG818L are nonfunctional owing to retention in the ER. These results also show that HERG823W is not normally processed, consistent with the report of Ficker et al. (Ficker et al., 2002) and contrary to Cui et al. (Cui et al., 2001). The normal trafficking phenotype can, however, be rescued by introducing a conservative mutation for R823 (HERG823R) (Bordo and Argos, 1991), as indicated by the appearance of a mature PK-sensitive HERG823R band (Fig. 3A,B). This observation is similar to that previously reported for HERGwt (Akhavan et al., 2003) and serves as a positive control. Furthermore, all LQT2 missense mutants that fall in the CNBD display currents similar to those found in mock-
transfected cells, demonstrating that the absence of cell-surface localization is reflected by a lack of HERG current (Fig. 3C). However, consistent with its cell-surface localization, the HERGR823K mutant was functional with similar biophysical properties to that of HERGwt (data not shown).

Disruption or deletion of the CNBD abolishes HERG trafficking

Previously, we reported that residues 860-899 of HERG (Fig. 4A, downward arrows) constitute a crucial trafficking segment (Akhavan et al., 2003). In view of the alignment of the CNBD presented above, ER retention associated with deletion of this segment might be a consequence of a disruption of the CNBD. To test this possibility, we generated a series of truncation constructs that incorporated a progressively larger fraction of the CNBD as indicated in Fig. 4A. Truncations extending to the terminal border of the CNBD at residue 870 (HERG870X) showed the presence of the slower-migrating band (Fig. 4B, lane 2) that was sensitive to PNGase (peptide N-glycanase) glycosidase digestion but not EndoH (Fig. 4C). All truncations extending to residue 860 (HERG860X) and beyond caused a loss of this band leaving only the more rapidly migrating band. This is consistent with the notion that truncations extending into the CNBD disrupt channel trafficking. To rule out the possibility that the trafficking-sensitive portion of the CNBD is relegated only to the 860-870 segment, we deleted all of the CNBD except for these residues (HERGΔ750-860). As shown in Fig. 4D, the deletion construct only generates a single rapidly migrating species. Taken together, these results indicate that HERG trafficking can be disrupted by deletion of multiple regions of the CNBD. To test this notion further, we systematically deleted each secondary structural motif of HERG, as predicted from the alignment in Fig. 1. As shown in Fig. 4E, deletion of any of the three α helices or eight β sheets resulted in ER retention of the ion channel.

Disrupted trafficking associated with mutations in the CNBD does not provide direct evidence that this domain is indeed crucial for trafficking. These mutations might simply cause misfolding of the CNBD and hence ER retention (for a review, see Ellgaard and Helenius, 2003), a promiscuous property that could apply to any other complex structural module. To assess whether the CNBD is a specific determinant of trafficking, we analysed the functional and biochemical phenotypes associated with the complete deletion of the putative CNBD (HERGΔ750-870). As shown in Fig. 5A, HERGΔ750-870 generates only the more rapidly migrating HERG species when produced in HEK cells. This was also true for cardiac HL-1 cells (Claycomb et al., 1998), neuronal PC12 cells and Cos-7 cells, (Fig. 5B), indicating that the abnormalities associated with deletion of the CNBD do not depend upon the specific cellular environment. The trafficking abnormalities were not rescued when MiRP, the putative auxiliary subunit of HERG (Abbott et al., 1999), was co-transfected with the mutant channels (not shown). In addition, we did not observe any change in trafficking of mutant HERGΔ750-870 at permissive temperatures known to rescue misfolded proteins (not shown). HERGΔ750-870 shows a perinuclear distribution associated with ER retention (Fig. 5C), is resistant to PK cleavage (Fig. 5D) and is nonfunctional (Fig. 3C), further confirming the lack of surface localization.

To test whether abnormal trafficking is a general consequence of any domain deletion, we analysed the effect of removing the PAS domain (HERGΔ26-135) from the N-terminus.
of HERG. HERGA26-135 generates two prominent bands (Fig. 5E), indicating that removal of this domain does not prevent ER exit.

CNBD is crucial for trafficking of other channels

**Trafficking of ERG3**

ERG3 is a CNBD-containing isoform of HERG (Shi et al., 1997) whose biochemical properties have not been previously characterized. Here, we demonstrate that recombinant wild-type ERG3 (ERG3wt) produced in HEK cells generates two prominent bands (Fig. 6A). To determine whether ERG3wt is a glycoprotein, we tested the sensitivity of the recombinant protein to glycosidase digestion. As shown in Fig. 6A, PNGase treatment resulted in a shift of both immunoreactive species, whereas EndoH shifted only the more rapidly migrating band. In addition, tunicamycin treatment of cells producing ERG3wt inhibited the generation of the more-slowly migrating form of ERG3wt (Fig. 6A). Pulse-chase experiments (Fig. 6B) demonstrate that ERG3wt is initially synthesized as a single immunoreactive species with a molecular weight corresponding to that of the immature protein (Fig. 6B, see chase times 0 and 0.5). Progressively longer chase times show that the disappearance of the lower molecular mass band is directly related to the appearance of the higher-molecular-mass band. Taken together, these results demonstrate that ERG3wt is indeed a glycoprotein that acquires differential glycosylation in the ER and Golgi.

The alignment of the putative CNBD of ERG3 is shown in Fig. 1 along with the highly conserved CNBD residues at V825, R826 and P849 of ERG3; V825 and R826 can be seen to correspond with the HERG missense residues V822M and R823W, respectively. Introducing corresponding missense mutations at V825 (ERG3V825M) and R826 (ERG3R826W) of ERG3 resulted in the disappearance of the more slowly migrating species (Fig. 6C). A similar result was found for ERG3P849M. The remaining more rapidly migrating band was EndoH sensitive, indicating ER retention. Furthermore, we demonstrate that, when the entire CNBD of ERG3 was deleted (ERG3A753-873), only an EndoH-sensitive band was generated (Fig. 6C), indicating that this band constitutes the immature species. By contrast, deletion of the PAS domain does not prevent ER exit of ERG3 (not shown).

**Trafficking of HCN2**

The HERG and ERG3 channels are closely related and so we decided to investigate the role of the CNBD in a distinct channel, HCN2. We confirmed that HCN2wt is a glycoprotein with a SDS-PAGE migration pattern associated with ER and Golgi glycosylation (not shown) (Much et al., 2003). As shown in Fig. 7A, the slower migrating band of HCN2wt is sensitive to PK in a dose-dependent manner, whereas the more rapidly migrating species is not. This result indicates that the higher-molecular-mass form is the mature species processed in the Golgi and localized to the cell surface. It has been reported that the αg region of CNBD is crucial for HCN2 trafficking to the plasma membrane (Proenza et al., 2002b). Our analysis with HERG channel indicates that multiple segments throughout the entire CNBD are important for normal trafficking. Consequently, we tested the effect of missense mutations at the highly conserved residues G560 (HCN2G560W) and P619 (HCN2P619M) of HCN2 that fall within the CNBD but outside the αg region (gray arrows in Fig. 1). As shown in Fig. 7B, in both cases, the mutations result in the disappearance of the more slowly migrating species. A similar result was obtained with deletion of the entire CNBD (HCN2A425-644) (Fig. 7B).

Abnormal assembly is a common cause of ER retention of multimeric proteins (for a review, see Ellgaard and Helenius, 2003) such as ion channels (for a review, see Papazian, 1999). To test this possibility, we analysed the sucrose-sedimentation profile of recombinant channels produced in HEK cells. Cells...
expressing HERGwt and HCN2wt as well as the corresponding CNBD deletion mutants were lysed using mild detergent conditions (0.5% NP40) and the soluble extracts were analysed on nondenaturing continuous sucrose gradients. As shown in Fig. 7C, most HERGwt channels sediment at a sucrose density close to the 669 kDa molecular-weight marker. This sedimentation profile is consistent with the molecular weight of tetrameric HERG (~540 kDa). We found a similar sedimentation profile and peak immunoreactivity for the HERGΔ750-860 mutant (~490 kDa). In a similar fashion, HCN2wt (~380 kDa) and HCN2Δ525-644 (~330 kDa) channels show similar peaks and sedimentation profiles (Fig. 7D). These findings indicate that the absence of CNBD does not preclude tetramer formation by either HERG or HCN2.

Discussion
In this study, we show that all LQT2 missense mutations that fall within the putative CNBD of HERG cause intracellular retention. In the course of this study, a novel mutation was reported that creates a stop codon at residue 863 of HERG (Teng et al., 2004). According to our alignment, this mutation eliminates part of the CNBD and is probably represented by our HERG 863X mutant (Fig. 4B, lane 3), which also fails to form functional channels (Teng et al., 2004), suggesting lack of surface localization. This finding is consistent with several previous studies (Zhou et al., 1998; Ficker et al., 2000; Ficker et al., 2002; Deslisle et al., 2003) and suggests that the putative CNBD is important for HERG trafficking. By analysing the effect of many truncations and deletions as well as complete domain removal (ΔCNBD), we provide direct evidence that an intact CNBD is indispensable for ER exit of HERG channels.

We extended our analysis to other channel types and showed that ERG3wt and HCN2wt also require an intact CNBD, broadly implicating this domain in channel trafficking. Our findings are consistent with those of Proenza et al. (Proenza et al., 2002b), 

Fig. 4. Truncation of the CNBD results in ER retention. (A) Schematic representation of HERG with six transmembrane segments (S1-S6). Downward arrow indicate residues of HERG corresponding to the putative CNBD, PAS domain (black arrows) and a previously identified segment involved in channel trafficking (gray arrows). HERG truncated constructs analysed in this study are indicated by the upward arrows. Maturation state corresponding to individual mutants is indicated as X (defective trafficking) or √ (normal trafficking). (B) Immunoblots of truncation constructs co-expressed with green fluorescent protein (GFP) to compare the transfection efficiency. Transiently transfected cells were lysed and equal amounts of solubilized protein were subjected to SDS-PAGE. The top membrane was probed with an anti-Myc antibody, stripped and reprobed for CASK to compare loading amounts (indicated as 15 µg and 30 µg). The bottom portion of the same membrane was probed for GFP. (C) Cells producing HERG870X were lysed and subjected to overnight treatment with EndoH and PNGase. Treated (+) and untreated (−) cell lysates were separated on SDS-PAGE and subjected to immunoblotting with an anti-Myc antibody (α-Myc). The arrow indicates the position of the mature band (M). (D) Cells transfected with the cDNAs encoding HERG3wt and HERGΔ750-860 were lysed 1 day after transfection and treated with EndoH (+) or untreated (−). Equal amounts of solubilized proteins were subjected to immunoblotting with an anti-HA antibody. (E) Cells producing different mutant constructs were co-transfected with GFP, lysed and subjected to immunoblotting. (top) The membrane was probed with anti-HA and anti-CASK antibodies. (bottom) The same membrane was probed with an anti-GFP antibody. The arrow indicates the position of CASK signal at the expected molecular weight.
who reported that the αB of the CNBD is indispensable for cell-surface localization and function of HCN2 channels (Proenza et al., 2002b). Moreover, the analysis of a point mutation upstream of αB in HCN2 argues that multiple regions within the CNBD can also be important for its trafficking.

Our results suggest that channel misassembly is not the cause of exit block of ∆CNBD and that ER retention is the likely mechanism associated with lack of surface localization of HERG and HCN2 mutants. This is consistent with the observation that a mutant of HCN2 that lacks the CNBD is able to form heteromers with wild-type HCN1 (Proenza et al., 2002b). However, this does not provide direct evidence that the channels are folded properly. In the case of Shaker potassium channels, channel assembly does not require prefolded monomers (Schulteis et al., 1998). Thus, it is fully conceivable that ∆CNBD mutants are misfolded channels that nonetheless can form tetramers. Alternatively, it is possible that the CNBD might function as an ER export signal without affecting overall structure and/or assembly of the ion channel (Ma et al., 2001).

Defective trafficking associated with some LQT2 mutations in the putative CNBD of HERG (Ficker et al., 2000; Ficker et al., 2002) can be rescued by lowering temperature, ostensibly by stabilizing misfolded proteins (for a review, see Kopito, 1999). This suggests that these mutations cause structural defects that, in turn, lead to ER retention. However, we did not notice any change in the trafficking of ∆CNBD HERG (HERG ∆750-870) at permissive temperatures. A similar finding has been reported for an HCN2 mutant channel harboring a truncation that completely eliminates the CNBD (Proenza et al., 2002a). Thus, different mechanisms might account for
mistracking of channels that contain mutations within the CNBD versus those that completely lack a CNBD. Alternatively, complete removal of the CNBD could result in gross structural changes that are not amenable to rescue at permissive temperatures.

Regardless of the mechanism involved, it seems that the trafficking of channels is particularly sensitive to mutations in the CNBD. Is this a unique feature of CNBD or will other highly structured domains have similar effects? It has been recently shown that a mutation in the PAS domain causes ER retention (Paulussen et al., 2002). However, it has also been reported that other mutations in the PAS domain or N-terminal truncations that eliminate this domain do not always preclude generation of functional HERG channels (Morais Cabral et al., 1998; Chen et al., 1999; Wang et al., 1998; Viloria et al., 2000; Gomez-Varela et al., 2002). Our results indicate that complete deletion of PAS is well tolerated for proper trafficking (Fig. 5E). These observations suggest that the CNBD has a more profound effect upon ion channel processing and trafficking than other highly structured modules. With this in mind, it will be very interesting to test whether binding of nucleotides is a prerequisite for proper folding and ER exit of CNBD-harboring ion channels. The accessibility of nascent proteins to the cytoplasmic environment (Hegde and Lingappa, 1996) and the involvement of trans-acting factors (Hedge et al., 1998) are becoming recognized as crucial criteria for early steps of protein biogenesis at the ER. Based on these observations, it is entirely possible that the CNBD is engaged with the cytosolic cyclic nucleotides in the vicinity of the ER membrane, which might promote proper folding of nascent channels. Consistent with this view, misfolded proteins that are retained in the ER are often rescued by specific ligands (Rajamani et al., 2002).

A conserved proline in the CNBD of ERG3, HCN2 and HERG (Fig. 1) at residues 849, 619 and 846, respectively is required for normal trafficking of these channels. We found that mutation of the conserved proline to methionine in the CNBD of ERG3 (ERG3P849M) and HCN2 (HCN2P619M) led to ER retention and an absence of cell-surface expression of these channels. Interestingly, mutation of the proline of HCN2 to an alanine does not affect the functional expression of the HCN2 channel (Zahynacz et al., 2003). This might suggest that alanine and proline can be "safely" substituted (Bordo and Argos, 1991), in a fashion analogous to the observation that HERGR823W results in ER retention, whereas the conservative mutant HERGR823K restores ER exit (Fig. 3A).

Many mutations that cause hereditary cone-photoreceptor disorders or achromatopsia fall within the CNBD of distinct CNG subunits (Kohl et al., 1998; Sundin et al., 2000; Wissinger et al., 2001). Considering the role of this domain in channel gating, it has been assumed that these mutations result in permanent closure of channels owing to their inability to bind cyclic nucleotides (Kohl et al., 1998). However, based on our findings, we speculate that some of these mutants might be associated with an absence of channels from the plasma membrane. Furthermore, we propose that abnormal trafficking caused by mutations in the putative CNBD might underlie diverse genetic ion channel disorders.

Fig. 7. CNBD is required for ER exit but not tetramer assembly. (A) Cells producing HERGWT were treated with PK (+) or left untreated (−) and subjected to immunoblotting with an anti-Myc antibody. PK concentrations are shown on top of each lane. Cells producing HCN2 mutant variants were lysed and subjected to immunoblotting with an anti-Myc antibody. (C) Cells producing HERGWT and HERG∆750-870 were lysed and fractionated on linear non-denaturing sucrose gradients, and samples from the indicated fractions were subjected to immunoblot analysis. Pixel intensities of signals were measured and normalized to the maximum signal intensity. HERGWT and HERG∆750-870 are indicated by ■ and □, respectively. The position of markers is shown by arrows (alcohol dehydrogenase and thyroglobulin, 150 kDa and 669 kDa, respectively). (D) Sucrose-gradient analysis of HCN2 as described for HERG in C.
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