Different Roles for the Cyclic Nucleotide Binding Domain and Amino Terminus in Assembly and Expression of Hyperpolarization-activated, Cyclic Nucleotide-gated Channels*

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In mammalian heart and brain, pacemaker currents are produced by hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels, which probably exist as heteromeric assemblies of different subunit isoforms. To investigate the molecular domains that participate in assembly and membrane trafficking of HCN channels, we have used the yeast two-hybrid system, patch clamp electrophysiology, and confocal microscopy. We show here that the N termini of the HCN1 and HCN2 isoforms interacted and were essential for expression of functional homo- or heteromeric channels on the plasma membrane of Chinese hamster ovary cells. We also show that the cyclic nucleotide binding domain (CNBD) of HCN2 was required for the expression of functional homomeric channels. This expression was dependent on a 12-amino acid domain corresponding to the B-helix in the CNBD of the catabolite activator protein. However, co-expression with HCN1 of an HCN2 deletion mutant lacking the CNBD rescued surface immunofluorescence and currents, indicating that a CNBD need not be present in each subunit of a heteromeric HCN channel. Furthermore, neither CNBDs nor other COOH-terminal domains of HCN1 and HCN2 interacted in yeast two-hybrid assays. Thus, interaction between NH2-terminal domains is important for HCN subunit assembly, whereas the CNBD is important for functional expression, but its absence from some subunits will still allow for the assembly of functional channels.

The hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels that produce Ih have been recently cloned (1–5). In addition to Ih, cloned HCN channels also produce a time-independent, instantaneous current (I\textsubscript{inst}), which is CAMP-sensitive and has a reversal potential similar to that of Ih (6). Based on their homology to the tetrameric voltage-gated potassium (Kv) channels and cyclic nucleotide-gated (CNG) channels, HCN channels are thought to be composed of four subunits, each having six transmembrane helices with cytoplasmic amino and carboxyl termini.

Four mammalian HCN isoforms (HCN1 to HCN4) have been cloned and heterologously expressed. The time-dependent (Ih) currents produced by these channels differ from each other mainly in their sensitivity to cAMP and rates of activation, with time constants for activation following the series HCN1 < HCN2 < HCN3 < HCN4 (2, 4, 5, 7). Native hyperpolarization-activated currents in brain and heart differ from the slowly activating currents produced by heterologously expressed homomeric HCN channels, and different subunit isoforms have overlapping expression patterns (8, 9). Thus, it is thought that native pacemaker currents may be produced by a combination of homo- and heteromeric channels. Indeed, the difference in activation rates between Ih produced by HCN1 and HCN2 has been used to demonstrate that these subunits can combine to form heteromeric HCN channels; co-expression of the two isoforms or expression of tandem dimers results in Ih with similar activation rates intermediate between those produced by homomeric HCN1 and HCN2 channels (10, 11).

HCN channels have a cyclic nucleotide binding domain (CNBD) in the COOH terminus. Direct binding of cAMP to the CNBD of native pacemaker channels in sino-atrial node myocytes (12) and to the CNBDs of the cloned HCN2 (4) and HCN4 (5) isoforms is thought to shift the voltage dependence of Ih activation to more positive potentials. The CNBDs of HCN channels are homologous to those of the related CNG channels and other cyclic nucleotide binding proteins, including the bacterial catabolite-activating protein for which the crystal structure has been solved (13, 14). This canonical CNBD is composed of three α-helices (A-, B-, and C-helix) and an eight-stranded anti-parallel β-barrel, which lies between the A-helix and the B-helix (14, 15). Although the sequence identity between the CNBDs of HCN or CNG channels and that of CAP is quite low (~20%), the predicted secondary structures are similar, and a structural model based on CAP has been used as a model for CNBDs of CNG channels (e.g. Refs. 16 and 17) and HCN channels (e.g. Refs. 18 and 19).

In other members of the six-transmembrane domain family of ion channels, the carboxyl and amino terminals are involved in subunit interactions and trafficking to the cell surface.
Domains Involved in Assembly and Expression of HCN Channels

Fig. 1. NH₂ termini of HCN1 and HCN2 interact in yeast two-hybrid assays. Shown are representative sections (~2 cm²) of nutritional selection medium (SD–Trp–Leu–His) plated with yeast strain AH109 that had been co-transformed with cDNA encoding the NH₂-terminal domains of HCN1 and HCN2 cloned in frame with the binding domain or activation domain of the GAL4 transcription factor. The yeast were incubated at 30 °C for 4–7 days. The presence of yeast colonies indicates an interaction between the fusion proteins. The numbers in parentheses indicate the number of positive interactions over the number of independent yeast transformations.

example, the NH₂ termini of K₁ channels have been shown to interact (20), to form tetramers in isolation from the rest of the channel (21, 22), and to play a role in assembly of channels (23, 24). COOH-terminal domains in K₁ channels (25, 26) and Ca²⁺ channels (27, 28) have been shown to be important for trafficking of the channels to the cell membrane. We have previously shown that deletion of the CNBD eliminated functional expression of HCN2 in CHO cells (6). This is similar to the loss of function observed upon deletion of CNBDs in the human ether-a-go-go-related (HERG) channel (29) and the plant KAT1 channel (30), which are similar to HCN channels.

In this paper, we have examined the roles of the NH₂ and COOH termini of HCN channels in subunit interactions and functional expression by using a combination of whole cell patch clamping, yeast two-hybrid assays, and confocal microscopy. We found that the NH₂-terminal domains of HCN1 and HCN2 interacted and were essential for expression of homo- or heteromeric channels on the cell surface. We also found that the putative B-helix of the CNBD was required for expression of homomeric HCN channels. However, no interactions were detected between CNBDs, and it was not necessary to have an intact CNBD in each subunit of a heteromeric HCN1–HCN2 channel for functional expression. These results are consistent with a role for the amino terminus in assembly of HCN channels and a role for the CNBD in trafficking of HCN channels to the plasma membrane.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assays—For yeast two-hybrid interaction tests, portions of HCN1 (1) and HCN2 (2) were inserted in-frame into both the GAL4 activation domain and binding domain plasmids, pGAD424 and pGBT9 (CLONTECH, Palo Alto, CA) by means of restriction sites added to the ends of the fragments generated by PCR. Inserts produced by PCR were confirmed by automated DNA sequencing. The following segments of HCN1 and HCN2 were assayed for interaction by expression in the yeast strain AH109; N terminus (HCN1 residues 1–129, HCN2 residues 1–182), COOH terminus (HCN1 387–910, HCN2 442–863), proximal COOH terminus (HCN1 387–471, HCN2 442–524), CNBD (HCN1 472–593, HCN2 525–646), and distal COOH terminus (HCN1 594–910, HCN2 647–863). Pooled yeast colonies expressing the fusion proteins were collected from plates containing synthetic medium lacking tryptophan and leucine (SD–Trp–Leu–His) and were then spread onto plates containing synthetic medium lacking tryptophan, leucine, and histidine (SD–Trp–Leu–His). The test plates were examined for the appearance of colonies after incubation for 3–7 days at 30 °C. Positive interactions were compared with a control interaction consisting of the interaction domains from the α and β subunits of the skeletal muscle L-type Ca²⁺ channel (27) and were defined as the appearance of colonies in >70% of the test transformations. Each interaction was tested in at least six independent transformations.

Mutagenesis and Expression—The NH₂-terminal deletion mutant, HCN2-ΔN, was constructed by replacing an EcoRI–AecI restriction fragment of the wild type channel with a PCR product lacking coding sequence for residues 2–182. The channels tagged with green fluorescent protein (GFP), GFP-HCN2 and GFP-HCN2-ΔN, were constructed by ligating coding sequence for wild type or mutant channels in-frame with sequence encoding a modified GFP in a mammalian expression plasmid (31), such that the GFP was expressed on the N terminus of the resulting fusion protein. COOH-terminal deletion mutants were constructed by engineering stop codons into the cDNA encoding mouse HCN2 (4) using overlapping PCR mutagenesis. The following COOH-terminal deletion mutants were constructed by replacing the codon indicated in parentheses with a stop codon: HCN2-ΔChelix (codon 620), HCN2-ΔBhelix (codon 608), HCN2-Δsheet (codon 533), and HCN2-ΔCNBD (codon 525). All constructs were confirmed by automated DNA sequencing (Biotechnology Laboratory, University of British Columbia). CHO-K1 cells were obtained from American Type Culture Collection (Manassas, VA), maintained in Ham’s F-12 medium supplemented with antibiotics and 10% fetal bovine serum, and incubated at 37 °C with 5% CO₂. Cells were plated onto glass coverslips in 35-mm dishes. One day after plating, mammalian expression vectors encoding wild type or mutant HCN channels (2 µg/dish) were transiently co-transfected into the cells along with the GFP reporter plasmid (0.3 µg/dish) using the FuGene6 transfection reagent (Roche Molecular Biochemicals). Expressing cells were identified by green fluorescence 24–48 h after transfection.

Electrophysiology—1–2 days following transfection, a shard of coverslip plated with cells was transferred to a recording chamber (~200-µl volume) and continually perfused (0.5–1.0 ml/min) with a low K⁺ extracellular solution (5.4 mM KCl, 135 mM NaCl, 0.5 mM MgCl₂, 1.9 mM CaCl₂, 5 mM HEPES, adjusted to pH 7.4 with NaOH). Following rupture of the patch membrane, the solution was changed to a high K⁺ recording solution (135 mM KCl, 5.4 mM NaCl, 0.5 mM MgCl₂, 1.9 mM CaCl₂, 5 mM HEPES, adjusted to pH 7.4 with KOH) to maximize current amplitude. The patch pipettes were filled with a solution containing 130 mM potassium aspartate, 10 mM NaCl, 0.5 mM MgCl₂, 5 mM HEPES, and 1 mM EGTA and adjusted to pH 7.4 with KOH, except in Fig. 4A, where 130 mM KCl instead of 130 mM potassium aspartate was used in the pipette solution. Whole cell currents were measured using borosilicate glass electrodes, which had a resistance of 2.0–4.0 MΩ when filled with the intracellular solution. Currents were recorded using an Axopatch 200B amplifier and Clampex software (Axon Instruments). Data were filtered at 2 kHz and were analyzed using Clampfit (Axon Instruments) and Origin (Microcal) software. All experiments were conducted at room temperature (20–22 °C). Series resistance was not compensated, and currents were not leak-subtracted. Instantaneous currents were taken as the peak current measured after the capacitive transient (6). The voltage dependence of activation was determined from tail currents at −35 mV following 4-s test pulses; interpulse intervals were 15 s to ensure complete deactivation of channels. Normalized tail current amplitudes were plotted as a function of test potential, and values were fit with a Boltzmann function.

\[
I_V = \frac{I_{\text{max}}}{1 + e^{(V/V_0 - V_1)/k}} \\
(\text{Eq. 1})
\]

to determine the midpoint of activation (\(V_0\)) and slope factor (\(k\)). Single test pulses were often followed by a 200–500-ms pulse to +5 mV to ensure complete channel deactivation, and the resting current was always allowed to return to its baseline value before subsequent voltage pulses. Statistical comparisons were performed using unpaired Student’s t tests or an analysis of variance with an appropriate post hoc test; significance was assumed if the p value was <0.05. Data are reported as mean ± S.E., and n values represent the number of cells measured, which were from a minimum of three separate transfections for each value reported.
FIG. 2. The N terminus of HCN2 is essential for functional expression. A, schematic illustration (top) and representative traces from cells expressing GFP, HCN2-ΔN, and HCN2-ΔN incubated at 28 °C, or HCN2 in response to voltage steps to −150 mV from a holding potential of −35 mV (bottom). Scale bars, 1 nA and 500 ms. Dashed line, zero current level. Average $I_h$ current density (B) and $I_{inst}$ current density (C) in response to voltage pulses to −150 mV from a holding potential of −35 mV in cells expressing HCN2 (n = 43), HCN2-ΔN (n = 11), HCN2-ΔN at 28 °C (n = 5), or GFP (n = 9). The asterisks indicate significant differences from currents in cells expressing HCN2. Confocal images of cells transfected with GFP–HCN2-ΔN (D), GFP–HCN2 (E), or HCN1 plus GFP–HCN2-ΔN (F). The arrows point to membrane regions conspicuous for the presence or absence of fluorescence. pF, picofarads.

FIG. 3. Schematic illustration of CNBD deletion mutants. Gray boxes S1–S6 indicate putative membrane-spanning domains; P, pore region; CNBD, cyclic nucleotide binding domain; A, α helix “A” of the CNBD (residues 525–532); B, β sheet domain of the CNBD (residues 533–607); B, α helix “B” of the CNBD (residues 608–619); C, α helix “C” of the CNBD (residues 620–646).
Immunocytochemistry and Confocal Microscopy—2–3 days after transfection, cells on coverslips were washed with phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde in PBS for 5 min. The cells were washed with PBS, permeabilized using 0.2% Triton X-100, and blocked with 10% normal goat serum (NGS). After one wash with PBS containing 1% NGS, cells were incubated with a rabbit polyclonal antibody specific to the HCN2 channel isoform (Alomone Laboratories, Jerusalem, Israel) at a dilution of 1:400 in PBS with 1% NGS for 48 h at 4°C. The antibody was removed, cells were again washed with PBS, and cells were then incubated with a donkey anti-rabbit secondary antibody tagged with Cy3 (Jackson Laboratories, West Grove, PA) at a dilution of 1:200 in PBS with 1% NGS for 1 h at room temperature in the dark. After washing, coverslips were mounted on slides using Permount (Fisher).

Cells were examined using wide field (Olympus BX 40) and confocal (Zeiss LSM 5 Pascal) microscopy. For confocal microscopy, serial sections were taken in 0.2–0.4-μm steps using a 63× oil immersion objective lens and an excitation wavelength of 488 nm for cells expressing GFP or 535 nm for cells incubated with the Cy3-tagged antibody.

**RESULTS**

The NH₂ Termini of HCN1 and HCN2 Interact in Yeast Two-hybrid Assays—An interaction of the NH₂-terminal domains has been shown to be important for assembly and heteromerization of Kᵥ channels (20, 23, 24). We thus investigated whether the NH₂-terminal domains of HCN channels play a similar role. We began by using the yeast two-hybrid system to test for interactions between the NH₂ termini of the HCN1 and HCN2 isoforms, which have been shown to form heteromeric channels in functional studies (10, 11). Fusion proteins were constructed between HCN NH₂ termini and the binding or activation domain of the GAL4 transcription factor. These fusion protein constructs were co-expressed in the yeast strain AH 109 and were assayed for interaction by activation of a HIS3 reporter gene. We found that the NH₂ termini of HCN1 and HCN2 interacted, both with themselves and with each other. The B-helix of the CNBD is required for production of Iₗ and Iₗ₉₉ by HCN2. Current families elicited by voltage steps from −40 to −150 mV in 10-mV increments from a holding potential of −35 mV in representative CHO cells expressing HCN2 (A) or HCN2-ΔChelix (B) and currents elicited by voltage steps to −150 mV in cells expressing GFP (n = 12), HCN2-ΔCNBD (n = 11), HCN2-Δβsheet (n = 11), HCN2-ΔChelix (n = 15), HCN2-ΔChelix (n = 15), or HCN2 (n = 43). The asterisks indicate a statistically significant difference from cells expressing only GFP. wtHCN2, wild type HCN2.
Domains Involved in Assembly and Expression of HCN Channels

Fig. 5. The B-helix of the CNBD is required for surface immunofluorescence. Confocal images of representative CHO cells transiently expressing HCN2 (A), HCN2-Δhelix (B), HCN2-ΔCNBD (C), HCN2-Δsheet (D), or HCN2-ΔCNBD (E). Cells were incubated with a primary antibody directed against the N terminus of HCN2 and a Cy3-labeled secondary antibody. The arrows indicate areas conspicuous for the presence or absence of fluorescence on the cell surface.

other, as indicated by the presence of yeast colonies on representative sections of SD/Trp−/−Leu− His nutritional selection medium (Fig. 1). This interaction was specific to the HCN NH2 termini and not due to cross-reactivity with the GAL4 portions of the fusion proteins, because colonies were never observed in negative control experiments in which the HCN NH2-terminal constructs were co-expressed with the “empty” GAL4 activation or binding domain plasmids (n = 6 transformations each; data not shown).

HCN2 Channels Lacking the N Terminus Do Not Express Currents or Surface Fluorescence—To investigate the functional consequences of the interactions between NH2-terminal domains observed in yeast two-hybrid experiments, we next constructed a mammalian expression plasmid, HCN2-ΔN, encoding HCN2 lacking the amino terminus (residues 2–182 were deleted; Fig. 2A). As illustrated in Fig. 2, A–C, neither instantaneous (I0) nor time-dependent (Ih) current was produced by CHO cells expressing HCN2-ΔN. Ih was taken as the peak current measured after the capacitive transient and thus includes contributions of currents endogenous to CHO cells and nonspecific leak. I0 amplitude was taken as the difference in current level between the peak current at the end of the capacitive transient and the steady state current at the end of a 2-s test pulse. Both I0 and Ih were significantly smaller in cells expressing HCN2-ΔN than in cells expressing the intact HCN2 channel and did not differ from currents produced by control cells expressing only the GFP transfection marker. Incubation of the transfected cells for 2–3 days at 28 °C did not rescue expression of currents (n = 5), as it has been reported to do for misfolded HERG channels (32).

To determine whether the yeast two-hybrid interaction between NH2-terminal domains underlies the ability of HCN1 and HCN2 isoforms to assemble as heteromers, we took advantage of the slower rate of activation produced by heteromeric HCN1-HCN2 channels compared with homomeric HCN1 channels (10, 11). Thus, we co-expressed HCN2-ΔN with the full-length HCN1 channel. Time-dependent Ih currents elicited by voltage pulses to −150 mV from cells expressing HCN1 alone or co-expressing HCN1 and HCN2-ΔN were fit with a double exponential function. If heteromeric channels are able to form between HCN1 and HCN2-ΔN, then the resulting Ih currents would be expected to have activation kinetics slower than those produced by homomeric HCN1 channels (10, 11). However, there was no difference in the time constants of activation for Ih produced by cells expressing HCN1 and HCN2-ΔN compared with those produced by cells expressing HCN1 alone (data not shown). Thus, HCN1 was unable to rescue expression of currents for HCN2-ΔN, suggesting that the N terminus is essential for the formation of functional heteromeric HCN channels.

A number of mechanisms could explain the failure of HCN2-ΔN to form functional channels. The HCN2-ΔN subunit may not be translated (or could be rapidly degraded), HCN2-ΔN subunits may not assemble, correctly assembled channels may not traffic to the plasma membrane, or correctly assembled channels in the plasma membrane may be nonfunctional. To begin to distinguish among these possibilities, we tagged HCN2-ΔN at the amino-terminal stump with GFP (GFP-HCN2-ΔN), expressed it either alone or with HCN1, and examined transfected cells by confocal microscopy. Fluorescence of cells transfected with GFP-HCN2-ΔN was much more intense than the background fluorescence of untransfected cells, indicating that the channel protein was being produced (Fig. 2D). However, the distribution of fluorescence was markedly different from that of a full-length HCN2 channel tagged with GFP (GFP-HCN2; Fig. 2E), with intense fluorescence in cells expressing the mutant channel limited to the intracellular
Domains Involved in Assembly and Expression of HCN Channels

FIG. 6. COOH-terminal domains of HCN1 and HCN2 do not interact in yeast two-hybrid assays. A, a schematic illustration of an HCN channel showing the regions of the COOH terminus that were tested for interaction in yeast two-hybrid assays. The entire COOH-terminal domain (C terminus), the region between S6 and the CNBD (Proximal C terminus), the CNBD itself (CNBD), or the region downstream from the CNBD (Distal C terminus) of both HCN1 and HCN2 were each cloned in frame with the binding domain (in plasmid pGAD9) and activation domain (in plasmid pGBT9) of the GAL4 transcription factor. B, representative sections of SD/-Trp/-Leu/-His nutritional selection media plated with yeast strain AH109 co-transformed with cDNA encoding the indicated portions of the HCN1 or HCN2 COOH termini expressed as fusion proteins with the binding domain or activation domain of the GAL4 transcription factor. Each test pair was assayed for interaction by activation of a HIS3 reporter gene after incubation of the yeast at 30 °C for 4–7 days on nutritional selection medium (SD/-Trp/-Leu/-His). pGAD and pGBT9 indicate the empty vectors (i.e. without inserts), which were used as negative controls. αID and βID are the interaction domains of the skeletal muscle L-type Ca²⁺ channel α and β subunits, respectively, which were used as positive controls. Negative and positive signs indicate the absence or presence of two-hybrid interactions. The numbers indicate the number of positive interactions over the number of independent transformations.

domains and absent from the periphery of the cell. This indicated that the HCN2ΔN subunit was largely localized to intracellular compartments, consistent with its inability to produce currents (Fig. 2, A–C). Co-expression of HCN1 with GFP- HCN2ΔN did not change the pattern of fluorescence (Fig. 2F), indicating that HCN1 was unable to rescue the surface expression of GFP-HCN2ΔN. Taken together, the yeast two-hybrid interactions, electrophysiology, and confocal microscopy are consistent with a role for the NH2 termini of HCN subunits in interactions, channel assembly and heteromerization.

The B-helix of the CNBD Is Required for Expression of Homomeric HCN2 Channels—We have previously described a COOH-terminal deletion mutant, HCN2-ΔCNBD, in which the CNBD and downstream portions of the COOH terminus were deleted, eliminating residues 525–863. When expressed in CHO cells, HCN2-ΔCNBD did not produce either HCN2-ΔBhelix (which produced current) and HCN2-Δhelix (which elicited by 4-s hyperpolarizing voltage steps from −40 to −150 mV from a holding potential of −35 mV. The midpoint activation voltages (V1/2) and slope factors (k), which were determined by fitting normalized tail currents with a Boltzmann function (see “Experimental Procedures”), did not differ significantly for cells expressing HCN2-ΔBhelix (V1/2 = −105.4 ± 3.4 mV, k = 8.4 ± 1.3 mV, n = 9) compared with cells expressing HCN2 (V1/2 = −101.0 ± 3.1 mV, k = 12.1 ± 1.7 mV, n = 7). Currents in cells expressing HCN2-ΔBhelix activated more slowly (p < 0.05) than in cells expressing HCN2; currents elicited by voltage steps to −150 mV and with a single exponential function had time constants of 350 ± 26 ms (n = 14) and 246 ± 22 ms (n = 11) for HCN2-ΔBhelix and wild type HCN2, respectively. The amplitudes of both Ii and Iinst produced by HCN2-ΔBhelix were compared with those produced by the full-length HCN2 channel. The average current densities for Ii and Iinst elicited by voltage pulses to −150 mV did not differ and were significantly larger than Ii and Iinst in control cells expressing only GFP (Fig. 4, F and G). These results indicate that the C-helix portion of the CNBD and downstream regions of the COOH terminus are not necessary for functional expression of HCN2 in CHO cells.

In contrast to the HCN2-ΔBhelix, none of the other CNBD deletion mutants (HCN2-ΔC helix, HCN2-Δβsheet, and HCN2-ΔCNBD) produced Ii or Iinst (Fig. 4, C-G). Thus, the 12-amino acid B-helix, the only difference between HCN2-ΔBhelix (which produced current) and HCN2-ΔBhelix (which did not produce current), appears to be important for the functional expression of HCN2 channels in CHO cells.

Altered Subcellular Distribution of HCN2 Channels Lacking the B-helix—The mechanism by which mutant channels lack-
Domains Involved in Assembly and Expression of HCN Channels

Fig. 7. Co-expression with HCN1 rescues HCN2–ΔCNBD. A, average values for the slow time constant of activation of currents elicited by pulses to −150 mV from a holding potential of −35 mV and fit with a double exponential function in cells expressing HCN1 (n = 26) or HCN1 + HCN2–ΔCNBD (n = 23). The asterisk indicates a significant difference from HCN2–ΔCNBD. Inset, representative currents scaled to the same peak I0 amplitude elicited by 1-s steps to −150 mV in cells expressing HCN1 (black trace) or HCN1 + HCN2–ΔCNBD (gray trace). Shown is a confocal micrograph of cells expressing HCN1 + HCN2–ΔCNBD (B) or HCN1 alone (C) and visualized with an anti-HCN2 primary antibody and Cy3-tagged secondary antibody. No cross-reactivity with HCN1 was observed.

Expression of HCN Channels Does Not Require a CNBD in Each Subunit—We next used a functional assay to determine whether all four CNBDs in a tetrameric HCN channel are required for functional expression. The mutant HCN2–ΔCNBD channel was co-expressed with wild type HCN1, and the resulting currents were fit with a double exponential function.
Co-expression of HCN2-ΔCNBD and HCN1 resulted in currents with slow time constants that were significantly slower than those produced by HCN1 alone (Fig. 7A). A similar result was obtained by comparing surface fluorescence in cells expressing HCN2-ΔCNBD with those co-expressing HCN1 and HCN2-ΔCNBD. Whereas HCN2-ΔCNBD was not localized to the surface membrane (Fig. 5E), co-expression with HCN1 restored HCN2 surface immunoreactivity (Fig. 7B). The surface fluorescence was not due to cross-reactivity of the HCN2 antibody with the HCN1 channel, because no staining was apparent when cells expressing only HCN1 were stained with the HCN2 antibody (Fig. 7C). These observations are consistent with the rescue of surface expression of HCN2-ΔCNBD by heteromerization with at least one HCN1 subunit. Furthermore, they indicate that surface expression of heteromeric HCN channels in CHO cells does not require a CNBD in each channel subunit, although at least one intact CNBD appears to be necessary for expression of homomeric HCN2 channels (Figs. 4 and 5).

**Discussion**

In this paper, we have shown that the NH2 termini of the HCN1 and HCN2 pacemaker channel isoforms interact, both with themselves and with each other, in yeast two-hybrid assays. This interaction appears to be obligatory for expression of homomeric and heteromeric HCN channels, because expression of an HCN2 channel lacking the N terminus was not rescued by HCN1. The lack of functional expression of HCN2-ΔN channels could be explained by a failure of subunits to assemble and form channels, a failure of assembled channels to move to the plasma membrane or an inability of assembled channels on the plasma membrane to conduct current. The latter possibility seems unlikely, because the surface immunofluorescence in cells expressing the HCN2-ΔN mutant was dramatically reduced compared with that in cells expressing the full-length HCN2, suggesting that only a small fraction, if any, of the mutant channels were on the plasma membrane. Co-expression with HCN1 did not change the localization of fluorescence for HCN2-ΔN, consistent with the absence of functional heteromers on the plasma membrane. Based on the data presented here, it seems reasonable to propose that direct interactions between the NH2 termini are important for HCN subunits to assemble and form functional channels.

The ~100-amino acid T1 “tetramerization domain” (21) adjacent to the first transmembrane segment in the N terminus of K+ channels is important for subunit assembly. The inability of subunits from different K+ subfamilies to assemble involves differences in this domain (35). The HCN NH2 termini are not similar in primary sequence to the T1 domains of K+ channels. However, the four mammalian HCN channel isoforms do have strong sequence conservation in the first 51 amino acids adjacent to the first transmembrane domain (the region is not as well conserved in the sea urchin channel, spHCN1). Sequences of the N terminus further from S1 are quite divergent among different HCN isoforms. It will be interesting to determine whether the conserved domain in the N terminus is responsible for the yeast two-hybrid interactions and whether the presence of this domain determines functional expression of the channel. If so, the conserved domain of HCN channels might play the same role in subunit assembly as the T1 domain of K+ channels, demonstrating a general pattern of organization of functional domains within the superfamiliy of voltage-gated potassium channels.

In the second part of this paper, we have shown that at least one subunit of a functional HCN channel must contain an intact CNBD. This differs from the finding that expression of HCN2 channels in Xenopus oocytes was independent of the CNBD (19), indicating that the expression of functional channels depends on the cellular system used. Regardless of the expression system, however, it is clear that subunits lacking the complete CNBD can assemble under the right circumstances. Indeed, we found that co-expression of HCN1 rescued expression of HCN2-ΔCNBD. This rescue probably reflects the formation of heteromeric channels as has been suggested previously (10, 11). Together, the functional heteromerization of HCN1 with HCN2-ΔCNBD and the lack of interactions between any COOH-terminal domains in yeast two-hybrid experiments suggest that assembly and functional expression of HCN channels does not require either a CNBD within each subunit or interaction between CNBDs of different subunits.

The mechanism by which the putative B-helix controls expression in CHO cells remains to be determined. One possible explanation is that the deletion may disrupt the tertiary structure of a functional subdomain within the CNBD. For instance, the CNBD in CNG channels is formed by two distinct structural and functional domains: a β-roll, consisting of the eight-stranded β-sheet flanked by the A- and B-helices, which stabilizes the ligand in a state-independent manner, and the C-helix, which stabilizes the ligand in the open state of the channel (36, 37). Thus, if the CNBD of HCN channels is organized in the same manner as that of CNG channels, it is possible that functional expression of HCN2 in CHO cells depends on an intact β-roll subdomain. Deletion of the B-helix may prevent expression by disrupting the tertiary structure of the CNBD and perhaps destabilize the binding of CAMP. In support of this notion, a similar dependence on an intact CNBD for functional expression is seen in HERG (38) and KAT1 (30) channels.

A second explanation for the inability of HCN2 channels lacking the putative B-helix to reach the plasma membrane is that this region may contain a primary sequence motif that promotes surface expression. Ma et al. (26) have recently described motifs that are responsible for localization of K+ channels to the plasma membrane and showed that this function can be transferred to other potassium channels or to a G-protein-coupled receptor. It will be interesting in further experiments to determine whether the putative B-helix contains such a sequence and whether this can restore function and surface expression of channels that otherwise lack a CNBD.

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