The Subfamily-specific Assembly of Eag and Erg K^+ Channels Is Determined by Both the Amino and the Carboxyl Recognition Domains*

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Background: K^+ channel subunits from different ether-à-go-go subfamilies cannot form heterotetramers.

Results: Reversal of subfamily specificity was only possible when we exchanged both amino and carboxyl termini between two ether-à-go-go subunits.

Conclusion: Both recognition domains are required for subfamily-specific assembly.

Significance: This is the first evidence suggesting that the amino terminus of ether-à-go-go K^+ channels governs subunit interaction specificity.

A functional voltage-gated K^+ (Kv) channel comprises four pore-forming α-subunits, and only members of the same Kv channel subfamily may co-assemble to form heterotetramers. The ether-à-go-go family of Kv channels (KCNH) encompasses three distinct subfamilies: Eag (Kv10), Erg (Kv11), and Elk (Kv12). Members of different ether-à-go-go subfamilies, such as Eag and Erg, fail to form heterotetramers. Although a short stretch of amino acid sequences in the distal C-terminal section has been implicated in subfamily-specific subunit assembly, it remains unclear whether this region serves as the sole and/or principal subfamily recognition domain for Eag and Erg. Here we aim to ascertain the structural basis underlying the subfamily specificity of ether-à-go-go channels by generating various chimeric constructs between rat Eag1 and human Erg subunits. Biochemical and electrophysiological characterizations of the subunit interaction properties of a series of different chimeric and truncation constructs over the C terminus suggested that the putative C-terminal recognition domain is dispensable for subfamily-specific assembly. Further chimeric analyses over the N terminus revealed that the N-terminal region may also harbor a subfamily recognition domain. Importantly, exchanging either the N-terminal or the C-terminal domain alone led to a virtual loss of the intersubfamily assembly boundary. By contrast, simultaneously swapping both recognition domains resulted in a reversal of subfamily specificity. Our observations are consistent with the notion that both the N-terminal and the C-terminal recognition domains are required to sustain the subfamily-specific assembly of rat Eag1 and human Erg.

In neurons, a variety of different voltage-gated K^+ (Kv) channels are essential for setting membrane excitability, controlling firing frequencies, and determining the falling phase of action potentials (1, 2). A functional Kv channel comprises four pore-forming α-subunits (3, 4). Based on the sequence analysis of α-subunits, Kv channels are divided into 12 major subfamilies (Kv1–Kv12) (5). In general, only members of the same Kv channel subfamily may co-assemble to form functional heterotetramers (6–10). This subfamily-specific formation of tetrameric K^+ channels requires specific intersubunit associations via recognition or stabilization domains located within individual α-subunits. In Kv1 channels, for example, the cytoplasmic N-terminal tetramerization domain (T1 domain) plays an important role in both subunit tetramerization and subfamily-specific assembly (8, 10, 11).

The ether-à-go-go family of K^+ channels (KCNH) encompasses three distinct Kv subfamilies: Eag (Kv10), Erg (Kv11), and Elk (Kv12) (12). Members of different ether-à-go-go subfamilies, such as Eag and Erg, fail to form heterotetramers (13). A stretch of 41 amino acids in the distal end of the C terminus of Eag α-subunits has been suggested to have a high probability of forming coiled-coil structure and may contribute to subunit assembly; this region is hence termed the carboxyl assembly domain (CAD) (14, 15). A similar tetramerizing coiled-coil (TCC) domain is also found in the distal end of the C terminus of Erg α-subunits (15). Interestingly, a chimeric Erg construct, whose TCC domain was replaced by the CAD of Eag, was able to form heteromultimeric channels with Eag (15), raising the possibility that CAD/TCC may serve as the subfamily recognition domain as well.

Much remains to be learned about the assembly of subunits in ether-à-go-go subfamilies. For instance, because the CAD

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3 The abbreviations used are: Kv, voltage-gated K^+; CAD, carboxyl assembly domain; CNBHD, cyclic nucleotide-binding homology domain; HEK, human embryonic kidney; hErg, human Erg; rEag1, rat Eag1; T1 domain, tetramerization domain; TCC, tetramerizing coiled-coil.
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A

Myc-rEag1

GFP-rEag1  Flag-rEag2  Flag-hErg

Input  α-Myc/IP  α-Myc/IP  α-Myc/IP

1  2  1  2  1  2

170  130  95  72  55

WB: α-GFP  α-rEag2  α-Flag

1  2  1  2

170  130  95  72  55

WB: α-Myc

1  2

Myc-rEag1 & Flag-rEag2

Input  Mouse IgG/IP  α-Myc/IP  Mouse IgG/IP  α-Myc/IP

1  2  1  2  1  2

170  130  95  72  55

WB: α-Myc  α-rEag2

1  2

B

rEag1-G440C

PAS  S450-G440  C-Linker  CNBD  CAO

Input & Co-IP

Normalized current amplitude

(69)  (74)  (88)

rEag1-WT  +H₂O  +rEag1-G440C

(74)  (88)

hErg-WT  +H₂O  +rEag1-G440C

hErg-G628S

PAS  S450-G628  C-Linker  CNBD  CAO

Input & IP

Normalized current amplitude

(52)  (57)  (42)

rEag1-WT  +H₂O  +hErg-G628S

(50)  (57)  (80)

hErg-WT  +H₂O  +hErg-G628S

**Note:** The histograms show normalized current amplitudes with error bars indicating standard deviation.
and the TCC domain are only about 25 and 85 residues away from the very end of the Eag and the Erg proteins, respectively, it is still unknown how a domain that is translated relatively late can effectively govern subunit assembly and subfamily recognition processes. Most importantly, is CAD/TCC the sole and/or principal assembly and recognition domain for Eag and Erg? Previous truncation analyses from our laboratory indicate that the CAD is not required for the assembly of Eag subunits (16).

**Figure 1.** Subfamily-specific assembly of rEag1 and hErg subunits. A, biochemical verification of the subfamily-specific interaction of the ether-à-go-go K⁺ channel subunits in HEK293T cells. cDNAs for Myc-tagged rEag1 and GFP-rEag1/FLAG-rEag2/FLAG-hErg were co-transfected into HEK293T cells. Cell lysates were immunoprecipitated with the anti-Myc antibody. Corresponding β-actin expression levels for each lane are displayed in the bottom panels. C, quantification of protein expression levels of Myc-rEag1 (top) and FLAG-hErg (bottom) chimeras in HEK293T cells. Protein density was standardized as the ratio of Myc or FLAG signal to cognate β-actin signal. Values from various chimeras were then normalized with respect to those of the corresponding WT construct. Densitometric scans of immunoblots were obtained from 3–6 independent experiments. Asterisks denote significant difference from the corresponding WT (*, Student’s t test, p < 0.05). Error bars, S.E.

**Figure 2.** Protein expression of the C-terminal chimeras between rEag1 and hErg channels. A, schematic representation of C-terminal chimeras (for more detail, see “Experimental Procedures”). B, representative immunoblots of various Myc-tagged rEag1 (top) and FLAG-tagged hErg (bottom) chimeras in HEK293T cells. Protein bands were detected with anti-Myc or anti-FLAG antibodies. Also shown are immunoblots of cells transfected with control vectors (–). Corresponding β-actin expression levels for each lane are displayed in the bottom panels. C, quantification of protein expression levels of Myc-rEag1 (top) and FLAG-hErg (bottom) chimeras in HEK293T cells. Protein density was standardized as the ratio of Myc or FLAG signal to cognate β-actin signal. Values from various chimeras were then normalized with respect to those of the corresponding WT construct. Densitometric scans of immunoblots were obtained from 3–6 independent experiments. Asterisks denote significant difference from the corresponding WT (*, Student’s t test, p < 0.05). Error bars, S.E.
Likewise, subunit interaction for Erg channels may take place in the absence of the TCC domain (17–21). It is therefore possible that other regions within Eag and Erg may also contribute to subunit assembly and subfamily recognition.

In this study, we aim to ascertain the structural basis underlying the subfamily-specific assembly of ether-à-go-go channels. We began by generating a series of different chimeric constructs over the C-terminal region of rat Eag1 (rEag1) and human Erg (hErg) subunits. By performing biochemical and electrophysiological characterizations of the subunit interactions of the chimera, we demonstrated that the putative assembly domains in the distal C terminus alone were not sufficient to explain their subfamily specificities. Further chimeric analyses over the N-terminal region revealed a hitherto unknown role in governing the specificity of subunit interactions. Our results suggest that both the N- and the C-terminal recognition domains are required for the subfamily-specific assembly of rEag1 and hErg K⁺ channels.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs**—rEag1 and rEag2 cDNAs subcloned into pcDNA3 (Invitrogen) were kindly provided by Dr. Olaf Pongs (Zentrum für Molekulare Neurobiologie, Hamburg, Germany). hErg (hErg 1a) cDNA subcloned into pSP64-poly(A) was kindly provided by Dr. Gail A. Robertson (University of Wisconsin, Madison, WI). To create epitope-tagged constructs for biochemical analyses, rEag1 cDNA was switched into either pcDNA3-Myc or pEGFP (Clontech), rEag2 cDNA was transferred into pcDNA3-FLAG, and hErg cDNA was swapped into either pFLAG-CMV2 (Sigma) or pEGFP. pcDNA3- and pSP64-based constructs were used for heterologous expression in Xenopus oocytes; pcDNA3-, pFLAG-CMV2-, and pEGFP-based constructs were chosen for heterologous expression in mammalian cells.

The QuikChange site-directed mutagenesis kit (Stratagene) was used to produce the following mutant constructs. G440C and G628S pore mutants were created for rEag1 and hErg, respectively. A premature stop codon (denoted by X) was introduced at Asn-861 or Arg-1032 to generate the truncated hErg constructs N861X and R1032X. As summarized below, chimeric constructs were generated by introduction of compatible restriction sites in both rEag1 and hErg through silent or missense mutations: chimera A, KpnI (rEag1-N481G/T482T; hErg-G669G/T670T) and EcoRV#1 (rEag1-A558I/S559S; hErg-K135F/D136K/M137L) and Pmel#2; chimera B, Pmel#1 (rEag1-A135A/K137K; hErg-A408L); chimera O, Pmel#1 (rEag1-V215V/F216F/K217K/T218L; hErg-A408L) and Pmel#2; chimera P, HindIII and Pmel#1; N-terminal deletion (∆N), double digestion with HindIII and Pmel#2, followed by insertion of the triamino acid linker sequence LAG. All constructs were subject to DNA sequencing verification.

**Cell Culture and Transfection**—Human embryonic kidney (HEK293T) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM l-glutamine, 100 units/ml penicillin/streptomycin, and 10% (v/v) fetal bovine serum (Hyclone). Cells were maintained at 37 °C in a 95% air and 5% CO₂ humidified incubator and passaged about every 4 days. Transient transfection was performed by standard calcium phosphate methods. Unless stated otherwise, for each construct, about 3 μg of cDNA was added to each well on a 6-well cell culture plate. For co-expression experiments, cDNA co-transfection was conducted in a 1:1 molar ratio (i.e. 3 μg + 3 μg of cDNA/well). Two days after transfection, cells were processed for biochemical experiments.

**Co-immunoprecipitation and Western Blotting**—Transfected HEK293T cells were solubilized in ice-cold immunoprecipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM Na₂HPO₄, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitor mixture (Roche Applied Science). Insolubilized materials were removed by centrifugation. Solubilized lysates were precleared with protein A/G-Sepharose beads (GE Healthcare) for 1 h at 4 °C and then incubated for 16 h at 4 °C with protein A/G-Sepharose beads precoated with appropriate antibodies. Beads were gently spun down and washed three times in immunoprecipitation buffer and twice with TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl). The immune complexes were eluted from the beads by boiling for 5 min in SDS sample buffer. Protein in the cell lysates or immunoprecipitated samples was separated on 7.5% SDS-PAGE; transferred to nitrocellulose membranes; and detected using mouse anti-Myc (clone 9E10), rabbit anti-GFP (1:5000; Abcam), mouse anti-FLAG lose membranes; and detected using mouse anti-Myc (clone 9E10), rabbit anti-GFP (1:5000; Abcam), mouse anti-FLAG (1:1000; Sigma, clone M2), rabbit anti-FLAG (1:5000; Sigma), mouse anti-β-actin (1:10,000; Sigma), rabbit anti-rEag1 (1:10,000; Alomone), or rabbit anti-rEag2 (1:5000; Alomone) antibodies. Blots were then exposed to horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5000; Jack-

**FIGURE 3. Functional expression of rEag1 and hErg C-terminal chimeric channels.** A, the relative mean K⁺ current amplitudes (3 mM external KCl) measured from oocytes injected with the cRNA (0.1 μg/μl) for various constructs of rEag1 (left) and hErg (right) channels. For rEag1, steady-state current amplitudes at ±60 mV were measured. For hErg, peak tail current amplitudes at ±120 mV (in response to ±50 mV test pulses) were measured. Asterisks denote significant difference from the corresponding WT (*, Student’s t test, p < 0.05). B, representative K⁺ current traces recorded from oocytes expressing various constructs of rEag1 (left panels) and hErg (right panels) channels. In order to perform detailed biophysical analyses, the cRNA concentrations for some of the low expressing constructs were increased up to 3 μg/μl. The bath solution contained 3 mM KCl. Depending on the steady-state voltage dependence properties of different constructs, the holding potential was set at −90, −100, or −120 mV. For rEag1, the pulse protocol comprised 370-ms depolarizing test pulses (with 10-mV increments) up to ±120 mV, followed by a tail potential at −100/−140 mV. The steady-state activation curves of various constructs of rEag1 (left) and hErg (right) channels. The fraction of open channels (Pₒ) was plotted against the corresponding test potential (V). Data points were fit with a Boltzmann equation (solid curves). For more detail, see Tables 1 and 2. Error bars, S.E.
Capped cRNA was transcribed in vitro from the linearized cDNA template with the Message mMachine T7 kit (Ambion). The concentration of cRNA was determined by gel electrophoresis and verified with spectrophotometry.

Adult female *Xenopus laevis* (African *Xenopus* Facility, Knysna, South Africa) were anesthetized by immersion in Tricaine (1.5 g/liter). All procedures were in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003) and approved by the Institutional Animal Care and Use Committee of National Yang-Ming University. Ovarian follicles were removed from *Xenopus* frogs, cut into small pieces, and incubated in ND96 solution (96 mm NaCl, 2 mM KCl, 1.8 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.5). To remove the follicular membrane, *Xenopus* oocytes were incubated in Ca²⁺-free ND96 containing collagenase (2 mg/ml) on an orbital shaker (~200 rpm) for about 60–90 min at room temperature. After several washes with collagenase-free, Ca²⁺-free ND96, oocytes were transferred to ND96. Stage V–VI oocytes were then selected for cRNA injection. Injected oocytes were stored at 16 °C in ND96 solution supplemented with 50 mg/liter gentamycin.

For all cRNA injection paradigms, the total volume of injection was always 41.4 nl/oocyte. To examine the phenotype of individual chimeric and mutant constructs, cRNA concentration up to about 3 μg/μl was used for oocyte injection (i.e. up to 124.2 ng of cRNA was injected into an oocyte). For co-expression experiments, it is imperative to find a submaximal cRNA concentration for WT rEag1 and hErg channels that allows us to add an extra amount of mutant cRNA. Therefore, we empirically fixed WT cRNA concentration at 0.1 μg/μl (i.e. 4.14 ng of cRNA/oocyte) (16), known as the standard cRNA concentration for oocyte injection. To implement a strict criterion for assessing dominant negative effects of non-functional mutants in the co-expression experiment, the cRNA concentration of mutant constructs was always equal to or less than that of the functional counterpart. Unless stated otherwise, a 1:1 molar ratio (i.e. 8.28 ng of cRNA/oocyte) was used for co-expression with hErg-WT. A 1:1 co-injection ratio was also applied for evaluating the dominant negative effects of rEag1 mutants on rEag1-WT. For co-expressing Eag1-WT and hErg mutants, however, the molar ratio 1:0.5 (i.e. 6.21 ng of cRNA/oocyte) was chosen because most hErg constructs displayed relatively higher protein expression levels.

### TABLE 1

**Steady-state voltage-dependent activation parameters of chimeric rEag1 hErg K⁺ channels**

| Construct                  | V₅₀ (mV) | k | n  |
|----------------------------|----------|---|----|
| rEag1-WT                   | −18.4 ± 0.1 | 9.1 ± 0.1 | 142 |
| hErg-chimera A             | −23.7 ± 0.6 | 9.1 ± 0.6 | 31  |
| hErg-chimera A             | −17.8 ± 0.5 | 13.3 ± 0.5a | 5   |
| hErg-chimera C             | −14.4 ± 0.2 | 10.3 ± 0.2 | 53  |
| hErg-chimera D             | −61.4 ± 1.7a | 16.0 ± 1.5a | 11  |
| hErg-chimera E             | −89.2 ± 0.4a | 8.4 ± 0.4 | 16  |
| hErg-chimera F             | −65.1 ± 0.8a | 12.3 ± 0.7a | 9   |
| hErg-chimera G             | −66.6 ± 0.6a | 11.3 ± 0.5 | 11  |
| hErg-chimera N             | 7.8 ± 0.8a | 11.4 ± 0.7 | 19  |
| hErg-chimera P             | 13.4 ± 0.4 | 10.5 ± 0.4 | 11  |
| hErg-chimera NC            | 5.2 ± 1.1 | 11.3 ± 1.0 | 11  |
| rEag1-chimera A            | −23.2 ± 0.4 | 9.6 ± 0.3 | 26  |
| rEag1-chimera B            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera C            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera D            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera E            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera F            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera G            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera N            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera P            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera NC           | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |

* Significantly different from rEag1-WT; Student’s t test, p < 0.05.

### TABLE 2

**Steady-state voltage-dependent activation parameters of chimeric hErg K⁺ channels**

| Construct                  | V₅₀ (mV) | k | n  |
|----------------------------|----------|---|----|
| hErg-WT                    | −18.4 ± 0.1 | 9.1 ± 0.1 | 142 |
| hErg-chimera A             | −23.7 ± 0.6 | 9.1 ± 0.6 | 31  |
| hErg-chimera B             | −17.8 ± 0.5 | 13.3 ± 0.5a | 5   |
| hErg-chimera C             | −14.4 ± 0.2 | 10.3 ± 0.2 | 53  |
| hErg-chimera D             | −61.4 ± 1.7a | 16.0 ± 1.5a | 11  |
| hErg-chimera E             | −89.2 ± 0.4a | 8.4 ± 0.4 | 16  |
| hErg-chimera F             | −65.1 ± 0.8a | 12.3 ± 0.7a | 9   |
| hErg-chimera G             | −66.6 ± 0.6a | 11.3 ± 0.5 | 11  |
| hErg-chimera N             | 7.8 ± 0.8a | 11.4 ± 0.7 | 19  |
| hErg-chimera P             | 13.4 ± 0.4 | 10.5 ± 0.4 | 11  |
| hErg-chimera NC            | 5.2 ± 1.1 | 11.3 ± 1.0 | 11  |
| rEag1-chimera A            | −23.2 ± 0.4 | 9.6 ± 0.3 | 26  |
| rEag1-chimera B            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera C            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera D            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera E            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera F            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera G            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera N            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera P            | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |
| rEag1-chimera NC           | 11.3 ± 0.3 | 11.5 ± 1.0 | 26  |

* Significantly different from hErg-WT; Student’s t test, p < 0.05.

Two-electrode Voltage Clamp Recording in *Xenopus* Oocytes—2–3 days after cRNA injection, oocytes were functionally assayed in a recording bath containing Ringer solution (in 115 mM NaCl, 3 mM KCl, 1.8 mM CaCl₂, 10 mM HEPES, pH 7.2). Where indicated, 60 mM KCl was employed (by replacing NaCl) to record tail currents. Niflumic acid (0.5 mM) was added to the bath to minimize the contribution of endogenous Ca²⁺-activated Cl⁻ currents. The bath volume was about 200 μl. An agarose bridge was used to connect the bath solution with a ground chamber (containing 3 mM KCl) into which two ground electrodes were inserted. Borosilicate electrodes (0.1–1 megaohms) used in voltage recording and current injection were filled with 3 M KCl. K⁺ currents through rEag1 channels were acquired, using the conventional two-electrode voltage clamp technique with an OC-725C oocyte clamp (Warner). For the majority of rEag1 channel constructs, passive membrane properties were compensated using the −P/4 leak subtraction method provided by the pCLAMP 8.2 software, whereas no leak subtraction was performed for rEag1-chimeras N and P as well as all hErg channel constructs. Data were filtered at 1 kHz (OC-725C oocyte clamp) and digitized at 100 μs/point (10 kHz) using the Digidata 1332A/pCLAMP 8.2 data acquisition system.
All recordings were performed at room temperature (20–22 °C). Data analyses were performed via built-in analytical functions of the pCLAMP 8.2 software. For the same batch of oocytes, mean current amplitudes recorded on the same day were normalized with respect to those of the corresponding control condition (e.g., WT construct; co-injection with the nuclease-free water). Normalized data from different batches of oocytes or different days of experiments were later pooled together for comprehensive analyses. Results shown are representative of at least 3–5 independent experiments. All values are presented as mean ± S.E. The significance of the difference between two means was tested by Student’s t test, whereas means from multiple groups were compared by one-way analysis of variance. Statistical analyses were performed with Origin 7.0 software (Microcal Software).

**RESULTS**

**Subfamily-specific Assembly of rEag1 and hErg K⁺ Channels**

To highlight the subunit interaction property of the ether-à-go-go family, we employed both biochemical and electrophysiological methods to verify the subunit assembly specificity of rEag1 and hErg channels. Asterisks denote significant difference from the water co-injection control (*, Student’s t test, p < 0.05). Error bars, S.E.
hErg K⁺ channels. For biochemical characterization, Myc-tagged rEag1 (Myc-rEag1) was co-expressed with either GFP-tagged rEag1 (GFP-rEag1) or FLAG-tagged hErg (FLAG-hErg) in HEK293T cells for the co-immunoprecipitation experiment. Despite the large size of GFP, previous observations from our laboratory indicated that adding the GFP tag fails to significantly affect the assembly and trafficking of Eag subunits (22). Fig. 1A shows that Myc-rEag1 co-existed with GFP-rEag1, but not with FLAG-hErg, in the same protein complex. By contrast, heteromultimer formation was clearly demonstrated upon co-expressing Myc-rEag1 with FLAG-tagged rat Eag2 (FLAG-rEag2), an isoform of the same Eag subfamily.

For electrophysiological characterization, we first generated the non-functional pore mutant G440C and G628S for rEag1 and hErg, respectively (23, 24). We then investigated whether the two non-functional mutants could exert a dominant negative effect on the functional expression of WT rEag1 and hErg K⁺ channels. As mentioned in the legend to Fig. 4C, pore mutations were introduced into the indicated low expressing rEag1 and hErg chimeras, followed by evaluation of their suppression effects on WT channels. Only chimera Gs exerted significant dominant negative effects on both rEag1 and hErg channels. Asterisks denote significant difference from the water co-injection control (*, Student's t test, p < 0.05). Error bars, S.E.

FIGURE 5. Loss of subfamily specificity for rEag1- and hErg-chimera G. Biochemical investigations of the subfamily specificity of Myc-rEag1- (A) and FLAG-hErg-chimera D, E, F, and G (B) were carried out in HEK293T cells. Individual chimeras were co-expressed with GFP-rEag1-WT or GFP-hErg-WT by following the same protocols as outlined in the legend to Fig. 4, A and B. Also shown are immunoblots of cells transfected with control vectors (-). Only chimera Gs were effectively co-immunoprecipitated with both GFP-rEag1-WT and GFP-hErg-WT. C, functional examination of the subfamily specificity of rEag1 and hErg chimeras in Xenopus oocytes by quantifying the dominant negative effects of the non-functional pore mutants rEag1-G440C and hErg-G628S. As mentioned in the legend to Fig. 4C, pore mutations were introduced into the indicated low expressing rEag1 and hErg chimeras, followed by evaluation of their suppression effects on WT channels. Only chimera Gs exerted significant dominant negative effects on both rEag1 and hErg channels. Asterisks denote significant difference from the water co-injection control (*, Student’s t test, p < 0.05). Error bars, S.E.
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A rEag1-K848X

Myc-rEag1-K848X

WB: α-Myc

Normalized current amplitude

+H₂O  +rEag1-G440C  +H₂O  +hErg-G628S

rEag1-K848X  rEag1-K848X

B rEag1-N673X

Myc-rEag1-N673X

WB: α-Myc

Normalized current amplitude

+H₂O  +rEag1-N673X  +H₂O  +rEag1-N673X

rEag1-WT  hErg-WT
with hErg-G628S resulted in the suppression of hErg-WT only (Fig. 1B). Identical patterns of dominant negative effect were observed when we repeated the same experiments in HEK293T cells (data not shown). Because cRNA injection provided a much more precise control of the cRNA molar ratio for co-expression of mutant and WT constructs, thereafter we focused on Xenopus oocytes for electrophysiological analyses.

**Generation of C-terminal Chimeras**—We began by creating a series of seven different chimeric constructs between rEag1 and hErg over the C terminus (Fig. 2A): chimera A involved switching the C-linker region; chimera B swapped both the C-linker and the cyclic nucleotide-binding homology domain (CNBHD); chimera C exchanged the post-CNBHD region; chimera D traded the S6 segment; and chimera E–G gradually extended the range of sequence substitution from “S6-C-linker” to “S6-post-CNBHD” (for more detail, see “Experimental Procedures”). Fig. 2B illustrates the representative protein expression patterns of Myc-tagged rEag1 chimeras harboring hErg sequences (Myc-rEag1-chimera A–G), as well as those of FLAG-tagged reverse chimeras (FLAG-hErg-chimera A–G) in HEK293T cells. All chimeric constructs generated a significant amount of channel protein, although some of them showed relatively lower expression levels. Like their WT counterpart, hErg chimeras A and D, and to a lesser extent chimera E, were heavily glycosylated, whereas the other hErg chimeras displayed a significantly reduced glycosylation pattern.

We also examined the functional phenotype of the chimeras. Upon expression in Xenopus oocytes (by injecting with the standard 0.1 μg/μl cRNA; for more detail, see “Experimental Procedures”), all chimeric constructs yielded functional K⁺ channels, albeit with a pronounced disparity in the relative current amplitude (Fig. 3A). For those chimeras with poor functional expression (rEag1-chimera C, E, G; hErg-chimera B, F, G), considerable K⁺ currents were recorded upon increasing the cRNA concentration up to 3 μg/μl (Fig. 3B). All chimeras were capable of forming functional channels in HEK293T cells as well (data not shown). The general current shapes of the chimeras were similar to those of their WT counterparts: all rEag1 chimeras displayed typical outward K⁺ currents with no apparent inactivation, whereas all hErg chimeras exhibited the characteristic inactivation in response to strong membrane depolarizations (Fig. 3B). Moreover, hyperpolarization-induced delay in channel activation, a trademark gating property of the Eag K⁺ channel subfamily, was also observed in all rEag1 chimeras (data not shown).

The impact of chimeric substitution on channel gating properties, however, differs between rEag1 and hErg. Compared with rEag1-WT, the steady-state activation curves of rEag1-chimera A–G were predominantly right-shifted (Fig. 3C and Table 1), with rEag1-chimera E showing a striking shift of about 60 mV. On the contrary, hErg chimera D–G showed left-shifted activation curves (Fig. 3C and Table 2), with hErg-chimera E demonstrating a conspicuous change of more than 70 mV. Accordingly, the apparent gating kinetics of some of the chimeras was notably affected. For example, introduction of hErg C-terminal sequences decelerated both the activation and the deactivation kinetics of rEag1, with rEag1-chimeras F displaying the most dramatic change (data not shown). In contrast, hErg chimera A and B exhibited accelerated deactivation kinetics, whereas hErg-chimera D and E showed remarkably slower deactivation kinetics (data not shown).

**C-terminal Assembly Domains Are Inadequate to Determine Subfamily-specific Assembly**—At least three structural domains in the C-terminal region may be involved in mediating subunit interactions: the C-linker (25), the CNBHD (26), and the post-CNBD region that contains the CAD or the TCC domain (14, 15). In particular, CAD/TCC was previously suggested to serve as the subunit assembly domain as well as the subfamily recognition domain (14, 15). We therefore asked whether the C-terminal region constitutes the sole or principal recognition domain for subfamily specificity of Eag and Erg channels. To address this issue, we studied the subunit interaction property of various chimeras. As mentioned above (see Fig. 1), upon co-expressing a chimeric channel with its WT counterpart in a 1:1 molar ratio, the presence of potential subunit assembly was verified with the co-immunoprecipitation experiment in HEK293T cells and with the dominant negative assay (in the standard cRNA concentration) in Xenopus oocytes. In order to acquire reliable non-functional subunits suitable for dominant negative assays, pore mutations (G440C for rEag1; G628S for hErg) were introduced into those chimeras with poor functional expression (e.g. rEag1-chimera C, E, G; hErg-chimera B, F, G) (see Fig. 3), which were subject to co-expression with WT constructs in oocytes. On the other hand, for the other C-terminal chimeras showing vigorous K⁺ channel activities, pore mutants in the WT background were employed for cRNA co-injections.

We first investigated the subunit interaction property of chimeras A (C-linker), B (C-linker and CNBHD), and C (post-CNBD). As illustrated in Fig. 4, both biochemical and electrophysiological analyses indicated that neither chimera A nor chimera B significantly altered the subfamily-specific assembly of rEag1 and hErg channels. By contrast, in agreement with the
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A

rEag1

hErg

chimera

B

GFP-rEag1

Myc-rEag1-chimera

WT N P O

GFP-hErg

Myc/rEag1-chimera

WT N P O

C

rEag1-chimera-N

hErg-chimera-N

rEag1-chimera-P

hErg-chimera-P

rEag1-chimera-O

hErg-chimera-O
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previous report by Jenke et al. (15) rEag1-chimera C, the chimeric rEag1 channel harboring the hErg post-CNBDH region co-existed in a protein complex with and exerted significant dominant negative effect on hErg-WT (Fig. 4, A and C). Similarly, hErg-chimeric C displayed a prominent interaction with rEag1-WT as well (Fig. 4, B and C). These results seemed to suggest that the two chimera Cs reversed their respective subfamily specificity. Nevertheless, as exemplified by their reduced but notable co-immunoprecipitation efficiencies and dominant negative effects, rEag1-chimera C and hErg-chimera C were still capable of interacting with rEag1-WT and hErg-WT, respectively (Fig. 4). These findings imply that rather than reversing specificity, the two chimera Cs may instead lose their subfamily specificity.

Given its role in the tetrameric organization of the pore region, the transmembrane S6 segment may also contribute to subunit assembly in rEag1 and hErg channels. Because none of chimeras A through C involved switching the S6 segment, an alternative explanation to the foregoing results is that the subfamily recognition capacity of the post-CNBDH region may somehow be ineffective or even defective in the absence of a homologous S6 segment. To test this hypothesis, we investigated the subunit interaction property of the chimeras that exchanged the S6 segment: chimera D (S6 only), E (from S6 through C-linker), F (from S6 through CNBHD), and G (from S6 through post-CNBDH). Both biochemical and electrophysiological analyses in Fig. 5 demonstrated that for rEag1-chimera D–G, only the post-CNBDH-switching chimera G showed a noteworthy interaction with hErg-WT. Likewise, for hErg-chimera D–G, only chimera G effectively co-existed in a protein complex with rEag1-WT (Fig. 5). Most importantly, exactly like chimera Cs, both chimera Gs still retained significant subunit interaction with their respective “native” subfamily (Fig. 5). Together these data strongly argue that exchanging the post-CNBDH region between the two ether-à-go-go K⁺ channel subfamilies does not result in a reversal of subfamily specificity but rather leads to a virtual loss of the intersubfamily boundary.

If the aforementioned inference is true, then it may imply that the post-CNBDH region is not the only or principal subfamily recognition domain for rEag1 and hErg channels. To address this possibility, we inspected the subfamily specificity of four truncation mutants lacking CAD/TCC; rEag1-K848X and hErg-R1032X retained the proximal portion of the post-CNBDH region (Fig. 6A), whereas rEag1-N673X and hErg-N861X contained virtually none of the post-CNBDH region (Fig. 6B). The truncation mutants rEag1-K848X and hErg-R1032X produced functional K⁺ channels with biophysical properties similar to those of respective WT (16) (data not shown). By contrast, neither rEag1-N673X nor hErg-N861X generated significant K⁺ currents (16, 17). Despite the absence of CAD/TCC, all of the four truncation mutants preserved their native subfamily-specific assembly properties (Fig. 6), suggesting that indeed CAD/TCC may be dispensable for the subunit-specific assembly of rEag1 and hErg channels.

N-terminal Regions Also Contribute to Subfamily-specific Assembly—If we define the role of a recognition or stabilization domain as being able to effectively promote the assembly of structurally compatible ion channel subunits, as has previously been proposed for the T1 domain in Kv1 channels (27), then a reasonable interpretation of the above results would be that both rEag1 and hErg channels may contain additional subfamily recognition domains outside the C-terminal region. The N-terminal region of both the Eag and the Erg subfamilies comprises three structural divisions: the cap sequence, the PAS domain, and the N-linker region that connects the PAS domain with the transmembrane S1 segment (28–31). Despite sharing homologous cap sequence and PAS domain (collectively known as the eag domain), Eag and Erg significantly differ in the length of the N-linker, with the latter being about 190 amino acids longer. Importantly, hErg 1a (the hErg clone employed herein) and the truncated hErg 1b, two isoforms with a size difference of more than 300 amino acids in the N terminus (32, 33), have been shown to form hetero-oligomers mediated by N-terminal interactions (34), raising the possibility that hErg channels may contain certain N-terminal assembly domains. Furthermore, the hErg PAS (eag) domain was suggested to regulate deactivation kinetics via intersubunit interactions (26). We therefore hypothesized that rEag1 and hErg may contain additional subfamily recognition domains in the N terminus. To test this idea, we generated three different types of N-terminal chimeras: chimera N exchanged the complete N terminus; chimera P switched the homologous eag domain; and chimera O swapped the divergent N-linker (Fig. 7A) (for more detail, see “Experimental Procedures”).

All of the N-terminal chimeras displayed notable protein expression (Fig. 7A). Furthermore, except for hErg-chimera O, the N-terminal chimeras produced functional K⁺ channels (Fig. 7A and Tables 1 and 2). Most importantly, both biochemical and electrophysiological analyses indicated that chimera Ns were effectively co-immunoprecipitated with both rEag1-WT and hErg-WT (Fig. 7, B and C), suggesting that, comparable with the consequence of switching CAD/TCC, exchanging the complete N terminus seemed to result in the collapse of the intersubfamily boundary. However,
chimera Ps and chimera Os, both of which involve swapping partial N-terminal sequences, displayed effective interaction with the WT subunit of the same subfamily only (Fig. 7, B and C). Collectively, the preceding results imply that the N terminus may also contain subfamily recognition domains that presumably comprise a subset of protein sequences entailing both the distal eag domain and the proximal N-linker region.

Exchanging Both the N-terminal and the C-terminal Regions Reverses Subfamily Assembly Specificity—We have already shown that the removal of CAD/TCC failed to affect the subfamily-specific assembly of rEag1 and hErg channels (see Fig. 6). To examine whether the N terminus is indispensable for the subfamily specificity, we also created N-terminal deletion mutants that obliterated the complete N terminus (ΔN) (Fig. 8). As depicted in Fig. 8, for both rEag1 and hErg, removal of...

FIGURE 8. Subfamily-specific assembly of N-terminal truncation mutants. A, left, schematic representation of N-terminal truncation (ΔN) constructs (for more detail, see “Experimental Procedures”). Right, relative mean K⁺ current amplitudes (3 mM external KCl) measured from oocytes (0.1 μg/μl cRNA). No significant K⁺ current was observed for rEag1-ΔN and hErg-ΔN when we increased the cRNA concentration up to 3 μg/μl. Asterisks denote significant difference from the corresponding WT (*, Student’s t test, p < 0.05). B, biochemical investigation of the subfamily specificity of Myc-rEag1-ΔN and FLAG-hErg-ΔN with GFP-tagged WT subunits in HEK293T cells. Immunoprecipitation experiments were performed with anti-Myc or anti-FLAG antibodies. Note that N-terminal truncation mutants were effectively co-immunoprecipitated with WT subunit of the same subfamily only. C, functional examination of the subfamily specificity of rEag1-ΔN and hErg-ΔN chimeras in Xenopus oocytes. The N-terminal mutants were evaluated for their dominant negative effects on WT channels. Both truncation mutants retained their native subfamily-specific suppression properties. Asterisks denote significant difference from the water co-injection control (*, Student’s t test, p < 0.05). Error bars, S.E.
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A, top, schematic representation of the double deletion mutants rEag1-ΔN-K848X and hErg-ΔN-R1032X (for more detail, see “Experimental Procedures”). Bottom, immunoprecipitation experiments of Myc-rEag1-ΔN/FLAG-hErg-ΔN with GFP-tagged WT subunits in HEK293T cells with anti-Myc or anti-FLAG antibodies. Neither mutants were effectively co-immunoprecipitated with WT subunits.

B, top, schematic representation of the double exchange NC chimeras (for more detail, see “Experimental Procedures”). Bottom, immunoprecipitation experiments of Myc-rEag1-ΔN and FLAG-hErg-ΔN. Chimera NCs were more effectively co-immunoprecipitated with the WT subunit from the opposite subfamily.

FIGURE 9. Biochemical characterization of double deletion and double exchange constructs. A, top, schematic representation of the double deletion mutants rEag1-ΔN-K848X and hErg-ΔN-R1032X (for more detail, see “Experimental Procedures”). Bottom, immunoprecipitation experiments of Myc-rEag1-ΔN/FLAG-hErg-ΔN with GFP-tagged WT subunits in HEK293T cells with anti-Myc or anti-FLAG antibodies. Neither mutants were effectively co-immunoprecipitated with WT subunits. B, top, schematic representation of the double exchange NC chimeras (for more detail, see “Experimental Procedures”). Bottom, immunoprecipitation experiments of Myc-rEag1-ΔN and FLAG-hErg-ΔN. Chimera NCs were more effectively co-immunoprecipitated with the WT subunit from the opposite subfamily.
the entire N-terminal region failed to abolish the subfamily-specific assembly property.

One interpretation of these results is that neither the N-terminal nor the C-terminal region serves as the principal subfamily recognition domain for rEag1 and hErg channels such that we should be looking for other targets over the S1–S6 transmembrane region. Alternatively, one may argue that both the N-terminal and the C-terminal regions may serve as principal and equally important subfamily recognition domains that can take over the subunit discrimination task in the absence of one or the other.

To address these important questions, we first generated double deletion mutants (rEag1-H9004N-K848X and hErg-H9004N-R1032X), in which the entire N terminus as well as CAD/TCC were removed (Fig. 9A). The results from co-immunoprecipitation experiments demonstrated that rEag1-ΔN-K848X and hErg-ΔN-R1032X did not appreciably interact with either rEag1-WT or hErg-WT (Fig. 9A). Moreover, functional assays indicated that both double deletion mutants were non-functional proteins that failed to exert discernible dominant negative effects on either WT channel (Fig. 10). These observations therefore do not support the idea that the S1–S6 transmembrane region may harbor a potential subfamily recognition domain for rEag1 and hErg channels.

Next, we generated double exchange chimeras (chimera NC), in which both the N terminus and the post-CNBHD region were swapped (Fig. 9B). Co-immunoprecipitation studies demonstrated that rEag1-chimera NC co-existed in the same protein complex with hErg-WT (Fig. 9B). By contrast, compared with rEag1-chimera N, rEag1-chimera NC was much less effective in interacting with rEag1-WT (Fig. 9B). Likewise, in terms of subunit association, hErg-chimera NC displayed a much more prominent preference for rEag1-WT over hErg-WT (Fig. 9B). In other words, the two double exchange chimeras seemed to exhibit "reversed" subfamily specificity. To further verify this notion, we went on to look for electrophysiological evidence. Despite the fact that the functional expression of rEag1-chimera NC and hErg-chimera NC was very small in the standard cRNA concentration (Fig. 10A), significant K⁺ currents were observed when we increased the cRNA concentration up to 3 μg/μl (data not shown). Instead of rendering the chimera NCs non-functional with the pore mutations, we decided to directly co-express the chimeras with WT channels by using the standard cRNA concentration. Therefore, in addition to performing dominant negative assays, we also asked whether chimera NCs could co-assemble with WT channels to generate novel K⁺ current phenotypes that cannot be explained by a simple summation of individual current traces. Fig. 11A shows that hErg-chimera NC, but not rEag1-chimera NC, exerted a significant current suppression effect on rEag1-WT. In addition, co-expression with hErg-chimera NC resulted in a prominent deceleration of the activation kinetics of rEag1-WT (Fig. 11A). By contrast, hErg-chimera NC did not display a notable dominant negative or kinetic effect on hErg-WT (Fig. 11B). Most strikingly, co-expression of hErg-WT with rEag1-chimera NC led to a novel K⁺ current phenotype that was...
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A rEag1-WT  rEag1-NC  rEag1-WT + rEag1-NC  hErg-NC  rEag1-WT + hErg-NC

- $\bullet$ rEag1-WT
- $\bullet$ rEag1-NC
- $\blackline$ SUM of rEag1-WT & rEag1-NC
- $\blackline$ rEag1-WT + rEag1-NC co-expression

B hErg-WT  rEag1-NC  hErg-WT + rEag1-NC  hErg-NC  hErg-WT + hErg-NC

- $\bullet$ hErg-WT
- $\bullet$ rEag1-NC
- $\blackline$ SUM of hErg-WT & rEag1-NC
- $\blackline$ hErg-WT + rEag1-NC co-expression
characterized by a dramatic reduction of hErg inactivation and a remarkable deceleration of hErg deactivation (Fig. 11B). A similar K⁺ current phenotype was also observed when hErg-WT was co-expressed with rEag1-chimera C (Fig. 12A), the chimera known to interact with both rEag1 and hErg (see Fig. 4). In comparison, both hErg-chimera B and rEag1-chimera E were unable to cross the boundary of subfamily specificity (see Figs. 4 and 5) and therefore failed to significantly alter the functional expression of rEag1-WT and hErg-WT, respectively (Fig. 12B). Taken together, our data are consistent with the idea that both the N-terminal and the C-terminal regions are required to confer the subfamily-specific assembly of rEag1 and hErg subunits.

**DISCUSSION**

In the current study, we investigated the mechanism underlying the subfamily-specific assembly of rEag1 and hErg K⁺ channels. By characterizing the subunit interaction properties of a series of different chimeric and truncation constructs over the C-terminal region, we confirmed the previous report that the CAD/TCC-containing post-CNBDH region may serve as a subfamily recognition domain. On the other hand, we also demonstrated that the C-terminal subfamily recognition domain is actually dispensable for the subunit-specific assembly of rEag1 and hErg. Furthermore, we provided several lines of evidence indicating the presence of a putative N-terminal subfamily recognition domain. Importantly, exchanging either the N-terminal or the C-terminal recognition domain alone did not result in a reversal of subfamily specificity but rather led to a virtual loss of the intersubfamily boundary between rEag1 and hErg. Finally, our biochemical and electrophysiological analyses strongly suggested that both the N-terminal and the C-terminal domains need to be present in order to execute the subunit discrimination task and thereby sustain the subfamily-specific assembly.

To the best of our knowledge, this is the first report showing that the N-terminal region may serve as a subfamily recognition domain for ether-à-go-go K⁺ channels. This N-terminal subunit interaction does not seem to be required for the homotetrameric assembly of ether-à-go-go K⁺ channel subunits because N-terminal deletions involving the complete eg domain and the majority of the adjacent N-linker region still produced functional K⁺ channels for both mouse Eaq1 and hErg (31, 35). Likewise, effective subunit interaction within the same subfamily was observed for rEag1 and hErg truncation mutants lacking the CAD and the TCC domain, respectively (16–21), indicating that the putative C-terminal recognition domain is not required for the homotetrameric assembly of ether-à-go-go K⁺ channel subunits either. In addition, at least in our heterologous expression system, the molecular control system for subfamily-specific interactions seems to be satisfied with the presence of either one of the two recognition domains, as exemplified by the observation that both the N-terminal and the C-terminal truncation mutants retained their native subfamily specificity.

Overall, we propose that, similar to the formation of heterologomers by hErg 1a and 1b (34), the subfamily recognition procedure for ether-à-go-go K⁺ channels takes place early in the biogenesis process. This subunit recognition task may require concerted interactions of N/C-terminal subfamily-specific sequences, such as the previously identified tetramerizing coiled-coil structure in CAD/TCC (14, 15). We speculate that both the N-terminal and the C-terminal regions serve as equally important subfamily recognition domains that can take over the subfamily discrimination duty in the absence of one or the other. This conjecture may also explain why the chimeras harboring mixed recognition domains from the two subfamilies (i.e., chimera Cs, chimera Gs, and chimera Ns) could break the intersubfamily boundary and co-assembled with both rEag1-WT and hErg-WT. For Kv1 channels, intersubunit associations via the T1 domain are considered the initial step of tetramerization during the biogenesis and may not be required for subsequent steps in channel assembly (27, 36). It is therefore likely that once the initial subfamily recognition process is accomplished, the ensuing protein maturation process leading to the tetrameric organization of a functional ether-à-go-go K⁺ channel may instead involve subunit assembly via other protein domains, such as the S6-C-linker region (16).

The detailed structural basis underlying the N-terminal recognition domain remains elusive. Based on our finding that the subfamily barrier-breaking phenotypes of chimera Ns could not be properly reproduced when we swapped partial N-terminal sequences only (i.e. chimera Ps and chimera Os), we speculate that the N-terminal subfamily recognition domain comprises a subset of protein sequences involving both the distal eg domain and the proximal N-linker region. Alternatively, one may also argue that the N-terminal recognition domain predominantly resides in either the eg domain or the N-linker region only and that the introduction of chimeric sequences in the other part of the N-terminal region may somehow indirectly disrupt the structure of the recognition domain per se.
thereby abolishing or reducing its subfamily differentiation efficacy. Regardless of its physical nature, the N-terminal recognition domain as well as the C-terminal counterpart should be able to effectively promote or stabilize the initial assembly of structurally compatible ether-à-go-go K⁺/H11001 channel subunits in a manner similar to what has previously been proposed for the T1 domain in Kv1 channels (27). It remains to be determined, however, how the N-terminal and the C-terminal domains coordinate with each other during the subunit recognition process in the endoplasmic reticulum. One possibility is that they accomplish this task in a sequential and perhaps independent manner, with the N-terminal domain initiating a co-translational association procedure. Alternatively, given that in mature tetrameric Eag1 and hErg proteins, the N-terminal eag domain

**FIGURE 12. Alteration of hErg-WT functional phenotype by rEag1-chimera C.** A, co-expression of hErg-WT with rEag1-chimera C in Xenopus oocytes; representative current traces (left) and current-voltage relationships (right). Co-expression of hErg-WT and rEag1-chimera C manifested non-inactivating outward currents and remarkably slower tail kinetics. B, co-expression of rEag1-WT and hErg-chimera B (left panels) and of hErg-WT and rEag1-chimera E (right panels). In either case, co-expression with the chimera failed to noticeably affect the current amplitude and kinetics of the corresponding WT channel. Data were collected from 5–12 oocytes. Error bars, S.E.
seems to be able to physically interact with the C-terminal CNBHHD (26, 30, 37, 38), the initial subunit recognition process mediated by the N-terminal domain may be subject to further modification dictated by the C-terminal domain.

In KCNQ (Kv7) channels, subunit interaction and subtype-specific assembly have been shown to be determined by two coiled-coil domains in the distal C-terminal region (39). Moreover, other members of the CNBHHD-containing channel family such as cyclic nucleotide-gated cation channels and plant inward rectifying AKT1/KAT1 K⁺ channels (40–42). As in the case for ether-à-go-go K⁺ channels, their N-terminal regions have long been postulated not to contribute to intersubunit assembly, although little experimental evidence was provided to support this assumption. The findings from our study would thus open up interesting perspectives for future investigations into the possibility that the subfamily-specific assembly of these channels may also employ both N-terminal and C-terminal recognition domains.

In summary, the current study provides compelling evidence showing that both the N-terminal and the C-terminal regions are required to confer the subfamily-specific assembly of rEag1 and hERG subunits. This requirement for compatible subfamily-specific interactions in both the N-terminal and the C-terminal recognition domains may ensure a better control against erroneous intersubfamily assembly. The physiological significance of our findings is highlighted by the fact that virtually all members of the ether-à-go-go K⁺ channel family display an abundant expression in the mammalian brain, in which a wide variety of different voltage-gated K⁺ channels contribute to diverse brain functions based on distinct subunit-specific subcellular targeting to precise sites and compartments in the neuronal membrane (43). The presence of a stringent molecular control system against intersubfamily heterotetramer formation is therefore crucial for the differential subcellular localization of discrete ether-à-go-go subunits in neurons.

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