

Assembly of Voltage-gated Potassium Channels

CONSERVED HYDROPHILIC MOTIFS DETERMINE SUBFAMILY-SPECIFIC INTERACTIONS BETWEEN THE α -SUBUNITS*

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Voltage-gated potassium (K^+) channels are assembled by four identical or homologous α -subunits to form a tetrameric complex with a central conduction pore for potassium ions. Most of the cloned genes for the α -subunits are classified into four subfamilies: Kv1 (Shaker), Kv2 (Shab), Kv3 (Shaw), and Kv4 (Shal). Subfamily-specific assembly of heteromeric K^+ channel complexes has been observed *in vitro* and *in vivo*, which contributes to the diversity of K^+ currents. However, the molecular codes that mediate the subfamily-specific association remain unknown. To understand the molecular basis of the subfamily-specific assembly, we tested the protein-protein interactions of different regions of α -subunits. We report here that the cytoplasmic NH_2 -terminal domains of Kv1, Kv2, Kv3, and Kv4 subfamilies each associate to form homomultimers. Using the yeast two-hybrid system and eight K^+ channel genes, two genes (one isolated from rat and one from *Drosophila*) from each subfamily, we demonstrated that the associations to form heteromultimers by the NH_2 -terminal domains are strictly subfamily-specific. These subfamily-specific associations suggest a molecular basis for the selective formation of heteromultimeric channels *in vivo*.

Potassium (K^+) channels comprise a large family of homologous membrane proteins. They regulate cardiac pacemaking, action potentials, and neurotransmitter release in excitable tissues (1–4). In non-excitable cells, they play important roles in hormone secretion, cell proliferation, cell volume regulation, and lymphocyte differentiation (5). Four membrane bound α -subunits can form a channel, either as a homotetramer or heterotetramer (6–8). The heterogeneity of the K^+ channel function is reflected in part by a large number of K^+ channel genes and their splice variants (9). The K^+ channel diversity may also arise from the formation of heteromultimeric channels with novel properties when compared with their parental homomultimers (10–12). A combination of differential gene expression and selective formation of heteromultimeric channels by different membrane-bound α -subunits and hydrophilic

β -subunits (13–16) may allow individual cells to acquire their own characteristic properties of K^+ currents.

The known α -subunits of voltage-gated K^+ channels share a common design composed of a hydrophobic core region with six putative transmembrane segments flanked by hydrophilic cytoplasmic amino (NH_2)- and carboxyl ($COOH$)-terminal domains (17, 18). There is about 40% amino acid identity within the transmembrane core regions between α -subunits of different subfamilies, compared with ~70% identity within each of the Kv1 (Shaker), Kv2 (Shab), Kv3 (Shaw), and Kv4 (Shal) subfamily (8, 9, 19). Studies of coexpressing different subunits in *Xenopus* oocytes have indicated that the formation of heteromultimeric channels is subfamily-specific (8, 10–12, 20, 21). In addition, coimmunoprecipitation of K^+ channel protein in culture cells and in rat brain has detected the physical association of different subunits in the Kv1 subfamily (22–24). The subfamily-specific association of α -subunits provides one mechanism to prevent different subunits expressed in a cell from random mixing, thereby permitting the cell to maintain several distinct K^+ current systems (8). However, the molecular determinant(s) for such selectivity of subunit assembly is unknown.

Analysis of homomultimer formation has resulted in the identification of regions in Kv1 α -subunits that play a role in subunit assembly (21, 25–27). Possible regions of K^+ channel protein involved in subfamily-specific association were inferred by electrophysiological studies on cells that heterologously co-express two different subunits. 1) Substitution of the NH_2 -terminal domain of a distantly related mammalian K^+ channel polypeptide Kv2.1 (DRK1, Ref. 34) with that of ShB in Kv1 subfamily permits the chimeric polypeptide to coassemble with ShB (21). 2) Deletion of the conserved regions within the NH_2 -terminal hydrophilic domains of Kv2.1 and hKv1.4 channels resulted in ~100- and ~20-fold decreases in channel expression in *Xenopus* oocytes (27, 28) and the loss of subfamily-specific assembly (27). Consistent with this hypothesis, Shen and Pfaffinger (29) recently showed that the NH_2 -terminal domains of AKv1.1 of the Kv1 subfamily and that of AKv3.1 (Kv3 subfamily) form only homomultimers, as determined by coimmunoprecipitation.

To systematically determine the regions that mediate the specific interaction between different subunits, we have studied eight K^+ channel genes from the four different subfamilies, two genes (one isolated from rat and one from *Drosophila*) from each subfamily. We report here that the NH_2 -terminal domains of Kv1, Kv2, Kv3, and Kv4 subfamilies each associate to form homomultimers. Using the yeast two-hybrid system, designed to analyze heteromultimeric interactions (30–32), and homomeric interactions (32, 33) of proteins in a physiological environment, we demonstrated that the associations to form heteromultimers by the NH_2 -terminal domains are strictly

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TABLE I
Sequences of oligonucleotide primers

Oligonucleotides	Sequences ^a	Genes	Amino acid positions
ML108	GGCGTCGACG <u>GAGGTGGCAATGGTGAG</u>	Kv1.4(RCK4)	2
ML109	GACGCGGCCGCACTA <u>GCCCTGGCAGGGCTGG</u>	Kv1.4(RCK4)	310
ML111	GACGCGGCCGCACTA <u>TCTGGCGGTTGCCGAA</u>	ShB	227
ML120	GGCCCCGGGG <u>GCCGCCGTTGCCGGC</u>	ShB	2
ML125	GACGGATCCGGTCGACG <u>ATGGCAGCCGGTGTTC</u>	Kv4.2(<i>rshal1</i>)	1
ML126	GACGCGGCCGCGGATCCTCA <u>CATGGTGTGGTGTGGG</u>	Kv4.2(<i>rshal1</i>)	183
ML127	GACGGATCCGGTCGACG <u>ATGACGAAGCATGGCTCG</u>	Kv2.1(DRK1)	1
ML128	GACGCGGCCGCGGATCCTCA <u>CACCGACGAGTTGGGC</u>	Kv2.1(DRK1)	182
ML129	GACGGATCCGGTCGACG <u>ATGGGCCAAGGGGACG</u>	Kv3.1(NGK2)	1
ML130	GACGCGGCCGCGGATCCTCA <u>CCGCGCATAGCCGATG</u>	Kv3.1(NGK2)	180
ML133	GACGGATCCGGTCGACG <u>GACGCTCTAATTGTGCTG</u>	Kv4.2(<i>rshal1</i>)	40
ML134	GACGGATCCGGTCGACG <u>CACCCAGAGACCCAAACAAT</u>	Kv4.2(<i>rshal1</i>)	77
ML135	GACGGATCCGGTCGACG <u>TTGCCCTTCTTTGGCCTC</u>	Kv4.2(<i>rshal1</i>)	119
ML136	GACGCGGCCGCGGATCCTCA <u>CTCTCTGTATTGTCACTG</u>	Kv4.2(<i>rshal1</i>)	159
ML137	GACGCGGCCGCGGATCCTCA <u>GGCCACTGCATACCTTT</u>	Kv4.2(<i>rshal1</i>)	228
ML900	CAGCCCGGAACC <u>ATGGTCGGCAATTGCAAG</u>	<i>fshab11</i>	1
ML901	CAGGCGGCCGCTTA <u>CCGGCGGCCGAAACTAG</u>	<i>fshab11</i>	435
ML902	CAGAGATCTTACC <u>ATGGCCTCGGTCGCCGC</u>	<i>fshal2</i>	1
ML903	CAGAGATCTTT <u>ACGGACGACGAGAAGCTCC</u>	<i>fshal2</i>	38
ML904	CAGGCGGCCGCTTA <u>CAGGCGCTCGTCGACG</u>	<i>fshal2</i>	185
ML905	CAGCCCGGTACC <u>ATGGATCTGATCAACATGGACTC</u>	<i>fshaw2</i>	3
ML906	CAGGCGGCCGCTTA <u>CTTTGGCTGCATTGGAAGTC</u>	<i>fshaw2</i>	174
ML1009	CAGGCGGCCGCTTA <u>CTTTGGGTGATAGCGGGC</u>	Kv2.1(DRK1)	136
ML1010	CAGCCCGGGA <u>TCCGCGCCGGTGGCC</u>	Kv2.1(DRK1)	26
ML1011	CAGCCCGGGA <u>GAAAACAGGGTGGTGCTC</u>	<i>fshaw2</i>	9
ML1012	CAGGCGGCCGCTTA <u>ATGCTGTGTAGGTATCC</u>	<i>fshaw2</i>	110

^a The underlined sequence represent coding sequence or complementary to coding sequence of the indicated potassium channel genes. For all antisense primers, a stop codon (UGA) was included.

subfamily-specific. Sequence analysis of the regions required for the associations suggests that the subfamily-specific associations are mediated by a motif of a common structural design. The subfamily-specific associations of the NH₂-terminal domains provide a molecular basis for the selective formation of heteromultimeric channels *in vivo*.

EXPERIMENTAL PROCEDURES

Yeast Strains—PCY2 (*MATaDgal4Dgal80URA3::GAL1-lacZ lys2-80^{amber} His3 Δ200trp1-Δ63ILEU2 ADE2-10^{leu}*) is a gift from Dr. D. Nathans. It contains a genomic *GAL1-lacZ* reporter gene, whereas the endogenous *GAL4* and *GAL80* genes are deleted (30). YGH1 (*LYS2::GAL1UAS-GAL1tata-HIS3, URA3::GAL1-lacZ*) is a gift from Dr. D. Beach (52). In addition to the deletion of endogenous *GAL1* and *GAL80* genes, it also contains a *GAL1-HIS3* reporter gene.

cDNAs and Plasmids—The full-length cDNA clones of Shab11, Shal2, fshaw2 are gifts from Dr. L. Salkoff (19), and Kv2.1 (or DRK1) was kindly provided by Dr. R. Joho and Dr. A. Brown (34). Two hybrid vectors that carry DNA binding domain (pPC97) and transcription activation domain (pPC86) are gifts from Dr. D. Nathans (30).

Vector Construction—Plasmid vector construction was carried out using standard recombinant DNA techniques (35). The desired partial cDNA fragments were obtained by low copy polymerase chain reaction according to the procedures described previously (21). Vectors expressing GST¹ fusion proteins were constructed using pG-IKS (provided by Dr. Lewis Williams of the Chiron Corp., Emeryville, CA), which contains the GST gene and one copy of 12CA5 epitope followed by *EcoRI* restriction enzyme site (SVPYDVPDYASLGPGF). The expression is driven by the human cytomegalovirus (CMV) immediate early promoter and the plasmid contains the SV40 large T antigen. The NH₂-terminal domain of ShBA(6–46) and that of Kv4.2 were cloned in frame into the *EcoRI* site COOH-terminal to the 12CA5 epitope, resulting in two constructs: pCMV.GST-NShBA(6–46) and pCMV.GST-NKv4.2. In the yeast two-hybrid tests, the vectors for expressing different GAL4 fusion proteins were constructed by inserting the coding fragments into the *SmaI-NotI* or *SalI-NotI* sites of pPC97 and pPC86 vectors (30). The expression of DNA binding domain and transcription activation domain fusion proteins were driven by a constitutive promoter of the alcohol dehydrogenase (*ADH*) gene. The stable expression of ShBA(6–46) in

CHO cells was obtained by transfecting with pRc/CMV vector (Invitrogen, San Diego, CA) carrying ShBA(6–46). Primers used for the cloning are listed in Table I.

CHO Cell Culture and Transfection—CHO cells were grown in Dulbecco's modified Eagle's medium-F-12 medium (Life Technologies, Inc.) supplemented with 5% fetal calf serum. The stable cell line was established by transfecting CHO cell using electroporation. Briefly, 10⁶ cells at 80% confluence were harvested, washed, and resuspended in 0.5 ml of phosphate-buffered saline containing 20 μg of pRc/CMV.ShBA(6–46) plasmid DNA. The electroporation transformation was done using Bio-Rad GenePulser system with voltage gradient of 650 V/cm. After electroporation, the cells were recovered by incubating with the normal medium. At 40 h after the transfection, the medium was replaced with fresh medium supplemented with 1 μg/ml of G418 (Life Technologies, Inc.). After selection for 14 days the survived cell colonies were isolated. The expression of ShBA(6–46) was tested by whole cell recording and by immunoblot using antibody against the COOH-terminal domain (amino acids 479–656) of ShBA(6–46) (21). One cell line (ShB-CHO4) with an expression level of ~5 × 10⁵ copies/cell was identified and used in this study. In the coexpression experiments, either the pCMV.GST-NShBA(6–46) or pCMV.GST-NKv4.2 was transfected by the standard calcium phosphate precipitation protocol (37) using 10 μg of plasmid DNA/transfection. The cells were harvested for later analysis 48–60 h after transfections.

Immunoblot—Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred on a nitrocellulose filter membrane. The membrane was then incubated in a blocking solution containing 10 mM Tris, pH 8, 150 mM NaCl, 1% Tween 20, 2% bovine serum albumin, 3% normal goat serum for 30 min at room temperature. Binding to primary antibodies was effected by adding the indicated rabbit anti-serum (1:5,000 dilution). The unbound material was removed by three washes using a solution containing 10 mM Tris, pH 8, 150 mM NaCl, and 1% Tween 20. The binding of primary antibody was detected by adding anti-rabbit peroxidase-conjugated secondary antibody (1:10,000) (Vector Laboratories, Inc., Burlingame, CA). The formed complexes were visualized by enhanced chemiluminescence (ECL, Amersham Corp.).

Immunoprecipitation—Cells at 70% confluence (typically 5 × 10⁶ cells) were transfected and allowed to grow in normal Dulbecco's modified Eagle's medium plus 5% fetal calf serum for 12 h before the S³⁵-labeled cysteine and methionine (Amersham) were added. After 24–36 h of labeling, the cells were washed and lysed in a buffer containing 30 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5 mM β-mercaptoethanol, plus 2.5% CHAPS. The antibody binding was carried out at 4 °C for 2 h, and the formed complexes were brought down by protein G-conjugated Sepharose (Sigma). The immunoprecipitated

¹ The abbreviations used are: GST, glutathione *S*-transferase; CMV, cytomegalovirus; CHO, Chinese hamster ovary; CHAPS, 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonic acid; SD, minimal selective medium (37).

polypeptides were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Methods for Yeast Two-hybrid System—Vectors (pPC86, pPC97, and their derivatives) carrying coding sequence of interest were introduced into yeast cells by lithium acetate transformation according to the procedures of Gietz *et al.* (36) using approximately 1 μ g of DNA. To test the association of the two fusion proteins, the cells having both plasmids were first selected using supplemented synthetic dextrose medium containing no leucine and tryptophan. The β -galactosidase activity of liquid yeast culture (either PCY2 or YGH1) was then quantified as described (37). All tests were carried out in triplicate, and mean values were shown. The standard errors were typically 20% of the mean. The growth selection tests were carried out using transformed YGH1 host. In this strain the GAL4 transcription factor controls the expression of the *HIS3* gene, which is required for the YGH1 cells to grow in synthetic medium lacking histidine.

Amino Acid Sequence Alignment— K^+ channel sequences of the cloned cDNAs were retrieved from data base (Swissprotein, GenBank[®], and EMBL) using a key word search. Nucleotide sequences that gave different restriction enzyme digest patterns were considered to be different genes. The deduced amino acid sequences were obtained and aligned. The gap assignments were guided by computer-based neural network alignment procedures (38).

RESULTS

Specific Association of ShB to NShB (Kv1 Subfamily) but Not NKv4.2 (Kv4 Subfamily)—The involvement of the NH_2 -terminal domains of the Kv1 K^+ channels in subunit assembly has been implicated by their ability to induce the dominant suppression of channel expression and their ability to mediate coassembly (21, 26). If indeed the association of the NH_2 -terminal domain with the full-length channel polypeptide inhibits proper channel assembly, one should be able to detect this physical association. To directly identify complexes that might be formed by the full-length ShB (Kv1 subfamily) and its NH_2 -terminal domain (NShB, amino acids 1–227), we transiently expressed GST-NShB Δ (6–46) (see “Experimental Procedures”) in a CHO cell line that stably expresses ShB Δ (6–46), a mutated ShB channel with a deletion of amino acids 6–46 that corresponds to the inactivation gate (39, 40). Thus any physical association to be detected could not be attributed to the possible binding between the inactivation gate and its acceptor (41). A 72-kDa polypeptide corresponding to the size of ShB Δ (6–46) polypeptide was coimmunoprecipitated using a monoclonal antibody 12CA5 specific to the tag only present in the GST fusion proteins (Fig. 1, lanes 2, and see “Experimental Procedures”). The identity of this 72-kDa polypeptide was further confirmed by a polyclonal antibody specific to the COOH-terminal domain of the ShB Δ (6–46) protein (Fig. 1, lane 5). This association of the NH_2 -terminal domain to ShB appears to be subfamily-specific, because an NH_2 -terminal domain GST fusion protein of Kv4.2 (*rshal1* or RK5, Refs. 50 and 51), NKv4.2 (amino acids 1–183, see “Experimental Procedures”), failed to interact with ShB Δ (6–46) (Fig. 1, lane 3). These results indicate that GST-NShB Δ (6–46) fusion protein, despite its lack of transmembrane segments, binds to ShB Δ (6–46), whereas the GST-NKv4.2 fusion protein of the Kv4 subfamily fails to bind ShB Δ (6–46).

Specific Homomultimeric Interaction of Shaker NH_2 -terminal Domain Detected by the Yeast Two-hybrid System—The yeast two-hybrid system (31) was employed as an efficient way to detect the interactions between K^+ channel α -subunit interaction. In this assay, the interacting fusion proteins have to be transported into the nucleus where their physical association will reunite the DNA binding domain (DB) and transcription activation domain (TA) of the yeast GAL4 transcription factor. This results in the activation of GAL4-mediated transcription machinery. To test whether this genetic method is suitable for studying the interaction of the NH_2 -terminal domains of voltage-gated K^+ channels, we constructed two plasmids, one of

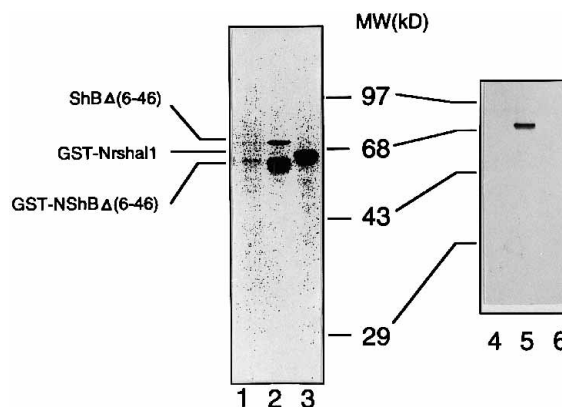


FIG. 1. Physical association of ShB Δ (6–46) with NShB Δ (6–46) detected by coimmunoprecipitation. CHO cells that express ShB Δ (6–46) were metabolically labeled after transfecting the cells with constructs that express either GST-NShB Δ (6–46) or GST-NKv4.2. The associated products were brought down by monoclonal antibody 12CA5 specific to the tag present in GST fusion proteins (see “Experimental Procedures”). The immunoprecipitated polypeptides were visualized by either autoradiography (lanes 1–3) or by immunoblot using rabbit antiserum against COOH-terminal domain of ShB (see “Experimental Procedures”) (lanes 4–6). Lanes 1 and 4, ShB Δ (6–46) expressing cells with no transfection; lanes 2 and 5, cells transfected with pCMV.GST-NShB Δ (6–46); lanes 3 and 6, cells transfected with pCMV.GST-NKv4.2.

which has the NShB fused in frame to the GAL4DB (amino acids 1–147) of GAL4, the other with NShB fused to the GAL4TA (amino acids 768–881). The expression of the fusion proteins is driven by the constitutive promoter of the alcohol dehydrogenase gene (*ADH*). These plasmids contain either the *LEU2* or *TRP1* gene for selection and *CEN2*, a yeast autonomously replicating sequence, to ensure one plasmid per cell (30). Yeast transformants were first selected using synthetic medium with supplement of histidine but no leucine and tryptophan (SDH) to identify YGH1 or PCY2 cells that carry both plasmids ($Leu^+ Trp^+$). The extent of GAL4-activated gene expression was determined by the activity of β -galactosidase in the transformed PCY2 or YGH1 cells using chromatogenic substrate, *o*-nitrophenyl- β -D-galactoside (see “Experimental Procedures”), and by the ability of the transformed YGH1 cells to grow in selection medium in the absence of histidine. Fig. 2 shows the selective growth of transformed YGH1 cells with different combinations of plasmids. Positive control cells containing GAL4DB-*c-fos* (amino acids 132–216) and GAL4TA-*c-jun* (amino acids 250–334) (*box 1*) grow on SDH and SD plates as expected (30). Transformants that carry NShB together with *c-fos*, *c-jun*, GAL4DB binding domain, or GAL4TA domains grew on SDH but not on SD medium (Fig. 2, boxes 2, 3, 5, and 6), indicating that NShB alone does not have transcription activity nor does it associate with GAL4 DB or TA domains. In contrast, coexpression of GAL4DB-NShB and GAL4TA-NShB resulted in the growth of the transformants on both SDH and SD plates (Fig. 2, box 4), which suggests that the homophilic interaction between NShB molecules could indeed bring together GAL4DB and GAL4TA to activate the expression of *HIS3*.

For a quantitative estimate of the extent of NShB-NShB interaction, a standard enzymatic assay for determining β -galactosidase activity in cell lysate was performed (Table II). The specific association of human *c-fos* and murine *c-jun* through the leucine zipper domains was used as an internal standard. The $Leu^+ Trp^+$ transformants expressing GAL4DB-NShB and GAL4TA-NShB exhibited $\sim 80\%$ of the β -galactosidase activity conferred by *c-jun* and *c-fos* interactions, which is at least 100-fold higher than the background as determined using

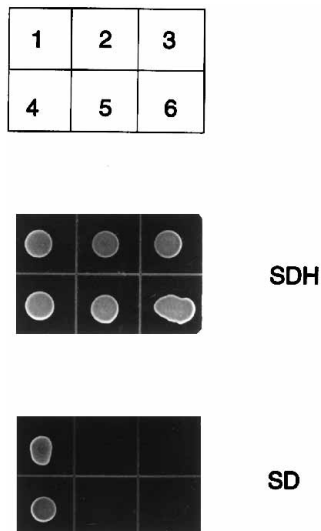


FIG. 2. Association of NShB detected by the yeast two-hybrid system. YGH1 yeast cells were transformed by different pairwise combinations of the two-hybrid constructs that express either fusion proteins of DNA binding domain of GAL4 (DB, pPC86) or transcription activation domain of GAL4 (TA, pPC97). The transformed cells expressing the two different fusion proteins were first selected by dextrose synthetic medium with supplement of histidine but no supplements of leucine and tryptophan (*SDH*). Identical numbers of cells in each combination were dotted on both SDH and SD (synthetic medium with no supplements of histidine) medium and allowed to grow at 30 °C for 65 h. *Box 1*, GAL4(DB)-*bz-fos* and GAL4(TA)-*bz-jun*; *box 2*, GAL4(DB)-*bz-fos* and GAL4(TA)-NShB; *box 3*, GAL4(DB)-NShB and GAL4(TA)-*bz-jun*; *box 4*, GAL4(DB)-NShB and GAL4(TA)-NShB; *box 5*, GAL4(DB) and GAL4(TA)-NShB; *box 6*, GAL4(DB)-NShB and GAL4 (TA).

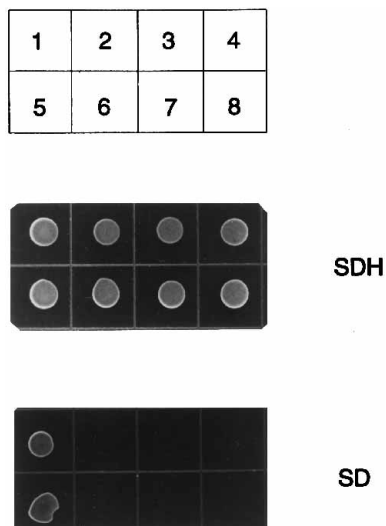


FIG. 3. Subfamily-specific association of NH_2 -terminal domains of the Kv1 subfamily. Associations of NShB with different NH_2 -terminal domains were tested by the two-hybrid system using growth selection as described in the legend to Fig. 2. In these experiments, GAL4(DB)-NShB fusion protein was coexpressed with one of the following GAL4(TA) fusion proteins: NShB (from *Drosophila*, Kv1 subfamily) (*box 1*), Nfshal2 (*Drosophila*, Kv4) (*box 2*), Nfshab11 (*Drosophila*, Kv2) (*box 3*), Nfshaw2 (*Drosophila*, Kv3) (*box 4*), NKv1.4 (rat, Kv1) (*box 5*), NKv4.2 (rat, Kv4) (*box 6*), NKv2.1 (rat, Kv2) (*box 7*), NKv3.1 (rat, Kv3) (*box 8*). The corresponding β -galactosidase activity was shown in Table II and Table III.

transformants of GAL4-DB and GAL4-TA (Table II, number 6). Transformants expressing either one of the NShB fusion proteins along with the complementary GAL4 domain showed only a background level of β -galactosidase activity consistent with the results from the tests of growth selection (Fig. 2). Coexpression of GAL4DB-NShB with GAL4TA-*c-jun* in either YGH1 or

TABLE II
Association of NH_2 -terminal domains of K^+ channels detected by the yeast two-hybrid system

DNA (5 μ g) from indicated pairs of plasmids were mixed and transformed into PCY2 strain by lithium acetate method (see "Experimental Procedures"). The cells were allowed to grow on glucose synthetic medium without leucine and/or tryptophan. Single colony (Leu+ and Trp+) from each cotransformation was picked and allowed to grow in 5 ml double selection medium. Equal number of cells were pelleted, lysed, and the β -galactosidase activity was quantified according to methods by Ausubel *et al.* (37) and calibrated against the control, *i.e.* the association between *bz-fos* and *bz-jun*, that was arbitrarily set at 100%.

No.	Double Transformants		β -Galactosidase activity
	GAL4(DB) fusion (pPC86)	GAL4(TA) fusion (pPC97)	
			%
1			<0.1
2	GAL4(DB)		<0.1
3		GAL(TA)	<0.1
4	GAL4(DB)-NShB		<0.1
5		GAL4(TA)	<0.1
6	GAL4(DB)	GAL4(TA)	<0.1
7	GAL4(DB)-NShB	GAL4(TA)	<0.1
8	GAL4(DB)-NShB	GAL4(TA)-NShB	80
9	GAL4(DB)-NKv1.4	GAL4(TA)	<0.5
10	GAL4(DB)-NKv1.4	GAL4(TA)-NKv1.4	112
11	GAL4(DB)-NKv1.4	GAL4(TA)-NShB	94
12	GAL4(DB)-NKv4.2	GAL4(TA)	<0.1
13	GAL4(DB)-NKv4.2	GAL4(TA)-NKv4.2	69
14	GAL4(DB)-NKv4.2	GAL4(TA)-NShB	<0.1
15	GAL4(DB)-NKv2.1	GAL4(TA)	<0.1
16	GAL4(DB)-NKv2.1	GAL4(TA)-NKv2.1	58
17	GAL4(DB)-NKv2.1	GAL4(TA)-NShB	<0.1
18	GAL4(DB)-NKv3.1	GAL4(TA)	<0.1
19	GAL4(DB)-NKv3.1	GAL4(TA)-NKv3.1	83
20	GAL4(DB)-NKv3.1	GAL4(TA)-NShB	<0.1
21	GAL4(DB)- <i>bz-jun</i>	GAL4(TA)- <i>bz-fos</i>	100

PCY2 cells led to no detectable growth on triple selection plates and only a background level of β -galactosidase activity (Table II and Fig. 2), revealing no nonspecific interaction between NShB to the GAL-mediated transcription machinery. Thus, it is most likely that association of NShB-GAL4 fusion proteins is responsible for the production of β -galactosidase activity and the activation of the *HIS3*.

Subfamily-specific Interactions of NH_2 -terminal Domains of K^+ Channels in Kv1, Kv2, Kv3, and Kv4 Subfamilies—In the Kv1 subfamily, subunits encoded by different genes coassemble to form heteromultimeric channels with novel properties. Using biochemical binding, we have found that their NH_2 -terminal domains also form heteromultimeric complexes (21). To determine whether this is true in the yeast two-hybrid system, we have constructed two additional plasmids expressing GAL4 fusion proteins of the NH_2 -terminal domain of Kv1.4 (or RCK4), NKv1.4 (amino acids 1–280), a rat homologue of ShB. When these fusion proteins, *i.e.* GAL4DB-NKv1.4 and GAL4TA-NKv1.4, were coexpressed in yeast, they conferred the ability of YGH1 cells to grow on SD plates and induced β -galactosidase activity (Table II). To determine whether the NKv1.4 interacts with NShB, we coexpressed either GAL4DB-NKv1.4 with GAL4TA-NShB or GAL4DB-NShB with GAL4TA-NKv1.4 in PCY2 and YGH1 cells. Indeed, the association of NShB and NKv1.4 was observed in both cases (Table II, numbers 9–11).

If the involvement of NH_2 -terminal domain in K^+ channel α -subunit assembly is a general mechanism, one would expect the interaction of NH_2 -terminal domains of other subfamilies. To test this hypothesis, we have subcloned cDNA fragments of the NH_2 -terminal domains of three rat K^+ channels into GAL4DB and GAL4TA vectors, which include NKv4.2 (amino acids 1–183 of Kv4.2, a member of the Kv4 or Shal subfamily), NKv2.1 (amino acids 1–182 of Kv2.1, Kv2, or Shab subfamily),

TABLE III
Subfamily specific association of NH₂-terminal domains detected by the yeast two-hybrid system

Double transformants (Leu⁺, Trp⁺) of YGH1 containing pair combinations of GAL4-TA fusion proteins and GAL4-DB fusion proteins were selected. The ability of association of the fusion proteins was tested by the growth on the same medium with no supplements of histidine. + indicates that the growth was detected; - indicates that no growth was detected. The number in the parenthesis indicates the relative β -galactosidase activity (%) using a procedure identical to that described in Table I. NShB represents the hydrophilic-NH₂-terminal domain (amino acids 1-227) of ShB; Nfshal2d (amino acids 38-185); Nfshabl1 (amino acids 1-435); Nfshaw2 (amino acids 3-174); NKv1.4 (amino acids 1-280); NKv2.1 (amino acids 1-182); NKv3.1 (amino acids 1-195), and NKv4.2 (amino acids 1-183).

GAL4-DB		GAL4-TA							
		NShB	Nfshabl1	Nfshaw2	Nfshal2d	NKv1.4	NKv2.1	NKv3.1	NKv4.2
NShB	(Kv1 subfamily)	+ (86)	- (<0.1)	- (<0.1)	- (<0.1)	+ (91)	- (<0.1)	- (<0.1)	- (<0.1)
Nfshabl1	(Kv2 subfamily)		+ (94)				+ (122)		
Nfshaw2	(Kv3 subfamily)			+ (78)				+ (89)	
Nfshal2d	(Kv4 subfamily)				+ (107)				+ (110)
NKv1.4	(Kv1 subfamily)	+ (97)				+ (122)			
NKv2.1	(Kv2 subfamily)		+ (144)				+ (64)	- (<0.1)	
NKv3.1	(Kv3 subfamily)			+ (131)			- (<0.1)	+ (87)	
NKv4.2	(Kv4 subfamily)	- (<0.1)			+ (119)		- (<0.1)	- (<0.1)	+ (76)

and Kv3.1 (amino acids 1-180 of Kv3.1, Kv3, or Shaw subfamily). To test the possible homophilic association of these NH₂-terminal domains, pairwise combinations of plasmids were co-transformed into PCY2 and the β -galactosidase activity was measured. Table II (numbers 12-20) summarizes the results from these experiments. Activity of β -galactosidase was detected when GAL4TA-NKv4.2 and GAL4DB-NKv4.2 were co-expressed in yeast cells. In contrast, coexpression of GAL4DB-NKv4.2 and GAL4TA produced only background β -galactosidase activity, indicating that the NH₂-terminal domain of Kv4.2 does not have transcription activation activity nor does it show nonspecific association with GAL4-TA domain. Results for the NH₂-terminal domains of Kv2.1 and Kv3.1 were also similar (Table II, numbers 15, 16, 18, and 19). Thus, the homophilic interactions of the hydrophilic NH₂-terminal domains were detected for each of the four subfamilies.

To test the ability and specificity of NH₂-terminal domains to form heteromultimers, eight K⁺ channel genes were selected as representatives of the four subfamilies: Kv1, Kv2, Kv3, and Kv4. Among them, four genes are from *Drosophila* (ShB, *fShabl1*, *fShaw2*, and *fShal2*) and four from rat (Kv1.4, Kv2.1, Kv3.1, and Kv4.2). The cDNA fragments that represent the coding sequences of the putative NH₂-terminal domains were cloned and fused in frame to GAL4TA and GAL4DB coding sequences. The ability of different fusion proteins to interact was determined by testing pairwise combinations of different NH₂-terminal domains. For example, GAL4DB-NShB was co-expressed with GAL4TA fusion proteins of NH₂-terminal domains of all eight genes, two genes (one isolated from rat and one from *Drosophila*) from each subfamily. Fig. 3 shows that these yeast transformants all grew in double selection of SDH medium with supplement of histidine, indicating the cells containing both plasmids. But the triple selection medium (SD) only allowed the growth of cells that have fusion proteins containing the NH₂-terminal domains of the same subfamily. Consistent results were also obtained using β -galactosidase assay (Table III). In summary, the heteromultimer formation of NH₂-terminal domains is strictly subfamily-specific. These results further strengthen the notion that the association of hydrophilic NH₂-terminal domains plays an important role in determining the specificity of α -subunit association to form heteromultimeric K⁺ channels.

Structural Motifs That Mediate the Subfamily-dependent Association of NH₂-terminal Domains—Detection of subfamily-specific associations of the hydrophilic domains of K⁺ channels using the yeast two-hybrid system facilitated a deletion analysis allowing the identification of regions responsible for these interactions. Fig. 4A shows that different coding segments of NKv4.2 were cloned in frame into the yeast two-hybrid vectors

to produce various GAL4TA fusion proteins. Their ability to associate with the GAL4DB-NKv4.2 was tested by the growth selection in the SD medium with no supplement of histidine and by testing β -galactosidase activity. This analysis has revealed that a fragment of 101 amino acid residues (amino acids 40-140) is sufficient for the association of NKv4.2.

Using a similar approach, regions responsible for associations of NH₂-terminal domains of other subfamilies were identified: Kv1.4 (Kv 1 subfamily), amino acids 177-279; Kv2.1 (Kv2 subfamily), amino acids 26-136; and fshaw2 (Kv3 subfamily), amino acids 9-110. Within the mapped regions, 20 amino acid residues were found to be conserved in these four α -subunits (Fig. 4B). These residues are present in the majority of the 56 members of the four subfamilies (Fig. 5).

Previous sequence comparison of K⁺ channels in different subfamilies has revealed significant conservation within the amino-terminal domains. Drewe *et al.* (42) have identified two such regions and named them A box and B box. We note that the A and B boxes are contained in our mapped regions for subfamily-specific association. Thus we name these four classes of motifs as NAB_{Kv1} (N, NH₂-terminal domain; AB, the AB boxes; Kv1, the subfamily), NAB_{Kv2}, NAB_{Kv3}, and NAB_{Kv4} (Fig. 5).

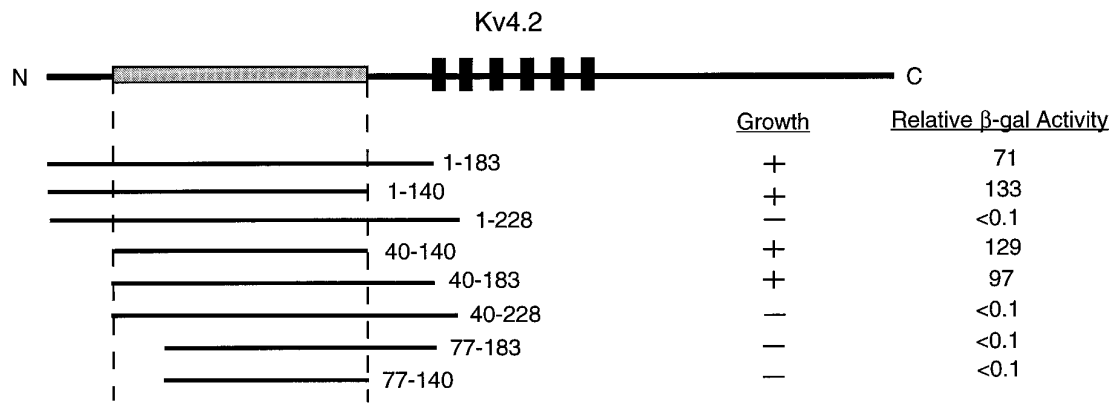
DISCUSSION

Using coimmunoprecipitation and the yeast two-hybrid system, we demonstrated that different NH₂-terminal domains interact to form heteromultimers only if they are from the same subfamily. These reported subfamily-specific associations of NH₂-terminal domains support the hypothesis that the specific interactions of NH₂-terminal domains determine the compatibility of different α -subunits in forming heterotetrameric channels.

Previous studies using pulse-chase metabolic labeling have suggested that the heteromultimeric assembly of Kv1 subfamily K⁺ channels is cotranslational (24). The extracellular NH₂-terminal domains of muscle nicotinic acetylcholine receptor (AChR), a ligand-gated ion channel, are necessary for channel assembly, and their interactions may precede the association of hydrophobic domains to form the functional channel (43). Perhaps the association of NH₂-terminal domains prior to the completion of translation helps to determine specificity and efficiency of K⁺ channel assembly. Consistent with this hypothesis, we have found that the coexpression of α -subunit of ShB together with its NH₂-terminal domain as a fusion protein leads to their physical association.

Subunit assembly of functional K⁺ channels involves two aspects: efficiency and specificity. Both are critical for cells to establish their characteristic current systems *in vivo*. Thus, a

A



B

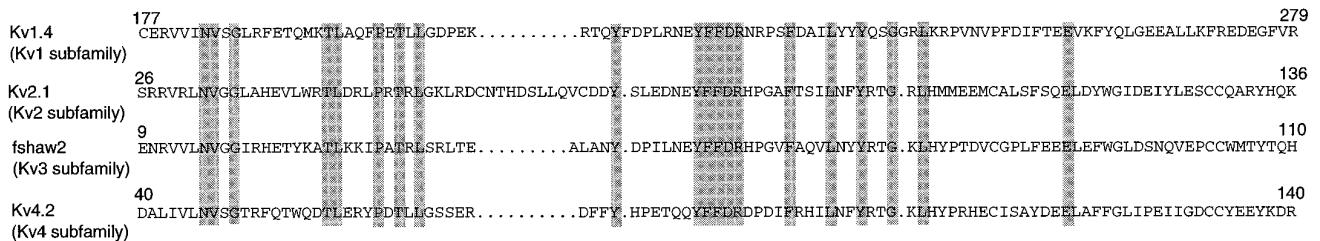


FIG. 4. Determination of four conserved motifs that mediate subfamily-specific association of NH_2 -terminal domains from Kv1, Kv2, Kv3, and Kv4 subfamilies. *A*, the schematic diagram of Kv4.2 coding region was shown. Black boxes represent the six putative transmembrane segments. The minimal region sufficient for NH_2 -terminal domain association of the Kv4.2 was determined by deletion analysis. The fragments representing different coding regions of Kv4.2 were obtained, expressed, and tested by the two-hybrid system. The ability to associate with NKv4.2 (amino acids 1–183) was determined by either growth selection or β -galactosidase activity. The + indicates that the growth; – indicates no growth. The results of β -galactosidase assay were shown as percent of the activity conferred by *jun* and *fos* interaction (Table II). A region of amino acids 40–159 (indicated by the gray box) is sufficient to mediate the association. *B*, minimal regions responsible for the associations of the other three subfamilies were mapped by deletion analysis as described above. Alignment of amino acid sequences of the four mapped motifs ($NAB_{Kv1.4}$, $NAB_{Kv2.1}$, NAB_{fshaw2} , and $NAB_{Kv4.2}$) corresponding to the four subfamilies of Kv1, Kv2, Kv3, and Kv4) was shown. The conserved positions were shaded. Single letter amino acid codes are: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; Q, Gln; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val.

region directly involved in subunit assembly should meet at least two criteria. 1) It is a site for protein-protein interaction(s). 2) Changes in this region alter the efficiency and/or specificity of assembly. The identified NAB domains meet this definition. First, the association of the NAB region has been demonstrated in Kv1 channels (21, 25) and AKv3.1 (29). In this report we have shown that the homomultimeric association of NH_2 -terminal domains can be generalized to all tested K^+ channels in all four subfamilies. Second, deletions of regions, including NABs, abolish the homomeric expression of ShB (9) and mKv1.1 (26) or reduce the expression of Kv2.1(Kv2.1) by more than 100-fold (28) and hKv1.4 by 20-fold (27). Finally, Kv2.1 of the Kv2 subfamily does not coassemble with ShB subunits of Kv1 subfamily unless its NH_2 -terminal domain is replaced with that of ShB (21). This observation is now supported at the molecular level by evidence in this report, namely, the NH_2 -terminal domains of the four subfamilies associate to form heteromultimers in a subfamily-specific manner. Hence, compatible association of NAB regions is important for both the efficiency and specificity of the K^+ channel assembly.

The yeast two-hybrid system detects the associated fusion protein complexes inside yeast nuclei. This system works only if the proteins to be tested do not possess a dominant signal that dictates their subcellular localization outside the nucleus.

We found no detectable difference in the ability of different NAB domains to reunite the two domains of GAL4 for the transcription activation regardless of whether they were attached to nuclear localization signal (NLS) of “MPKKKRKV” from simian virus 40 large tumor antigen. In contrast, if the first putative transmembrane region (S1) was included as a part of fusion protein, the association was no longer detectable (Fig. 4A). These results suggest that the NAB regions do not contain any dominant signals for membrane binding or cell surface localization in yeast. Thus, the ability and specificity of the NH_2 -terminal domain to associate do not appear to involve membrane binding but rather the specific interactions between these hydrophilic portions of α -subunits.

To directly compare the amino acid sequences of the mapped regions of the known K^+ channels, we have searched protein and/or DNA data bases. A total of 56 genes that encode K^+ channels of the four subfamilies have been retrieved and aligned using FFDR sequence that is conserved in all cloned genes listed. Guided by the computer-based neural network alignment procedures (38), several gaps were introduced to allow higher scores in alignment between different subfamilies. Three major features were seen from this analysis. First, the percentage of residue identity of the alignment (IDE) scores for the different genes encoding NAB of a given subfamily indicates remarkably high sequence conservation: IDE scores are

TABLE IV
Amino acid sequence alignment scores of NABs

NAB regions of the four *Drosophila* potassium channels (ShB, *fshab11*, *fshaw2*, and *fshal2*) were submitted for amino acid alignment against available protein sequences in the EMBL/Swissprotein data bank. The alignment procedures were done according to those established by Rost and Sander (38) (see "Experimental Procedures"). The percentage of residue identity of the alignment (%IDE) and weighted similarity of the alignment (%WSIM) of cross-alignment for the four NABs were listed.

	%IDE/%WSIM			
	NAB _{Kv1-NShB}	NAB _{Kv2-Nfshab11}	NAB _{Kv3-Nfshaw2}	NAB _{Kv4-Nfshal2}
NAB _{Kv1-NShB}	1.00/1.00	0.33/0.44	0.43/0.57	0.43/0.54
NAB _{Kv2-Nfshab11}	0.33/0.43	1.00/1.00	0.40/0.52	0.40/0.51
NAB _{Kv3-Nfshaw2}	0.40/0.53	0.50/0.59	1.00/1.00	0.43/0.58
NAB _{Kv4-Nfshal2}	0.43/0.53	0.41/0.50	0.49/0.60	1.00/1.00

NABs, analysis of a large number of NAB regions of different subfamilies using a neural network prediction program (38) suggests that NABs from different subfamilies exhibit similar secondary structure or pattern of side chain properties. This secondary structural pattern is also shared by the newly cloned Kv5.1 K⁺ channel that represents yet the fifth subfamily (46) (data not shown). Hence, a three-dimensional structure obtained from any one NAB would be likely to help our understanding on the NAB motifs of different subfamilies of K⁺ channels.

Since mutations within the regions involved in subunit assembly are likely to result in the loss of channel expression, analysis of protein interactions using the yeast two-hybrid system provides an alternative method to study the interactions of NH₂-terminal domains in the subunit assembly of K⁺ channels. This approach would allow one to screen a large number of mutants that have altered properties in K⁺ channel subunit interaction without the requirements for such mutants to form functional channels. Combining this mutational analysis with electrophysiological and cell biological methods may make it possible to analyze the molecular basis and physiological functions of subunit interactions of K⁺ channels.

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