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Assembly of Potassium Channels^a

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INTRODUCTION

Voltage-gated potassium channels are present in both excitable and non-excitable cells and serve a variety of biological functions.²⁻⁴ Unlike other channels, e.g., sodium channels and acetylcholine receptor (AChR) channels, a combination of low abundance, high heterogeneity, and lack of high affinity ligands makes the purification and biochemical analysis of potassium channels very difficult. The first potassium channel gene to be isolated was cloned from *Drosophila* and based on a mutant phenotype, the loss of an A-type potassium current in both neurons and muscles.⁷⁻¹² Subsequently, a large number of the genes and their splice variants that encode related potassium channels have been isolated from a variety of species and tissues. Electrophysiological studies of the channels expressed in *Xenopus* oocytes have shown that the functional form of the potassium channel is a multimeric protein complex, likely a tetramer.^{12,13} The cloned channels are classified into four subfamilies: Shaker, Shaw, Shal, and Shab. Only polypeptides in the same subfamily can form heteromultimeric channels when coexpressed in *Xenopus* oocytes.¹⁴ Their distinct but overlapping expression patterns in mammalian brain are compatible with the idea that the characteristics of the excitability of specific neurons derive from the particular subset of potassium channel genes that they express and the types of heteromultimeric and homomultimeric channels that are thus formed in the cell.¹⁵⁻¹⁸

Based on sequence comparison, all cloned potassium channels share a common design. Each subunit consists of a single polypeptide that can be divided into three domains: a hydrophobic domain with six putative transmembrane segments flanked by two (amino and carboxyl) cytoplasmic hydrophilic domains. The molecular events involved in the formation of functional channels are poorly understood. Here we discuss experiments indicating that the interaction between amino-terminal domains of subunits is critical for channel assembly and determines the compatibility of polypeptides in the formation of heteromultimeric channels.

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Specific Association between the Hydrophilic Amino-Terminal Domains

Comparison of amino acid sequence of potassium channels in the Shaker subfamily reveals a high degree of amino acid sequence similarity within the hydrophilic amino-terminal domains.^{18,19} This region is not dispensable since the deletion of this region in ShB (a splice variant *Shaker* gene) eliminates the functional expression.²⁰ To test whether the hydrophilic domain is involved in subunit assembly, we first asked whether this domain associates and forms multimers. The amino-terminal domain was cloned, expressed in bacteria, and purified. It behaved in gel filtration column as two discrete species corresponding to monomers (36 kD) and multimers (140 kD). The size of the multimer is consistent with that of a tetramer.¹ This indicates that there is a specific homophilic interaction involving the amino-terminal domain.

If the association is physiologically relevant, coexpression of the amino-terminal hydrophilic domain with the full length potassium channel polypeptide should result in association between amino-terminal domains that do not form channels with the full length subunits, thus reducing the number of functional channels formed by the full length polypeptides as homomultimers. To test this hypothesis, the cRNAs that encode either amino-terminal domain or the full length ShB polypeptide were coexpressed in *Xenopus* oocytes. These cRNAs have identical 5' and 3' untranslated sequences and the amount of cRNA injected was adjusted to avoid saturation of the translation machinery of the oocyte. The formation of functional channels was tested by two-electrode voltage clamp. The current amplitude decreased by at least a factor of ten when equal mass of cRNA for the amino-terminal domain and cRNA for ShB was coexpressed.¹ The probable explanation is that the amino-terminal domain co-assembles with full length polypeptides and results in nonfunctional channels.

To identify the sequences required for the homophilic interaction of the amino-terminal domain, we used a filter binding assay to test the interaction between ³²P-labeled polypeptides and immobilized polypeptides. The development of the filter binding assay is based upon the observation that many unfolded proteins can be refolded into their native conformation either in solution or after immobilization on a solid support. The specific interaction of the ³²P-labeled amino-terminal domain, but not the carboxyl-terminal domain, with the full length ShB polypeptides was detected¹ (Fig. 1). The immobilized protein preparation was the total cell lysate. Although the ShB polypeptides constituted less than 2% of the total protein loaded, they account for the only detected interaction with ³²P-labeled amino-terminal do-

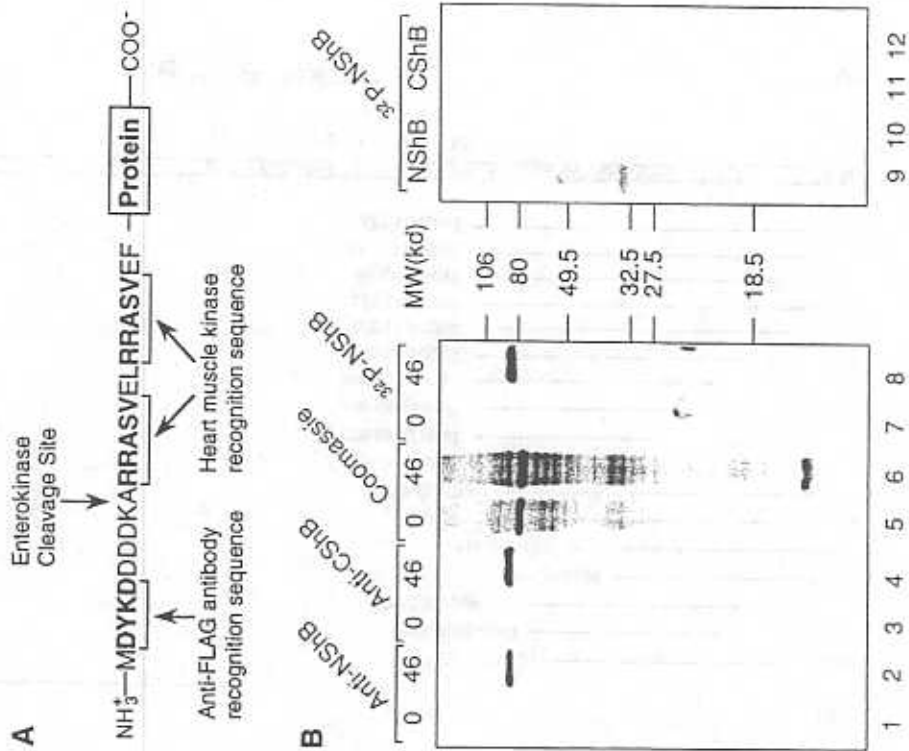


FIGURE 1. Homophilic association of ShB amino-terminal hydrophilic domains (NShB) revealed by binding ³²P-labeled NShB fusion protein to immobilized ShB and NShB. (A) Diagrammatic representation of the fusion protein expressed in bacteria. At the amino-terminus of the fusion protein is a short peptide (the "FLAG"), which is recognized by the commercially available antibody to FLAG (Inmunex Corporation), and two heart muscle kinase sites. The transcription was driven by T7 polymerase. 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. (B) Binding of NShB to ShB and NShB, but not to the carboxyl-terminal domain of ShB (CSHB). Proteins (5 µg/lane) were fractionated by SDS-PAGE (lanes 1 to 12) and either visualized by Coomassie blue staining (lanes 5 and 6) or transferred onto a nitrocellulose filter (lanes 1 to 4, 7 to 12). 0 and 46 represent total protein of Sf9 cells at 0 and 46 hours after infection by the JAI strain of recombinant baculovirus carrying ShB cDNA. The labels NShB and CShB above the blot on the right indicate lysates of IPTG (isopropyl β-D-thiogalactoside)-induced (lanes 9 and 11) and noninduced (lanes 10 and 12) bacteria that contain the expression vector for IPTG-induced expression of NShB (amino acids 1 to 227) and CShB (amino acids 479 to 656) fusion protein, respectively. Rabbit antisera to NShB (anti-NShB) and CShB (anti-CShB) were obtained by immunizing the rabbits with purified NShB and CShB fusion protein, respectively. These antibodies (dilution: 1/10,000 and 1/500) were used in immunoblots. They specifically recognize the ShB polypeptide (82 kD) expressed in Sf9 cells (lanes 1 to 4), as does ³²P-labeled NShB (lanes 7 and 8). The NShB but not the CShB fusion protein is recognized by ³²P-labeled NShB (lanes 9 to 12). (From Li *et al.* Reprinted with permission.)

FIGURE 2. A 114-amino acid fragment within the Shb amino-terminal domain (NSHB) is required for the homophilic interaction. This was determined by using ¹²⁵I-labeled NSHB as a probe to test for binding to various fragments of NSHB(B) or by using these fragments as probes to test for their binding to Shb(C). (A) (Top): A diagrammatic representation of the full-length Shb polypeptide. The shaded boxes represent the six putative transmembrane segments. The slashed box located in the hydrophilic amino-terminal domain represents amino acids 83 to 196. (Bottom) NSHB, CShb, and fragments of NSHB are produced by using expression plasmids, which include the coding sequences for the segments marked by the horizontal lines beneath the diagram of Shb. [p, plasmid; N, amino-terminal domain; C, carboxyl-terminal domain; numbers indicate the first and last amino acid residues in the fragment; pNSHBΔ17-25 and pNSHBΔ6-46 represent the amino-terminal domain (amino acids 1 to 227) with an internal deletion of amino acids 17 to 25 and amino acids 6 to 46, respectively.] (+) indicates that the fragment binds to both Shb and NSHB when used either as a probe or as an immobilized substrate. (-) indicates that no binding is detectable in either case. The smallest fragment that shows binding contains amino acids 83 to 196. (B) Sequence conservation of mapped association region among genes in the Shaker subfamily. The sequence required for NSHB homophilic association is shown by single letter code. The amino acid sequences of known K⁺ channel genes of the Shaker subfamily from different species (*Drosophila*, rat, mouse and human) are aligned with the Shb sequence from *Drosophila*. A rat gene (DRK1, Shb subfamily) is also included at the bottom. (-) indicates that the amino acid at that position is identical to that in the Shb sequence. Spaces in DRK1 sequence indicate gaps introduced. The name of each potassium channel gene is given on the left of the sequence. The position of the last amino acid is identified by the number on the right. The numbers in parentheses indicate the percentage of identity to Shb sequence in the region shown. (From Li et al. Reprinted with permission.)

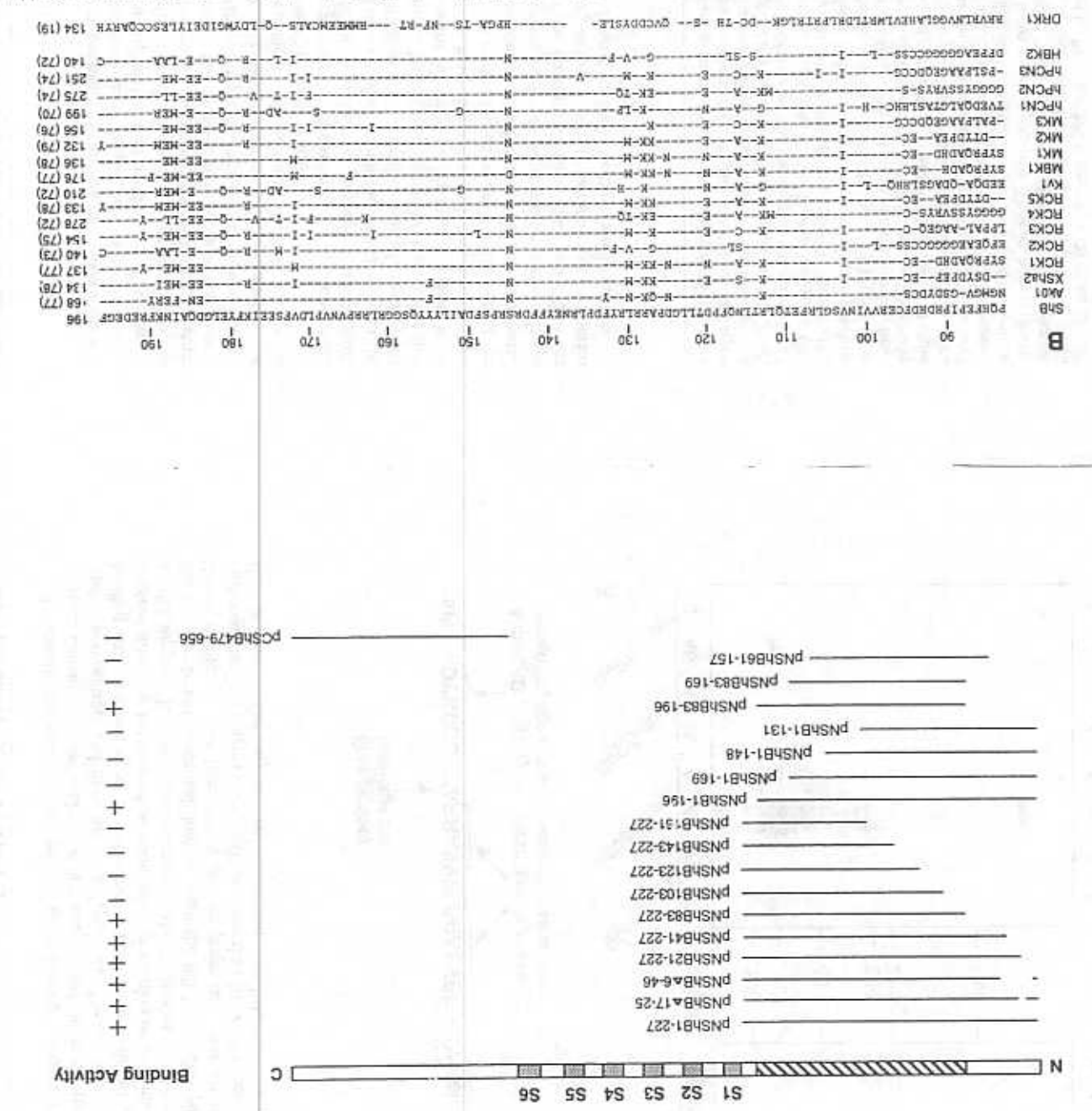


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means. The sensitivity of assay is within 100 picogram range and is partly dependent on immobilization efficiency. The minimum sequence requirement for the interaction was mapped within a fragment of 114 amino acid residues (amino acids 83 to 196 of ShB) (Fig. 2).¹

Specification of the Formation of Heteromultimeric Channels

Among genes in the Shaker subfamily, there is more than 70% amino acid identity within the 114 amino acid fragment critical for association (Fig. 2, B).¹ Most of the 30% divergence is located on the amino end of the region; it remains to be tested whether most residues between position 83 and 196 are necessary for the homophilic interaction. To test whether the compatibility between the different members of Shaker subfamily in forming heteromultimers is reflected by the ability of their hydrophilic amino-terminal domains to interact, we expressed the amino-terminal domain of a mammalian homolog of ShB, RCK1. Indeed this fusion protein can specifically associate with itself, with the full length ShB polypeptide, and with the ShB amino-terminal domain.¹

The cloned potassium channel genes have been classified into four subfamilies: Shaker, Shal, Shab, and Shaw. The amino acid identity in the hydrophobic domain is 70% for genes within a given subfamily, while this number drops to about 40% between genes in different subfamilies. Electrophysiological studies have shown that only coexpression of genes from the same subfamily in *Xenopus* oocytes will result in the formation of heteromultimeric channels. To test whether the incompatibility between potassium channels from different subfamilies could be due to incompatible interactions between their hydrophilic amino-terminal domains, we constructed a chimeric cDNA

that carries the ShB (Shaker subfamily) amino-terminal domain and DRK1 (Shab subfamily) hydrophobic and carboxyl-terminal domains. This cDNA induced a current similar to that of DRK1. Unlike DRK1, however, the chimeric channel polypeptide was able to associate with ShB polypeptides to form heteromultimers with novel kinetic properties (Fig. 3).

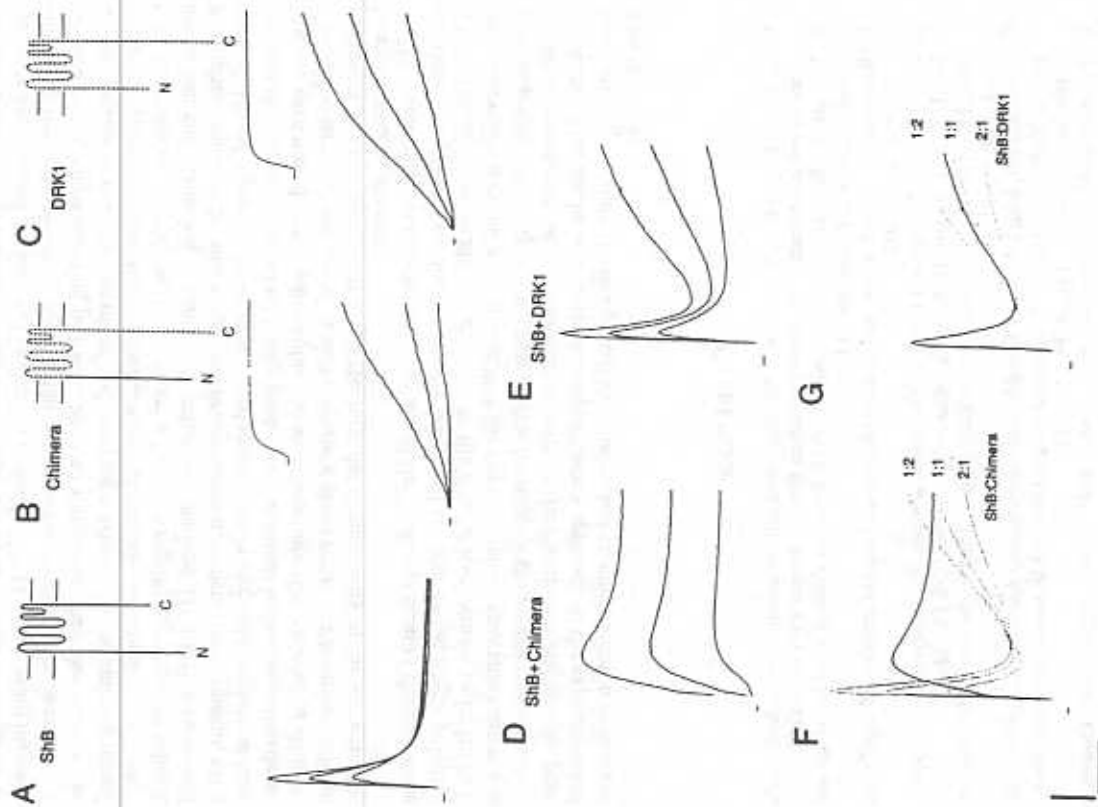


FIGURE 3. Formation of functional heteromultimeric channels by ShB and a chimera of ShB and DRK1. This chimera, NSaBΔ6-46/TmCDRK1, has the hydrophobic core region and the carboxyl-terminal domain of DRK1; the hydrophilic amino-terminal domain of DRK1 (amino acids 1 to 180) is replaced with that of ShB (amino acids 1 to 226), and an internal deletion of amino acids 6 to 46 of ShB is introduced to remove fast inactivation. This is shown schematically in the diagrams in (A) through (G). ShB: solid line, DRK1: dotted line. The chimera was functionally expressed in *Xenopus* oocytes and produced currents (B) that resembled the DRK1 K⁺ current (C); in both cases the current activated much more slowly than the ShB K⁺ current (A). Coexpression of ShB and the chimera gave rise to currents of different waveform (D) from those due to co-expression of ShB and DRK1 (E). (G) The current due to co-expression of ShB and DRK1 (elicited at +60 mV, solid line) is similar in waveform to that generated by digital addition of ShB and DRK1 currents (dotted lines, with ratios of the two currents indicated on the right of the traces); it matches the simulation of ShB:DRK1 = 1:1.2. (F) The current due to co-expression of ShB and the chimera (at +60 mV, solid line) does not match a simulation at any ratios. The currents were elicited by 85-msec test pulses at +20 mV, +40 mV, and +60 mV from a holding potential of -100 mV. The top traces in (B) and (C) were generated by 900-msec test pulses at +60 mV. The interval between test pulses was 3 seconds. The horizontal scale bar is 300 msec for inserted panels in (B) and (C) and 20 msec for all other traces. The vertical scale bar is 0.5 μA for (A), (D), (E), 0.14 μA for (B); 0.24 μA for (C); 1.1 μA for the inserted panel in (B); 1.9 μA for the inserted panel in (C). Each trace shown is representative of records from at least 4 oocytes. (From Li *et al.*, Reprinted with permission.)

DISCUSSION

Biochemical and physiological studies demonstrate that the highly conserved region in ShB hydrophilic amino-terminal domain is critical for the formation of functional channels. In addition the amino-terminal domain is the determinant for specifying the formation of heteromultimeric channels. Studies from mutagenesis in conjunction with electrophysiological analysis have shown that the hydrophobic core regions of the potassium channel are involved in channel assembly.^{20,21} The interaction between subunits in the hydrophobic domain should be critical for preserving the integrity and function of the ion conducting pathway. Such an interaction may also account for the observation that a null mutant of ShB carrying a deletion of the amino-terminal domain suppresses the formation of the functional channels by full length ShB polypeptides. In summary, we propose that there are at least two regions important for subunit interaction and formation of functional potassium channels: (1) a highly conserved hydrophilic sequence located before the first putative transmembrane segment; (2) a less well defined region in the hydrophobic domain. The hydrophobic regions, at least between the Shaker and Shab subfamily genes, are compatible in forming a functional channel, even though their hydrophobic domains show only 40% amino acid identity. The amino-terminal domain of Shaker subfamily genes *per se* can assemble to form multimers and this association may determine the compatibility in the formation of heteromultimeric channels.

The hydrophilic amino-terminal domain of the Shaker potassium channel alone dominantly suppresses the formation of a functional channel by full length polypeptides.¹ Although the detailed mechanisms at the cellular level remain to be studied, this observation itself indicates that cDNA for the hydrophilic association domain is a useful reagent for studying the physiological function of the potassium channels. It should be possible to use this construct to remove the function of endogenous gene product(s). This can be an important complementary approach to the experiments of knockout of potassium channel gene(s) by homologous recombination in embryonic stem (ES) cells.

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