

[1] Methods Used to Study Subunit Assembly of Potassium Channels

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Introduction

Expression of ion channels *in vivo* requires the specific association of various subunits. The composition and stoichiometry of various subunits in the assembled channel complexes determine many aspects of channel properties including biophysical kinetics, subcellular localization, and, ultimately, the ion conduction properties of a given cell.

Potassium channels play important roles in the cellular functions of both excitable and nonexcitable cells. The diversity of potassium currents is thought to be greatly enhanced as a result of the heteromeric association of different subunits that are encoded by more than 100 known potassium channel genes. Because each individual subunit may have distinct kinetic and conducting properties, the combinatorial assembly of various subunits provides a means of creating vast biophysical heterogeneity in both excitable and nonexcitable cells.

Thus, studies of subunit interaction are essential for understanding the molecular steps of channel expression at the posttranslational level. In addition, detailed biochemical and biophysical analyses of subunit interaction will provide important insights into the molecular coupling of subunit assembly with ion conduction in the cellular membrane. In this article, we describe several methods that we have previously used to study the subunit assembly of the Shaker-type potassium channels.¹⁻³ Both the rationale and techniques should be applicable to other ion channels.

Technical Consideration and Rationale

One powerful experimental paradigm commonly used in the structure and function studies of ion channels is to introduce specific changes into a channel subunit, for example, by site-directed mutagenesis. Comparison of the kinetic properties of mutated subunits with those of wild-type subunits allows one to infer the specific function of the mutated residue. Using this approach, elegant studies have been undertaken to identify residues or regions involved in voltage-sensing, inactivation, ion permeation/selectivity,

¹ J. Xu, W. Yu, Y. Jan, L. Jan, and M. Li, *J. Biol. Chem.* **270**, 24761 (1995).

² J. Xu and M. Li, *J. Biol. Chem.* **272**, 11728 (1997).

³ W. Yu, J. Xu, and M. Li, *Neuron* **16**, 441 (1996).

and shape of the ion channel pore.⁴⁻⁶ However, one prerequisite for the use of this approach is that the mutated channel has to possess measurable properties, for example, formation of conducting channels. If the same approach were applied to identification of residues that are important for mediating subunit assembly, critical amino acid substitutions may render the expression nonfunctional and thereby result in a null phenotype. Thus, site-directed mutagenesis alone would not be most informative as initial experiments to determine regions that are involved in subunit assembly.

To identify regions that are critical for subunit assembly, experiments are often designed on the basis of one assumption; that is, the region involved in subunit interaction is likely to play a role in subunit assembly. The first step is to identify a region(s) that is capable of binding to another subunit. After such regions are identified, the second step of an experiment can then be formulated to obtain additional evidence to test the hypothesis further. In this article, we describe three techniques: the yeast two-hybrid system, protein overlay binding, and chimeric construction/assay. The first two approaches are used to identify regions that are involved in subunit interaction. The third approach is used to probe the functional role of certain subunit associations.

Yeast Two-Hybrid System

The yeast two-hybrid system is an efficient genetic assay to test the protein-protein interaction of two potential interacting polypeptides.⁷ The method takes advantage of the yeast GAL4 transcription factor, which is composed of two functionally essential protein modules: the DNA binding (DB) domain and the transcription activating (TA) domain. To test a potential interaction between two proteins, one first needs to express them as either TA or DB fusion protein. If the two testing proteins associate, their physical association in the yeast nuclei will link the TA and DB domains to form a functional transcription factor. As a result, it permits the expression of reporter genes. Two commonly used reporter genes are *HIS3* and *LacZ*. The *HIS3* facilitates a positive growth selection, whereas *LacZ* allows a convenient enzymatic β -galactosidase assay. Thus, the detection of growth and/or β -galactosidase activity allows a genetic readout of the fusion protein interaction. We have used the yeast two-hybrid system to study the subunit interactions of the Shaker-type voltage-gated potassium

⁴ C. Miller, *Science* **252**, 1092 (1991).

⁵ L. Y. Jan and Y. N. Jan, *Annu. Rev. Physiol.* **54**, 537 (1992).

⁶ L. Salkoff, K. Baker, A. Butler, M. Covarrubias, M. D. Pak, and A. Wei, *Trends Neurosci.* **15**, 161 (1992).

⁷ S. Fields and S.-K. Song, *Nature* **340**, 245 (1989).

channels. Because nuclear localization is critical for the yeast two-hybrid system to work, the full-length membrane-bound α subunits are not suitable for this assay. However, since a key protein domain (NAB) that is involved in subunit assembly is localized in the hydrophilic cytoplasmic N terminus, expression of truncated potassium channels without their transmembrane domains has enabled us to test various α - α , α - β , and β - β interactions.¹⁻³

Strains and Reagents

Yeast Strain. HF7c [HF7c (*MAT α ura3-52 his-200 ade 2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3 URA3::GAL4_{17mer(x3)}-Cycl_{TATA}-lacZ*), a gift from Dr. David Beach, Cold Spring Harbor Laboratory].⁸

Drop-Out Mix. Combine the following dry ingredients minus the appropriate supplements (e.g., leucine, tryptophan, and/or histidine) and mix thoroughly by shaking in a 250 ml flask for 1 hr:

Adenine	0.5 g	Lysine	2.0 g
Alanine	2.0 g	Methionine	2.0 g
Arginine	2.0 g	<i>myo</i> -Inositol	2.0 g
Asparagine	2.0 g	Phenylalanine	2.0 g
Aspartic Acid	2.0 g	Proline	2.0 g
Cysteine	2.0 g	Serine	2.0 g
Glutamine	2.0 g	Threonine	2.0 g
Glutamic Acid	2.0 g	<u>Tryptophan</u>	2.0 g
Glycine	2.0 g	Tyrosine	2.0 g
<u>Histidine</u>	2.0 g	Uracil	2.0 g
Isoleucine	2.0 g	Valine	2.0 g
<u>Leucine</u>	4.0 g		

Drop-Out Media (One Liter)

6.7 g Yeast nitrogen base without amino acids (DIFCO, Detroit, MI)

2 g Appropriate drop-out mixture (His-/Trp-/Leu- or His+/Trp-/Leu-)

20 g Glucose (2% final)

(add 20 g Bacto-agar for plates)

YPD Medium. See *Current Protocols*.⁹

⁸ H. E. Feilotter, G. J. Hannon, C. J. Ruddell, and D. Beach, *Nucleic Acids Res.* **22**, 1502 (1994).

⁹ F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, "Current Protocol." Greens Publishing Associates, Inc., and John Wiley & Sons, New York, 1993.

Buffer Z (for One Liter)

100 mM NaPO ₄	16.1 g Na ₂ HPO ₄ · 7H ₂ O; 5.5 g
10 mM KCl	NaH ₂ PO ₄ · H ₂ O
1 mM MgSO ₄	0.75 g
50 mM 2-Mercaptoethanol	0.12 g
(pH to 7.0)	2.7 ml

X-Gal. Dissolve 5-bromo-4-chloro-3-indolyl- β -D-galactoside in *N,N*-dimethylformamide (DMF) at a concentration of 100 mg/ml. It can be stored at -20° for up to 1 month.

10× *TE*. 100 mM Tris (pH 7.5); 10 mM ethylenediaminetetraacetic acid (EDTA)

10× *Li-acetate*. 1.0 M Lithium acetate (adjust pH with dilute acetic acid to pH 7.5).

TE/Li-acetate. Mix 10× *TE*, 10× *Li-acetate*, and H₂O in a ratio of 1:1:8 (v/v).

TE/Li-acetate/8% glycerol. Mix 10× *TE*, 10× *Li-acetate*, glycerol, and H₂O in a ratio of 1:1:0.8:7.2 (v/v).

PLATE mix. Mix 10× *TE*, 10× *Li-acetate*, 50% polyethylene glycol (PEG 3350, Sigma, St. Louis, MO) in a ratio of 1:1:8 (v/v).

Construction of Fusion Protein Vectors

Vectors and Host Cells. We use a GAL4 centrometic vector system. For a detailed description of the DNA binding domain vector (pPC97) and the transcription activation domain vector (pPC86), please refer to Chevray and Nathans.¹⁰ This vector system maintains one copy of plasmid per cell. The HF7c strain was chosen because it gives the more stringent growth selection and higher transformation efficiency.

Procedure for Subcloning N-Terminal Domains of Shaker Potassium Channels into Yeast Fusion Protein Vectors

1. To prepare linearized template DNA, add one unit of *Eco*RI to 20 μ l restriction digestion buffer containing 2 μ g of pSK.ShB plasmid DNA (a gift of Dr. Lily Jan, University of California at San Francisco). Incubate at 37° for 30 min. Transfer 5 μ l of digestion mixture to 45 μ l of a final polymerase chain reaction (PCR) mixture (Boehringer Mannheim, Germany), containing 1 unit of *Taq* DNA polymerase and 100 nmol each of ML111 and ML120 (ML111: 5' GAC GCG GCC GCA CTA TCT GGC GGC TTG CGA AC 3'; ML120; 5' GGC CCC GGG GGC CGC CGT TGC CGG C 3'). Denature the template DNA for 5 min at 94°. Amplify the DNA fragment with 15 cycles of PCR: 94° for 1 min, 55° for 1 min,

¹⁰ P. M. Chevray and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5789 (1992).

and 72° for 1 min. Separate the PCR products on 1% agarose gel. Cut a small gel slice containing a DNA fragment ~700 bp in size that corresponds to the coding region of amino acids 2 to 227 of the N-terminal ShB (NShB). Recover the DNA fragment using the standard GeneClean protocol (Bio 101, Vista, CA). Digest the recovered DNA in a 200- μ l final volume overnight with *Sma*I at room temperature. After adjusting the buffer composition, digest the DNA with *Not*I enzyme. Precipitate the DNA fragment with ethanol, wash the pellet once with 80% cold ethanol, and resuspend the DNA pellet in 10 μ l water. Estimate the DNA concentration by running 1 μ l on a gel alongside 0.5 μ g of *Hind*III marker of known DNA concentration.

2. To prepare vector DNA, digest 2 μ g each of pPC97 and pPC86 individually in 200 μ l containing 20 units of *Sma*I at room temperature for 3 hr. Add 11 μ l of *Sal*I digestion buffer and 20 units of *Sal*I. After incubation at 37° overnight, add 5 units of calf intestine phosphatase (CIP) and incubate the mixture at 55° for exactly 30 min. Add ethylene glycol bis(β -aminoethyl ether) *N,N,N,N*-tetraacetic acid (EGTA) to a final concentration of 20 mM. Inactivate the enzyme by heating the mixture at 75° for 15 min. Extract the mixture once with an equal volume of chloroform. Precipitate the DNA with ethanol. Wash the pellet once with cold 80% (v/v) ethanol and allow the pellet to air dry. Resuspend the vector DNA pellet in 20 μ l doubly distilled H₂O. Estimate the DNA concentration as described earlier.

3. Mix 1 μ l (50 ng) of digested vector with 7 μ l (100 ng) of the digested NShB insert, and perform a 10-min ligation with a rapid DNA ligation kit (Boehringer Mannheim, GmbH, Germany). Transform 100 μ l of DH5 α cells with 10 μ l of the ligated product. Digest miniprep DNA and determine insert sequence. The resultant constructs are pPC97-NShB and pPC86-NShB.

Preparation of Yeast Competent Cells

1. Inoculate HF7c cells into 20 ml liquid YPD medium and grow overnight to $1-4 \times 10^7$ cells/ml (OD₆₀₀ 0.1–0.5) at 30° with a shaking speed of 240 rpm. Note that HF7c cells grow poorly at a higher shaking speed.
2. Dilute cells 1 : 10 in 200 ml of fresh, warm YPD medium. Growth to an OD₆₀₀ of 0.2–0.4.
3. Harvest cells by centrifugation for 8 min at 1500g at 4°. Wash once with 200 ml cold sterile water, once with 100 ml cold TE/Li-acetate, and once with 50 ml cold TE/Li-acetate/8% glycerol solution (see previous page for composition).
4. Resuspend the cell pellet with TE/Li-acetate/glycerol to a final density of 2×10^9 cells/ml. You will find that the volume of yeast cell

pellet may contribute to more than 50% of the total volume. Note that the concentration of glycerol is critical: A higher percentage will significantly decrease the competency.

5. Divide the yeast cells into aliquots of 300–1000 μl and freeze immediately on dry ice. Note that we found that storage in smaller aliquots significantly reduces the competency. The competent cells can be stored at -80° for several months at least without a significant decrease in transformation competency. We recommend that the cells always be frozen in TE/Li-acetate/8% glycerol even if transformation is going to be performed on the same day.

Yeast Transformation

1. Thaw yeast competent cells at room temperature. Vortex to make a homogeneous suspension. Thawing the competent cells on ice is not recommended, since these steps in the protocol are meant to further improve transformation efficiency.
2. Mix 50 μl of the yeast cell suspension with 0.5 μg of pPC97-NShB, 0.5 μg of pPC86-NShB plasmid DNA, and 50 μg of single-stranded salmon sperm carrier DNA in 1.5-ml microfuge tubes by vortexing at top speed for 2–5 sec. The best transformation efficiency is achieved with single-stranded carrier DNA of size range 7–9 kb.
3. Add 300 μl sterile PLATE mix, vortex thoroughly at top speed for 2 sec.
4. Incubate at 30° with a shaking speed of 240 rpm for 30 min.
5. Heat shock at 42° for 15 min.
6. The transformation mixture can be plated directly on selective medium or spun down and resuspended in an appropriate volume of sterile TE for plating.

Testing Domain Interactions by Growth

The growth assay of the yeast two-hybrid system uses the *HIS3* reporter gene as a selective marker. Interaction between the two fusion proteins allows for the expression of *HIS3* product. As a result, the yeast will be able to grow on media lacking histidine when the two testing proteins interact. If, however, the protein products of the two constructs cannot associate, the yeast will fail to grow on medium lacking histidine. In general, yeast transformant should be selected on Trp⁻/Leu⁻ medium first to ensure that the transformants carry two plasmid constructs. To test the potential NShB interaction, experimental design should include the suggested positive and negative controls of following plasmid combinations: Control 1, pPC86 + pPC97.NShB; Control 2, pPC86.NShB + pPC97; Control 3, pPC86.NShB + pPC97.NShB and Control 4, pPC86.bzJun + pPC97.bzFos.¹⁰

To test the NShB–NShB interaction, transform yeast with plasmid combination control 3. The controls include two negative controls (Controls 1 and 2) in which the yeast is cotransformed with either one of the fusion constructs and the corresponding empty vector of the other domain of GAL4, and a positive control (control 4) to rule out potential problems in the overall transformation procedures. The following two protocols describe two slightly different techniques of growth selection. The plate assay is simple and fast, while the assay of growth on liquid medium may be more quantitative and have potentially higher sensitivity to resolve variations in affinity.

Growth Assay on Plates

1. Cotransform HF7c with pPC97-NShB and pPC86-NShB as described in the previous section. Plate the transformation mixture on His⁺/Trp⁻/Leu⁻ plates, and allow yeast transformants to grow at 30° for 72 hr. Usually, yeast colonies become visible after 48 hr.
2. Inoculate a single colony from the plates into 3 ml His⁺/Trp⁻/Leu⁻ liquid medium. Allow to grow overnight at 30° at a speed of 240 rpm. Note that yeast may settle on the bottom of the culture tube by the next day.
3. Vortex to resuspend the yeast culture. Dilute it 1 : 10 with sterile TE and dot 3 μ l of diluted mixture on both His⁺/Trp⁻/Leu⁻ and His⁻/Trp⁻/Leu⁻ plates.
4. Allow to grow at 30° for 48 hr.

Procedure for Liquid Medium Growth Curve Assay

1. Follow steps 1 and 2 described in the section on Growth Assay on Plates.
2. After overnight culture, take an aliquot of the culture and measure the cell density at 600 nm. Stop the culture at an OD₆₀₀ value of 0.1–0.5. For HF7c, 1 ml of yeast cells at an OD₆₀₀ value of 0.25 has approximately 1×10^7 cells.
3. Inoculate 2×10^7 cells into two flasks containing 50 ml of either His⁻/Trp⁻/Leu⁻ or His⁺/Trp⁻/Leu⁻ medium. The starting OD₆₀₀ value should be about 0.01.
4. Take the OD₆₀₀ measurement and/or cell counts every 2–3 hr until 15 hr after cultures reach the stationary stage.
5. Plot logarithmic OD versus time.
6. Determine the doubling rate at log phase and the OD at stationary stage. (Note that HF7c cells with a plasmid combination of pPC97-NShB/pPC86-NShB have a doubling rate of 2.3 hr and the saturation OD₆₀₀ value is about 1.6 in His⁻/Trp⁻/Leu⁻ medium.)

Testing Domain Interactions by β -Gal Assay

Two protocols have been commonly used to test β -Gal activity. Here we describe the procedure for performing the β -Gal assay on a membrane support. Refer to *Current Protocols* for the liquid culture β -Gal assay.⁹

Screen Yeast Colonies with β -Gal Assay on a Nitrocellulose Filter

1. Wear gloves for all steps. Label the nitrocellulose filter with a fine marker. Carefully place the filter onto the plate to be assayed.
2. Slightly bend the filter in half, allowing first the center and then the edges to touch the plate. This will help to prevent air bubbles between the plate and the filter.
3. Mark with a syringe needle to orient the filter.
4. Lift the filter with a blunt-ended forceps. Submerge immediately in liquid nitrogen (for 5 sec to 5 min). The best way to do this is to place the filter into the liquid nitrogen vertically at a slight angle, so that the colonies face down and hit the liquid nitrogen first. This seems to keep the filter from cracking into pieces, and is particularly important when lifting large colonies.
5. Remove the filter from liquid nitrogen, and place the filter on Whatman 3 MM (Clifton, NJ) paper with colony side up. Allow to thaw for about 2 min, but do not allow the filter to dry out completely. They are sometimes difficult to rewet.
6. Place the filter (with the colony side up) on Whatman 3 MM papers that are presoaked with 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) in buffer Z (about 7–8 ml is needed for a 150-mm-diameter filter).
7. Incubate the filter in a sealed container at 37°. Visible blue color may develop in 30 min to 3 hr.

Protein Overlay Binding

The protein overlay assay tests the direct interaction between a soluble protein (domain) of interest and a target protein immobilized on membrane support. This method was first developed for studying DNA–protein interaction.¹¹ We have expressed the N-terminal domain of ShB (NShB) as a fusion protein containing two heart muscle kinase sites (RRASV) to allow radioactive labeling, and a Flag monoclonal tag sequence (DYKD) for affinity purification and immunodetection.^{12,13} The binding partner in this

¹¹ B. Bowen, J. Steinberg, U. K. Laemmli, and H. Weintraub, *Nucleic Acids Res.* **8**, 1 (1980).

¹² M. Li, Y. Jan, and L. Jan, *Science* **257**, 1225 (1992).

¹³ M. A. Blonar and W. J. Rutter, *Science* **256**, 1014 (1992).

case is either the full-length ShB protein or another N-terminal domain from a homologous K⁺ channel subunit. Protein-protein interaction can be detected using a radioactively labeled NShB probe or by immunodetection using an antibody specific to the probe. The binding can then be visualized with HRP-conjugated secondary antibody followed by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL).

Radioactive Labeling of NShB

1. Express and purify the NShB in *Escherichia coli* as a fusion protein using a standard protocol. The purified protein is diluted to 100 μg per ml in PBS. Carry out the labeling reaction as follows:

6 μl	10 \times heart muscle kinase reaction buffer [200 mM Tris pH 7.8, 10 mM dithiothreitol (DTT), 1 M NaCl, 120 mM MgCl ₂]
10 μl	[γ - ³² P]ATP 10 $\mu\text{Ci}/\mu\text{l}$ (Amersham, Arlington Heights, IL)
15 μl	Purified NShB fusion protein
27 μl	Deionized H ₂ O
2 μl	10 units/ μl heart muscle kinase (Sigma). The enzyme should be freshly made in 40 mM DTT

60 μl

2. Incubate the mixture at 37° for 1.5 hr, then dilute to 500 μl with HKE buffer containing 10 mM HEPES (pH 7.5), 60 mM KCl, and 1 mM EDTA.

3. To remove the free radioactive material, dialyze the labeling mixture against 200 ml of HKE buffer for 5 hr with five buffer changes. Count an aliquot of labeled protein sample and estimate the specific activity. We use probes with specific activity above 5×10^4 cpm/pmol.

Procedure for Using Radioactive Protein Probes

1. Protein preparations [e.g., crude lysate of baculovirus infected *Spodoptera frugiperda* fall armyworm ovary (Sf9) cells] are fractionated by SDS-PAGE. The separated proteins are electrophoretically transferred to a nitrocellulose filter using a standard transfer procedure. All remaining steps should be carried out at 4° and all buffers should be chilled on ice prior to use.
2. Rinse the filter for 5 min with buffer A containing 10 mM HEPES-KOH, pH 7.5, 100 mM KCl; 1 mM EDTA, 1 mM 2-mercaptoethanol.
3. To initiate the denaturation and renaturation, treat the filter for 15 min with buffer A supplemented with 6 M guanidine hydrochloride.

4. To renature the protein, treat the filter stepwise with buffer A supplemented with a decreasing concentration of guanidine hydrochloride (3, 1.5, 0.75, 0.38, 0.19, and 0.09 M). Each wash lasts for 5 min.
5. Rinse the filter with buffer A twice, for 10 min each time.
6. To block nonspecific binding, incubate the filter with buffer A, 5% (w/v) Carnation nonfat milk, 0.05% (w/v) Nonidet P-40 (NP-40), 60 min at 4°. Transfer the filter to buffer A, 1% Carnation milk, 0.05% NP-40, and incubate for 30 min.
7. To carry out protein binding, transfer the filter to buffer A containing 0.05% NP-40 and supplement with ³²P- or ³⁵S-labeled protein probe. Incubate for 12 hr. It is necessary to remove free radioactive material by either dialysis or chromatography.
8. Wash the filter for 30 min with buffer A supplemented with 1% Carnation milk and 0.05% NP-40 with three buffer changes.
9. Wrap the filter with Saran wrap. Visualize the binding signal by autoradiography.

Procedure for Using Probe-Specific Antibodies

1. Follow the procedures for using radioactive protein probes from steps 1 to 6.
2. Incubate the filter with purified protein probe for 12 hr in buffer A containing 1% Carnation milk and 0.05% NP-40. The preferred probe concentration is 2 µg/ml.
3. Wash the filter twice with buffer A containing 1% Carnation milk and 0.05% NP-40.
4. Incubate with first antibody 1:5000 specific to the probe in buffer A containing 1% Carnation milk and 0.05% NP-40, for 30 min.
5. Wash three times with buffer A containing 1% Carnation milk and 0.05% NP-40.
6. Incubate with horseradish peroxidase (HRP)-conjugated secondary antibody 1:5000 specific to the first antibody in buffer A containing 1% Carnation milk and 0.05% NP-40, for 30 min.
7. Wash three times with buffer A containing 1% Carnation milk and 0.05% NP-40.
8. Incubate the filter with chemiluminescence reagents (Amersham).

Construction of Chimeric Channels

After identification of a protein-protein interaction region within a given subunit, it is essential to establish its functional role(s) in channel assembly or properties. In the Shaker-like potassium channels, different α

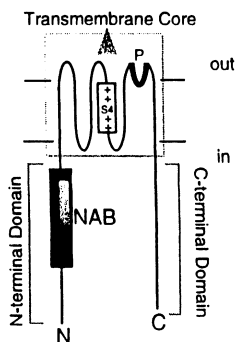


FIG. 1. Schematic diagram of the Shaker-type potassium channel.

subunits coassemble in a subfamily-specific manner, consistent with the binding specificity of the NAB–NAB interaction (Fig. 1). The chimeric channels are designed to test whether the NAB–NAB association plays a role in specifying the interacting subunit (see article in this volume dedicated to chimeric construction¹⁴).

Vector

The pShBΔ(6-46).2H3 plasmid is a pSK⁺-based vector that contains ShBΔ(6-46) cDNA (a gift from Dr. Richard Aldrich, Stanford University). Two *Hind*III restriction sites were introduced by site-directed mutagenesis using the following oligonucleotides: ShBhind3s: 5' GAA AGT TCG CAA GAT TCC AGA GTT GTA GCC 3', and ShBch3s: 5' CGT CGG TCT CGA TAC AAG CTT CCA GGG CAT TAT TGT G 3'. One *Hind*III site is located at amino acid 226 immediately prior to the S1 region; the other is located at amino acid 650 six amino acid residues prior to the stop codon. Introduction of these restriction sites produces a functional channel indistinguishable from the wild-type ShB.¹²

Procedure for Constructing Chimera

1. To prepare vector DNA, digest 10 μg of pShBΔ(6-46).2H3 in 100 μl containing 5 units of *Hind*III at 37° for 2 hr.
2. Add 5 units of CIP and incubate the mixture at 55° for exactly 30 min. Add EGTA to a final concentration of 20 mM. Inactivate the enzyme by heating the mixture at 75° for 30 min.

¹⁴ T. M. Ishii, P. Zarr, X. Xia, C. T. Bond, J. Maylie, and J. P. Adelman, *Methods Enzymol.* **293**, [4], 1998 (this volume).

TABLE I
METHODS USED TO TEST SUBUNIT INTERACTIONS IN ION CHANNELS

Method	Reagent requirements	Advantages	Disadvantages	Additional comments
Yeast two-hybrid system	Hydrophilic protein/donors; no toxicity to nuclear transport; need to have cDNA of two interacting partners	No biochemical assays involved	Negative results are not informative	Amenable for large-scale genetic screen
Protein overlay	Soluble purified probe protein is preferable; probe can be obtained by <i>in vitro</i> translation	Simultaneous testing of multiple interacting proteins including unknown proteins from crude cell extracts	Not all protein-protein interactions can be detected presumably due to denaturation from filter immobilization	It tests direct protein-protein interaction
Coimmunoprecipitation	Antibodies against both testing proteins are required	Can be done with native preparation. May be used for studying multimeric complex	Interaction could be indirect	Solubilization step is critical and sometimes tricky
Gel-filtration chromatography	Purified soluble protein preparation	Can be used to determine stoichiometry in combination with sedimentation	Homogeneous biochemical behavior is necessary	Specific antibody is required if there is no adequate amount of proteins
Sucrose gradient sedimentation	Purified soluble protein preparation	(Same as gel filtration)	Homogeneous biochemical behavior is necessary	(Same as gel filtration)
Chemical cross-linking	Purified soluble protein preparation	Can provide information of stoichiometry	Cross-linking conditions varied with different protein interactions and amino acid composition	Careful titrations are needed to determine the type and concentration of chemical cross-linker
Column binding/pull down	Soluble on column should be purified (not necessarily soluble)	Can use tissue extract (requires antibody)	One of the test proteins needs to be soluble	Antibody specific to testing protein may be necessary
Dominant suppression	Electrophysiologic or ligand binding assays	Direct functional test	Ability to suppress expression may not directly correlate its physiological association with functional subunits	Conclusion is based on negative result

3. Extract the denatured protein once with an equal volume of chloroform. Precipitate the DNA with ethanol. Resuspend the vector DNA pellet with 20 μl water.
4. Estimate the vector DNA concentration by running 1 μl on a gel alongside 0.5 μg of λ *Hind*III marker.
5. To prepare linearized DNA for PCR amplification, add one unit of *Eco*RI to 20 μl restriction digestion buffer containing 2 μg plasmid DNA of pSK.DRK1 (a gift of Drs. Rolf Joho at Univ. of Texas Southwestern, and Arthur Brown at Case Western Reserve Univ.). Incubate at 37° for 30 min.
6. Transfer 5 μl of the digestion mixture to 95 μl of PCR reaction mixture containing 100 nmol of DrkTM1s and Cdrk primers (DrkTM1s: 5' GAT GAA TTC CAA GCT TCG GTG GCC GCC AAG A 3'; Cdrk: 5' GGA TAA GCT TCC CGG GGG GAG CTC AGA TAC TCT G 3'). Denature the template DNA for 5 min at 94°. Amplify the DNA fragment with 15 cycles of PCR: 94° for 1 min, 55° for 1 min, and 72° for 2 min.
7. Fractionate the PCR products on 1% agarose gel. Cut a small gel slice containing a fragment ~2.0 kb in size. Recover the DNA fragment using the standard GeneClean protocol as described earlier. Digest the recovered DNA overnight with *Hind*III enzyme in a 200- μl final volume. Precipitate the DNA fragment with ethanol and resuspend the DNA pellet in 10 μl water. Estimate the DNA concentration by running 1 μl on a gel alongside 0.5 μg of λ *Hind*III marker.
8. To ligate, mix 200 ng of linearized, phosphatase-treated pShB Δ (6-46).2H3 with a twofold molar excess of the insert DNA. Add ligase buffer, 1 unit of ligase (New England Biolabs, Beverly, MA), and water to a final volume of 10 μl . As a control, set up an identical reaction without the insert DNA. Incubate overnight at 14°.
9. Transform ligated DNA into *E. coli*. Prepare miniprep DNA with the Promega (Madison, WI) Wizard system. Determine the insert and orientation by restriction enzyme digestion. Confirm the PCR fragment by DNA sequencing. This construct is now ready for preparation of cRNA for oocyte expression. The resultant chimeric channels can then be tested by electrophysiologic analyses and by toxin-binding studies.

Conclusion

The methods described in this article are part of a long list of methods that has been used to study subunit interaction in ion channels (see summary

in Table I). Some of the listed methods have been discussed in other articles in this volume.¹⁴⁻¹⁶

The methods in our chapter are particularly suitable for testing the interaction between two hydrophilic polypeptides, such as the N-terminal interaction domains of the Shaker-type potassium channels. The yeast two-hybrid system is a powerful genetic assay and can be modified for a genetic screen to identify interesting mutations that modulate protein-protein interactions, such as temperature-sensitive mutants and genetic enhancers and suppressors. The overlay binding assay tests the direct interaction between two proteins. Although it may not work for every protein, positive results are usually very convincing since it requires relatively high affinity association to be detectable. Finally chimeric construction and testing assembly specificity is a functional approach applicable to both hydrophilic and hydrophobic regions by directly studying their roles in the subunit assembly of a functional channel.

¹⁵ Z. Sheng and C. Deutsch, *Methods Enzymol.* **293**, [2], 1998 (this volume).

¹⁶ J. S. Trimmer, *Methods Enzymol.* **293**, [3], 1998 (this volume).