7

Overlay and Bead Assay

Determination of Calcium Channel Subunit Interaction Domains

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1. Introduction

Based on electrophysiological and pharmacological properties, voltage-dependent Ca^{2+} channels are classified as L-, N-, T-, and P/Q- type Ca^{2+} channels *(1,2)*. The brain N-type Ca^{2+} channels *(3)* and the skeletal muscle L-type *(4,5)* have both been purified and shown to have a similar subunit composition (α_1-, α_2/δ-, and β-subunits).

Complementary DNA studies have identified six different genes encoding the α_1-subunits (S, A, B, C, D, and E) and four β-subunit genes, each of which can form multiple-splice variants (reviewed in **ref. *6***). Electrophysiological studies have shown that all of the α_1- and β-subunits cloned to date from different tissues can associate and yield Ca^{2+} channels with characteristic current amplitude, voltage dependencies of activation, and inactivation properties (reviewed in **ref.** *7*). This suggests that these two proteins are directly interacting, possibly through a common binding domain. Several complementary approaches have recently been developed and used in our laboratory to examine these binding events in greater detail. The first approach that was taken to characterize the subunit–subunit interactions, and to determine the interaction motif on the α_1-subunit for the smaller β component in voltage-dependent Ca^{2+} channels, exploited the overlay binding assay *(8)*. Radiolabeled in vitro-translated β-subunit probes were prepared and shown to bind to the α_{1S} of the purified dihydropyridine receptor after electrophoresis and transfer onto nitrocellulose membranes. The subsequent screening of an α_{1S} epitope expression library with similar radiolabeled β-subunit probes facilitated the identification of a conserved binding motif that is present on all of the α_1-subunits cloned to date,

From: *Methods in Molecular Biology, Vol. 88: Protein Targeting Protocols*
Edited by: R. A. Clegg Humana Press Inc., Totowa, NJ

namely, the α interaction domain (AID). Site-directed mutagenesis of residues within the AID consequently revealed precise amino acids that are essential for this association, in addition to being critical for β-subunit-induced current stimulation.

To investigate the complementary binding motif on the β-subunit or β interaction domain (BID), the strategy taken was quite different (*9*). Preliminary experiments showed that it was not possible to overlay in vitro-translated α_1-subunits onto the denatured β-subunits immobilized on nitrocellulose; thus, screening a β-subunit epitope library to identify the minimum sequence could not be performed. As an alternative approach, truncated [^{35}S]-labeled β-subunit fragments were generated and these were overlaid on the AID_A GST-fusion protein on nitrocellulose membrane. After narrowing the BID to approx 40 amino acids, the essential residues that were required for this interaction were also established by site-directed mutagenesis of amino acids within the BID motif. This interaction was characterized further by expression of truncated β-subunits in *Xenopus* oocytes, together with the full-length α_{1A} and $\alpha_2\delta$-subunits and the measurement of functional changes in the current stimulation, inactivation kinetics and voltage-dependence of the resultant current. Since the functional assays that were used to test the effects of the β-subunit and truncated mutants on the α_1-induced currents can only be applied to ion channel-subunit interactions, and have been previously well-described (*10*), these methods will not be described here. The aim of this chapter is to describe the different experimental approaches that were used to determine the interaction domains through which these two Ca^{2+} channel subunits associate (*see* **Note 1**).

2. Materials

2.1. Overlay Binding Assay

1. $\beta_{1\beta}$-subunit in pcDNA3 vector (construct of interest containing a T7, SP6, or T3 RNA polymerase recognition site).
2. [^{35}S]-Methionine (Amersham [Arlington Heights, IL]).
3. Rabbit reticulocyte TNT in vitro translation kit (Promega [Madison, WI]).
4. RNasin Ribonuclease inhibitor (BMB [Indianapolis, IN]).
5. Nuclease free H_2O.
6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 3–12% gradient gel (*11*).
7. Purified dihydropyridine receptor prepared as detailed elsewhere (*5*).
8. 30°C Heating block.
9. Cocktail of protease inhibitors: Pepstatin A, chymostatin, aprotinin, antipain, and leupeptin, each at a final concentration of 0.1 μg/mL (Sigma [St. Louis, MO]).
10. Calf liver tRNA, final concentration 40 μg/mL (Sigma).
11. PD 10 column (Pharmacia [Piscataway, NJ]) contains G-25 Sephadex.
12. G-50 Sephadex resin (Pharmacia).

13. Nitrocellulose membrane (Pharmacia).
14. Phosphate-buffered saline (PBS): 150 m*M* NaCl, 50 m*M* sodium phosphate, pH 7.5.
15. Blocking solution I: 5% nonfat dry milk prepared in PBS, pH 7.5.
16. Blocking solution II: 0.1% gelatin, 5% bovine serum albumin (BSA), 0.1% Tween-20 in PBS, pH 7.5.
17. Blocking solution III: 5% BSA, 0.5% nonfat dry milk in PBS, pH 7.5.
18. Overlay buffer I: same as blocking solution III.
19. Overlay buffer II: 150 m*M* NaCl, 20 m*M* HEPES, 2 m*M* $MgCl_2$, 2 m*M* dithiothreitol, and 5% BSA, pH 7.5.
20. X-ray film (Kodak).

2.2. Preparation and Use of an α_1 Epitope Library to Identify the AID

1. 10X DNase reaction buffer: 100 m*M* $MgCl_2$, 200 m*M* Tris-HCl, pH 7.5, 0.25 mg/mL BSA.
2. RNase free DNase (Promega).
3. Seakem LE agarose gel (FMC Bioproducts [Rockland, ME]).
4. 1-kb DNA markers (BRL).
5. Qiaex gel extraction kit (Qiagen).
6. Phosphorylated *Eco*RI linkers (8-mer) (NEB).
7. T4 DNA ligase (1 U/μL) (BMB).
8. *Eco*RI enzyme (20 U/μL) (NEB).
9. Phenol-chloroform-isoamyl alcohol (25:24:1) (U.S. Biochem).
10. Spectrophotometer.
11. λ-gt11/*Eco*RI/CIAP-treated vector and packaging kit (Gigapack II Gold; Stratagene).
12. Suspension media (SM): 5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 mL 1*M* Tris-HCl, pH 7.5, 5 mL 2% (w/v) gelatin, H_2O to 1 L. Sterilize.
13. Chloroform (Kodak).
14. Y1090 *E. coli* cells.

2.3. Expression, Growth, and Purification of GST-Fusion Proteins

1. 50 mL LB media containing 50 μg/mL ampicillin.
2. 300-500 mL LB media containing 50 μg/mL ampicillin.
3. 1*M* IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma).
4. JA 10 centrifuge bottles.
5. Stock protease inhibitors: 0.2*M* benzamidine and 0.1*M* phenylmethylsulfonylfluoride (PMSF) prepared in ethanol and stored in a brown bottle.
6. Ice-cold PBS: 150 m*M* NaCl, 50 m*M* sodium phosphate, pH 7.5, containing 0.83 m*M* benzamidine, 0.23 m*M* PMSF.
7. 10% (w/v) Triton X-100.
8. JA 17 centrifuge tubes.
9. 2 mL Glutathione–Sepharose (Pharmacia) in a 10-mL plastic syringe plugged with a glass fiber filter.

10. Ice-cold 1% Triton X-100 in PBS containing 0.83 m*M* benzamidine and 0.23 m*M* PMSF, pH 7.5.
11. Ice-cold 50 m*M* Tris-HCl, pH 8.0, containing 0.83 m*M* benzamidine and 0.23 m*M* PMSF, with and without 10 m*M* glutathione.
12. 3–12% SDS polyacrylamide gel *(11)*.
13. 20% (w/v) sarcosyl in H_2O.

2.4. Identification of the BID, Using the Overlay Assay

1. β_{1b} plasmid *(12)*.
2. In vitro-translated truncated β_{1b}-subunit in pGEM3 expression vector.
3. Qiaex DNA purification kit (Qiagen).
4. 3–12% SDS polyacrylamide gel *(11)*.
5. Nitrocellulose membrane (Pharmacia).
6. X-ray film (Kodak).

2.5. Measurement of the Affinity of the Interaction Between the α_1- and β-Subunits

1. Glutathione–Sepharose (Pharmacia).
2. In vitro-translated [^{35}S]-labeled-β_{1b}-subunit.
3. Ice-cold PBS: 150 m*M* NaCl, 50 m*M* sodium phosphate, pH 7.5, containing protease inhibitors.
4. Grafit data-fitting program (Sigma).
5. Scintillation counter for quantifying [^{35}S]-β-subunit.

2.6. Purification of Native Ca^{2+} Channel β-Subunits with the AID

1. CNBr-activated Sepharose (Sigma).
2. Purified AID GST-fusion protein (purified as detailed in **Subheading 2.3.**).
3. Cocktail of protease inhibitors: 76.8 μ*M* aprotinin, 0.83 m*M* benzamidine, 1.1 μ*M* leupeptin, 0.7 μ*M* pepstatin, 0.23 m*M* PMSF.
4. 3–12% SDS polyacrylamide gel and nitrocellulose membrane.
5. β-subunit-specific antibodies *(13)*.
6. Homogenization buffer: 10 m*M* HEPES buffer, pH 7.4, 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 1*M* NaCl containing cocktail of protease inhibitors.

3. Methods

3.1. Overlay Binding Assay

The principle of the overlay binding assay for determining subunit–subunit interactions requires that the binding conformation is maintained after separation of either one of the proteins in question by SDS-PAGE. The preparation of a radiolabeled derivative of the other subunit facilitates detection of the interaction following association. This section describes the development of an overlay binding assay to characterize the interaction of the α_1- and β-subunit of Ca^{2+} channels.

3.1.1. Synthesis of Labeled β-Subunit Probe

Although newer methods of protein biotinylation are being developed, we utilized [^{35}S]-methionine-labeled in vitro translation products. It is possible, however, to incorporate other radiolabeled amino acids during translation (i.e., [^{35}S]-cysteine), if the probe of interest does not contain any methionine residues.

3.1.1.1. Designing a Construct for In Vitro Translation

When designing a construct for efficient in vitro translation, it is of utmost importance that the cDNA sequence contains a strong translation-initiation site (i.e., Kozak consensus sequence *[14]*). In addition, a promotor/enhancer such as Alfalfa mosaic virus (AMV) or cytomegalovirus (CMV), is required for good synthesis. Often, the transcription of probes is from a T7 polymerase recognition site, although SP6 or T3 RNA polymerase-driven transcription is adequate.

3.1.1.2. In Vitro Translation Procedure

All the reagents for translation are thawed after storage at –70°C and immediately placed on ice. A typical reaction is performed as follows (*see* **Notes 2–5**):

TNT Rabbit reticulocyte lysate	25 µL
TNT Reaction buffer	2 µL
TNT RNA polymerase (SP6, T3, or T7)	1 µL
Amino acid mixture minus methionine, 1 m*M*	1 µL
[^{35}S]-methionine (1000 Ci/mmol) at 10 mCi/mL	4 µL
RNasin ribonuclease inhibitor 40U/µL	1 µL
DNA template (pGEM3-β_{1b})	1 µg
Nucl ease free H_2O	to a final volume of 50 µL

The success of the translation can readily be tested by analysis of the translated β-subunit by SDS-PAGE, followed by autoradiography **(Fig. 1A)**.

3.1.2. Determining the Interaction of the α_1–β-Subunits by the Overlay Assay

Assessing the interaction between the two Ca^{2+} channel proteins is achieved after separation of the components of the purified skeletal muscle dihydropyridine receptor on a 3–12% SDS gel, electrophoretically transferring the gel onto nitrocellulose membrane and incubating the resulting membrane with the in vitro-translated β-subunit **(Fig. 1B)**.

1. Resolve aliquots (5–10 µg) of the purified dihydropyrdine receptor on 3–12% SDS polyacrylamide gels under reduced conditions *(11)* and transfer electrophoretically onto nitrocellulose, as described in **ref. *5***.
2. Block by incubating the membrane with blocking solution I, II, or III for 1 h at room temperature.

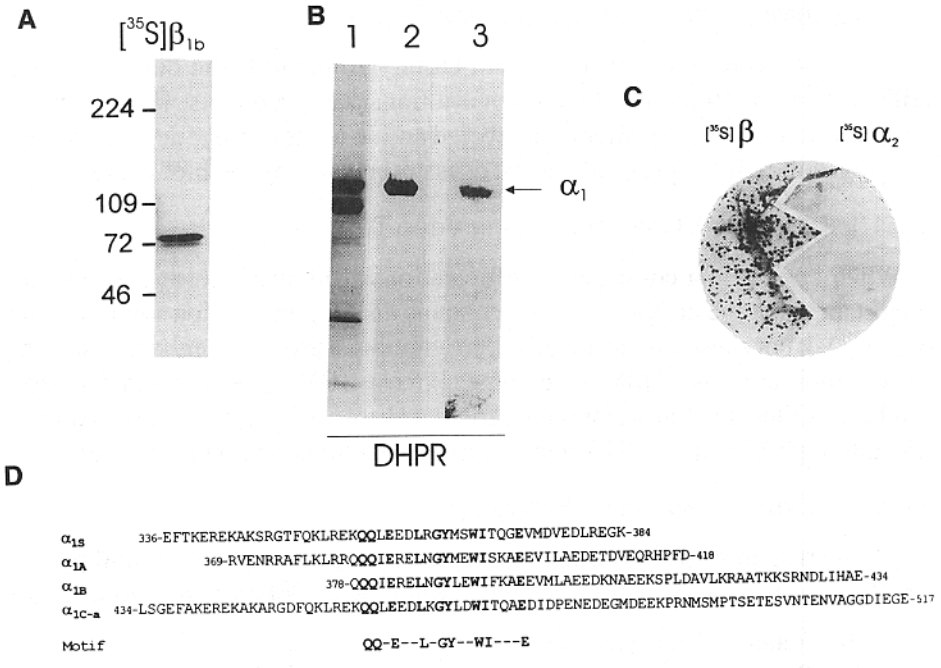

Fig. 1. Experimental approaches used to identify the interaction sites between the α_1- and β-subunits of the voltage dependent Ca^{2+} channel. **(A)** Autoradiogram of full-length in vitro-translated [^{35}S]-met labeled β_{1b}-subunit probe resolved using 3–12% SDS-PAGE. Mol wts are listed on the left. **(B)** Overlay technique demonstrates an interaction between the two proteins. Shown in lane 1 is Coomassie blue-stained gel of purified dihydropyridine receptor (DHPR) from skeletal muscle. Lane 2 shows an identical nitrocellulose blot stained with IIF7 monoclonal antibody to identify the α_1-subunit. Lane 3 is an autoradiogram of an identical nitrocellulose blot that has been incubated with the [^{35}S]-met labeled β_{1b} in vitro-translated probe, washed extensively, and exposed to film. Arrow indicates the binding of the β_{1b}-subunit probe to the α_1-subunit immobilized on nitrocellulose. **(C)** Demonstration of the specificity of the interaction of the β_{1b}-subunit probe with the plaque-purified α_1-subunit epitope clone. The voltage-dependent Ca^{2+} channel α_2-subunit probe was unable to bind the purified clone. **(D)** Sequence alignment of multiple α_1-subunit clones identified from screening four different α_1-subunit epitope libraries (α_{1A} ,α_{1B}, α_{1C}, α_{1D}) with the same β-subunit probe. Regions of overlap were then used to map and characterize the interaction motif between the α_1- and β-subunits. All parts of figure are modified from **ref. *8***.

3. Incubate the membrane in overlay buffer I for 1 h with the addition of the labeled probe (1 µL/mL overlay buffer), mixing gently for 12 h at 4°C.
4. Wash blots, subsequent to overlay incubations, for 1 h with 5% BSA in PBS at

In the experiment illustrated in **Fig. 1B** autoradiography revealed that the β-subunit interacted with the α_1-subunit of the Ca^{2+} channel (**Fig. 1B**) and this was confirmed by comparison with Coomassie blue staining of the purified dihydropyridine receptor and immunoblot analysis with an α_1-specific antibody that migrated at the same position.

Notably, the overlay technique is limited in its usefulness, since large amounts of protein may be required to measure good interaction. For example, in the case of the calcium channel, relatively large quantities of the purified skeletal muscle dihydropyridine-sensitive receptors are required to detect the interaction between the [^{35}S]-labeled β-subunit and the α_{1S}-subunit. Another limitation of this technique is that the interaction site on the protein present on the blot must be relatively unaltered by treatment with SDS, or renatured after removal of SDS, once transferred onto nitrocellulose. The binding motif relies on the tertiary structure of the protein, but is more likely to be identified if it is in a linear sequence (*see* **Note 8**).

3.2. Preparation and Use of an α_1 Epitope Library to Identify the AID

To establish the precise location of the AID, we have generated α_1 epitope expression libraries and screened them with the in vitro-translated [^{35}S]-labeled β-subunit probe. Epitope library screening is particularly useful in identifying protein–protein interaction sites on large proteins (>5000 bp cDNA size) where production of individual fusion proteins for the entire protein would be extremely time- and cost-consuming. An outline of the protocols for making and screening an epitope library are shown in **Figs. 2** and **3**, respectively.

1. Digest approx 15 µg of purified α_1-subunit cDNA in DNase reaction buffer (1X final concentration) with 1 U DNase I. Remove aliquots (5 µg) at three time-points (i.e., 3, 6, and 9 min), in order to achieve differential digestions. Resolve digested DNA on a 1% agarose gel along with size markers. Excise the smear of digested DNA from the gel between 0 and 500 bases with a clean razorblade. Isolate the digested DNA from the gel using the Qiagen gel extraction kit.
2. Ligate the phosphorylated *Eco*RI linkers to the ends of the randomly digested α_1-subunit DNA fragments, using T4 DNA ligase (1 U) in 30 µL vol in T4 DNA ligase buffer overnight at 15°C. Inactivate the ligase enzyme by heating at 65°C for 10 min.
3. Digest the DNA with *Eco*RI for 3 h at 37°C. Purify the DNA by phenol-chloroform extraction (twice), followed by ethanol precipitation (*see* **Note 9**). DNA was quantified by determining $A_{260/280}$ ratio using spectrophotometer.
4. Ligate the purified digested DNA into λgt11 *Eco*RI-digested and CIAP-treated λ arms (1 µg) at a molar ratio of 5:1 insert to vector.
5. Package the ligated λgt11 arms using Gigapack Gold phage. Keep the packaging reagents at –80°C until use, then thaw the reagents rapidly. Add half the ligated

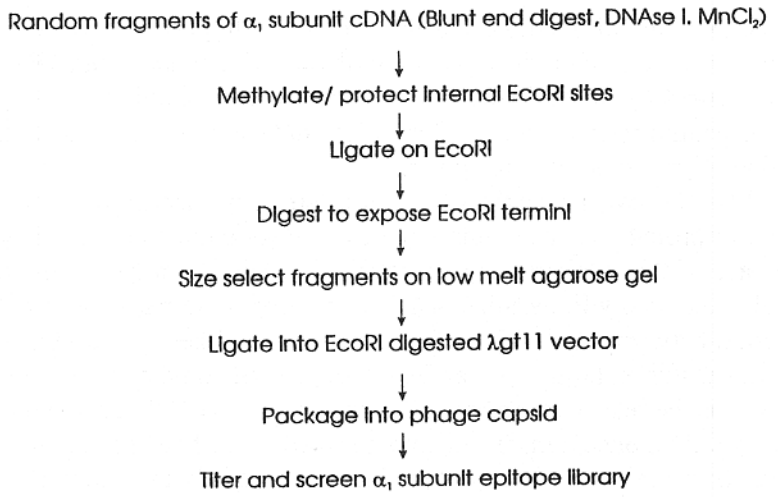

Fig. 2. Flow diagram for constructing an α_1-subunit epitope library. Epitope libraries of the α_1-subunit of the voltage dependent Ca^{2+} channel have been used for identification of protein interaction sites ***(8)*** and antibody recognition sites (Gurnett and Campbell, unpublished observations).

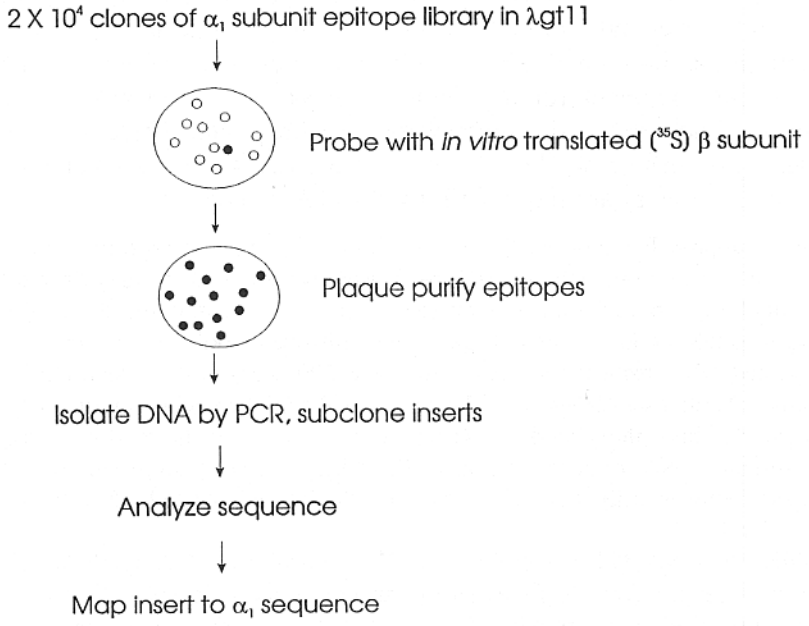

Fig. 3. Flow diagram for identification of protein–protein interaction sites using an epitope library. The α_1-subunit epitope library was screened with [^{35}S]-met labeled β-subunit. Purification and screening of positive clones has been used to map the site of interaction on the α_1-subunit ***(8)***.

*Eco*RI-digested λ arms to the kit extract, then add 15 µL sonic extract from the packaging kit. Incubated the samples for 2 h at room temperature. After incubation, add 500 µL SM media and 20 µL chloroform. This yields the unamplified library stock.

6. Titer and amplify the library immediately (*see* **Note 10**).
7. Use the β-subunit probe to screen 2×10^4 clones of each α_1-subunit epitope library in Y1090 *E. coli* (**Fig. 1C**). Amplify inserts from pure phage positives by PCR, using primers directed to λgt11-phage arms. Subclone these directly into a T-vector (prepared from Bluescript SK-plasmid) for sequencing, or digest with *Eco*RI and ligate into pGEX-1 vector for GST fusion protein production. Sequence all inserts in both directions. Perform peptide sequence homology searches using the BLAST network service provided by the National Center for Biotechnology Information (**Fig. 1D**).

3.3. Expression, Growth, and Purification of GST-Fusion Proteins

To verify and subsequently test the interactions identified by the library screen, we have prepared GST-fusion proteins of the AID by subcloning each of the positive clones after epitope screening into pGEX plasmids, transforming these constructs into *E. coli* DH5α cells and inducing the fusion protein production.

1. Inoculate 50 µL of glycerol stock of the AID fusion protein into 50 mL LB media containing ampicillin, to a final concentration of 50 µg/mL.
2. Incubate overnight at 37°C in an orbital shaker at 300 rpm.
3. Dilute the culture 10-fold, i.e., 50 mL in 300–500 mL LB media, and incubate at 37°C in the orbital shaker again for a further 1–2 h.
4. Add isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M* (at mid-log phase of growth) and incubate for an additional 3 h at 37°C shaking at 300 rpm.
5. Transfer cultures to JA10 bottles and add protease inhibitors (benzamidine [0.83 m*M*] and PMSF [0.23 m*M*]); sediment the bacteria at 5000*g* for 10 min at 4°C.
6. Decant off the supernatant; resuspend the whole cell pellet in a final volume of 9 mL PBS containing two protease inhibitors (PMSF and benzamidine), and add 1 mL of 10% Triton X-100 (to a final concentration of 1%).
7. Lyse the cells by sonication of the resuspended bacteria for a maximum of 30 s in 3×10 s bursts, to minimize proteolysis.
8. Prepare the bacterial lysate by centrifugation of the lysed cells in a JA 17 rotor at 13,800*g* for 10 min at 4°C (*see* **Note 11**).
9. To purify the GST-fusion protein, first equilibrate a glutathione–Sepharose column (1–2 mL) prepared in a 10-mL syringe plugged with a glass filter, with three bed volumes of 1% Triton X-100 in PBS containing two protease inhibitors, then transfer the glutathione–Sepharose to a 50-mL falcon tube and incubate with the bacterial lysate for 30 min at 4°C.

10. Pour the resin back into the column and discard the void. Wash the column extensively with 7 bed vol of 1% Triton X-100 in PBS containing two protease inhibitors and then with 10 bed vol of PBS containing the protease inhibitors. Finally, wash the column with 50 m*M* Tris-HCl, pH 8.0, and elute fusion proteins in the same buffer containing 10 m*M* reduced glutathione by collecting 10 bed volume fractions. Use analysis of the fractions by SDS-PAGE to identify, for subsequent pooling, those containing the most protein.
11. Determine the protein content by the Lowry protein assay *(16)*, using BSA as a standard, after removal of the glutathione by gel filtration or dialysis.

3.4. Identification of the BID Using the Overlay Assay

The AID GST-fusion proteins have been instrumental in determining the complementary interaction domain on the β-subunit (BID), using the following protocol:

1. Amplify truncated β-subunit constructs by PCR (**Fig. 4A**) from cDNA-encoding β_{1b}, using specific primers, purify DNA products using the Qiaex extraction kit (Qiagen) and subclone them into pGEM3 vector, modified to contain a 5' alfalfa mosaic virus enhancer region and a poly(A)$^+$ tail for enhanced expression.
2. Electrophorese aliquots (100 µL) of bacterial lysates containing a control GST-fusion protein and AID-GST-fusion proteins on 3–12% SDS gels (**Fig. 4B**).
3. Transfer proteins electrophoretically transferred onto nitrocellulose membrane and incubate in overlay buffer I with each of the in vitro-translated truncated β-subunits overnight at 4°C.
4. After extensive washing for 1 h at 22°C as described in **Subheading 3.1.2., step 4**, for the intact [^{35}S]-labeled β-subunit interaction, expose the membranes to X-ray film to enable detection of the specific interaction by autoradiography (**Fig. 4B**).

The specimen results illustrated in **Fig. 4B** reveal that a region that is present on the second conserved domain of the β-subunit, contained within amino acid residues 211–418, is required for maintaining this interaction (**Fig. 4B**). In further experiments, beyond the scope of this chapter, these interactions were further assayed electrophysiologically to identify a very small portion of the β-subunit encompassing residues 211–265 as being essential for α_1–β interaction. Furthermore, we have found that this truncated β-subunit is also capable of modulating the kinetics of the channels, thereby narrowing not only the α_1–β interaction domain, but also the region on the β-subunit responsible for Ca^{2+} current stimulation, down to approx 40 amino acids *(9)*. Site-directed mutagenesis of residues within this motif has allowed us to identify those amino acids that are essential for this interaction *(9)*.

3.5. Measurement of Affinity of the Interaction Between the α_1- and β-Subunits

Having identified both interaction domains on the two-subunits, additional properties of this interaction can now be examined. How strong is this inter-

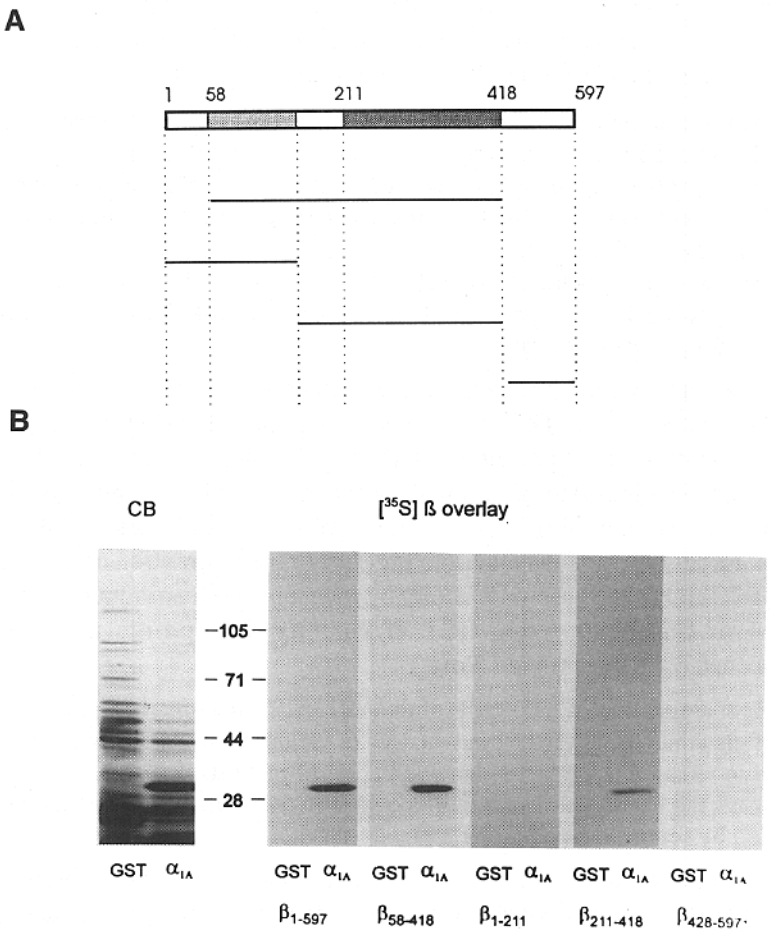

Fig. 4. Truncated probes can be used to identify the minimal sites required for protein interactions. **(A)** Truncated β-subunit constructs were generated for use as probes in the in the overlay assay ***(9)***. Shaded areas of the full-length construct indicate two structurally conserved regions on all β-subunit genes. Amino acids are numbered above the full-length construct. **(B)** Coomassie blue stained gel of 3–12% SDS-PAGE resolved crude bacterial lysate containing GST fusion protein (lane 1) and GST-α_{1A} I-II cytoplasmic linker (AID) fusion protein (lane 2). To the right is an autoradiogram of an identical nitrocellulose blot demonstrating that the full-length [^{35}S]-β-subunit overlays only on the GST-α_{1A} AID fusion protein. Four truncated [^{35}S]-β-subunit probes were then tested for their ability to overlay on the immobilized GST-α_{1A} AID fusion protein. The region between amino acid residues 211–418 of the β-subunit, which consists of the highly conserved second domain, was shown to be the minimal region required for binding to the α_{1A} -AID. Mol-wt markers are indicated. Both parts of the figure are modified from **ref. *9***.

action? Is this association reversible? These questions may be addressed using GST-fusion proteins of the AID and in vitro-translated β-subunits, with the development of a GST-glutathione–Sepharose bead assay.

1. Incubate aliquot of [^{35}S]- labeled β-subunit (0.7–1.3 p*M*) overnight at 4°C in PBS (1 mL) with increasing concentrations (100 p*M*–1 m*M*) of an AID-GST-fusion protein noncovalently coupled to glutathione–Sepharose beads.
2. Sediment the beads at 13,000 rpm in a bench centrifuge for 2 min and wash them four times with ice-cold PBS.
3. Determine the quantified bound [^{35}S]- labeled β-subunit by scintillation counting.
4. Analyze the data using the data-fitting program Grafit (Sigma) and derive the K_D and B_{MAX} for the interaction.

Experiments of this type reveal that the interaction is indeed very strong, with a K_D of 5.8 n*M* for the AID_A GST-fusion protein and β_{1b}. However, the affinity of this interaction was 10 times greater than that of the β_3-subunit, suggesting that although certain conserved residues is required for interaction, other amino acids within and around these domains also determine the affinity of the association *(17)*.

The association and dissociation kinetics of the interaction can also be tested using the AID_A-GST-fusion protein, by measuring the amount of [^{35}S]-labeled β-subunit bound to AID_A-GST at various time-points as the reaction approaches equilibrium (*see* **Note 12**).

1. Incubate an aliquot (~500 n*M*) of the AID_A-GST-fusion protein coupled to 40 µL of glutathione–Sepharose beads at 4°C for various times (0–300 min), with 0.32 p*M* of in vitro-translated [^{35}S]-labeled β_{1b} probe in 1 mL PBS.
2. Wash the beads four times with ice-cold PBS and measure the relative association by scintillation counting.

The reversibility of this association may also be addressed in competition experiments using an 18-amino acid synthetic AID_A peptide. This shows that 100 µ*M* of this peptide is sufficient to completely prevent the association of [^{35}S]-labeled β with any of the AID GST-fusion proteins. Dissociation is measured by allowing the association to reach equilibrium by overnight incubation of the reactants, as described above, and then measuring the binding over a period of time (up to 8 h) at 4°C in the presence of 500 µ*M* AID_A peptide. At 4°C, the interaction between AID_A and β_{1b} is almost irreversible, because no measurable decrease in the amount of β_{1b} bound to the beads is observed within the time-period *(9)*.

In conclusion, the GST-glutathione-bead assay using fusion proteins to specific interaction regions, together with the in vitro-translated probes, may be used to characterize certain properties of that association.

3.6. Purification of Native Ca^{2+} Channel β-Subunits with the AID

Because of the high affinity of this interaction, a GST-glutathione–Sepharose bead assay has been developed that has been used for identifying

β-subunits that are free or not associated with α_1-subunits of voltage dependent Ca^{2+} channels from both skeletal muscle and brain tissue ***(18)***.

1. Covalently couple the purified AID_A-GST fusion proteins to CNBr-activated Sepharose at a concentration of 0.5 mg/mL resin, according to the manufacturer's instructions.
2. Prepare tissue extracts from homogenates in 50 m*M* HEPES, pH 7.4, containing 100 m*M* NaCl and no detergent in the presence of a cocktail of protease inhibitors: aprotinin (76.8 n*M*), benzamidine (0.83 m*M*), leupeptin (1.1 μ*M*), pepstatin A (0.7 μ*M*) and PMSF (0.23 m*M*) by incubation for 2 h at 4°C and centrifuged at 37,000 rpm (100,000*g*) for 35 min (*see* **Note 13**).
3. Incubate the resultant supernatant with the AID_A-covalently attached to Sepharose overnight at 4°C. Significant binding of free β-subunits can be detected by Western blot analysis ***(17)*** with subunit-specific antibodies ***(13)***.

The use of fusion proteins, coupled to GST-glutathione–Sepharose beads as an affinity matrix, clearly has very important implications for aiding in deciphering the association of these proteins with other unknown cellular components (i.e., cytoskeleton).

4. Notes

1. Subunit–subunit interactions may be investigated using both biochemical and molecular techniques alone or in concert with functional assays, as was the case for the identification of the AID and BID of Ca^{2+} channel subunits. These approaches have also recently been successfully applied to characterizing the protein–protein interactions among a number of the components of the dystrophin glycoprotein complex ***(19,20)***. Clearly these methodologies can readily be used for investigating the molecular interactions between a wide variety of different proteins.
2. Small-scale reactions may be performed by reducing volumes proportionately. Notably, multiple proteins can be expressed from the same or different promotors in the same reaction by adding appropriate TNT RNA polymerases.
3. In order to minimize proteolysis, translation should be performed in the presence of a cocktail of protease inhibitors: pepstatin A, chymostatin, aprotinin, antipain, and leupeptin, at a final concentration of 0.1 μg/mL. Calf liver tRNA may also be added, to a final concentration of 40 μg/mL to reduce background translation.
4. The advantage of using this kit over conventionally preparing RNA, and subsequently utilizing this RNA for protein translation in a rabbit reticulocyte lysate, is that the handling time is minimized (from 10 to 5 h) and the reaction occurs in a single tube. However, a disadvantage of this coupled system may be lower yields of translated material, although we have not performed direct comparisons of the two methodologies.
5. Free [^{35}S]-methionine may readily be removed from the labeled probe using a PD 10 column (Pharmacia, G-25 Sephadex), especially if binding assays are being performed using the radiolabeled probe. If dilution of the probe is a problem, the

[^{35}S]-methionine removal can be accomplished by rapid gel filtration using G-50 Sephadex (Pharmacia) prepared in a 1-mL syringe column.

6. The blocking step for the proteins on the nitrocellulose is critical for reducing background and maximizing the interaction between the two proteins. Different blocking buffers can be used to reduce nonspecific binding of probe.
7. Overlay buffer II may be also be used for incubation with the radiolabeled probe overnight at 4°C with gentle agitation. Different blocking buffers and incubation conditions may be tested, since the interaction may be very sensitive to ionic strength, presence of cations, and reducing conditions.
8. In an attempt to alleviate this problem, the blot may be incubated with decreasing amounts of guanidine-HCl, which should renature the immobilized proteins somewhat, as described for the K^+ channel Shaker B α-subunit *(15)*.
9. DNA was quantified by determining $A_{260}/_{280}$ ratio using a spectrophotometer.
10. The integrity of the library should be tested by direct immunoscreening with a monoclonal antibody directed toward the α_1-subunit.
11. Fusion proteins that contain hydrophobic domains are more difficult to purify, since their hydrophobicity causes them to be incorporated into inclusion bodies; they usually yield Triton X-100 insoluble preparations. These fusion proteins may be solubilized using 10% sarcosyl, after initial solubilization with 1% Triton X-100. After 30 min incubation and centrifugation to remove all insoluble material, followed by the addition of fivefold concentration Triton X-100, the fusion protein is purified on a glutathione–Sepharose column, as detailed above for the soluble fusion proteins. Transmembrane regions should be avoided when designing fusion proteins.
12. At a given AID_A-GST-fusion protein concentration, the association rate constant corresponds to a half-time of approx 20 min. The maximum association is not affected by changes in Ca^{2+} concentration (1 n*M*–1 m*M*), ionic strength (0–2*M* NaCl), or phosphorylation by protein kinase C. However, large variations in pH (4–10) could decrease the maximal association by ~40%, and the interaction was totally abolished at pH 12.0.
13. Treatment of tissue with homogenization buffer containing protease inhibitors induced some dissociation of the β-subunit from the channel complex and allowed purification of native, free β-subunits. This method of purifying the native Ca^{2+} channel β-subunits can be exploited in isolating large amounts of native protein that would be required for complex structural studies (i.e., NMR and X-ray crystallography).

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