Generation of Functional Fluorescent BK Channels by Random Insertion of GFP Variants

Teresa Giraldez, Thomas E. Hughes, and Fred J. Sigworth

1Department of Cellular and Molecular Physiology and 2Kavli Institute for Neuroscience, Yale School of Medicine, New Haven, CT 06520
3Department of Cell Biology and Neuroscience, Montana State University, Bozeman, MT 59717

The yellow and cyan variants of green fluorescent protein (GFP) constitute an excellent pair for fluorescence resonance energy transfer (FRET) and can be used to study conformational rearrangements of proteins. Our aim was to develop a library of fluorescent large conductance voltage- and Ca\(^{2+}\)-gated channels (BK or slo channels) for future use in FRET studies. We report the results of a random insertion of YFP and CFP into multiple sites of the α subunit of the hsl0 channel using a Tn5 transposon-based technique. 55 unique fluorescent fusion proteins were obtained and tested for cell surface expression and channel function. 19 constructs are expressed at the plasma membrane and show voltage and Ca\(^{2+}\)-dependent currents. In 16 of them the voltage and Ca\(^{2+}\) dependence is very similar to the wild-type channel. Two insertions in the Ca\(^{2+}\)-bowl and one in the RCK2 domain showed a strong shift in the G-V curve. The remaining 36 constructs were retained intracellularly; a solubility assay suggests that these proteins are not forming intracellular aggregates. The “success rate” of 19 out of 55 hsl0 insertion constructs compares very favorably with other studies of random GFP fusions.

INTRODUCTION

Large-conductance voltage- and Ca\(^{2+}\)-activated K\(^{+}\) channels (BK channels) are modulated synergistically by voltage and Ca\(^{2+}\) (Horrigan and Aldrich, 2002), providing a unique link between chemical and electrical signaling in the cell (Toro et al., 1998). BK channels are found in a wide range of tissues, where they play diverse roles. They contribute to the feedback control of Ca\(^{2+}\) influx and neurotransmission, the repolarization of action potentials and the frequency adaptation processes in the nervous system (Crest and Gola, 1993; Hu et al., 2001), and to the tuning of the electrical resonance in hair cells (Rosenblatt et al., 1997).

BK channels are formed as tetramers of the pore-forming α subunits that sometimes form a complex with regulatory β subunits (Waller et al., 1995). The α subunits are coded by the slo gene. Cloning the cDNA that encodes the slo α subunit revealed that the predicted protein has six transmembrane regions (S1–S6), placing it in the superfamily of voltage-gated ion channels. Nevertheless, slo differs from the K\(^{+}\) family in having an additional transmembrane domain (S0) at the NH\(_2\) terminus, and a large COOH terminus with four hydrophobic regions originally designated S7–S10 (Butler et al., 1993; Meera et al., 1997). Subsequent studies have proposed the existence of several functional domains in the COOH terminus.

Jiang et al. (2001) described a new class of domains present in some K\(^{+}\) channels, named RCK domains for their possible role in the regulation of the conductance to K\(^{+}\). In the bacterial MthK channel tetramer, eight RCK domains have been proposed to form a “gating ring” that transduces Ca\(^{2+}\) binding into opening of the channel pore (Jiang et al., 2002). BK α subunits present two RCK domains in the COOH-terminal region (Jiang et al., 2002; Roosild et al., 2004), separated by a non-conserved linker (Schreiber and Salkoff, 1997). It is tempting to conclude that a similar gating ring mechanism is applicable in BK channels, but the differences between MthK and BK channels are substantial: the Ca\(^{2+}\) binding sites in MthK are not conserved in BK channels; MthK has a relatively low Ca\(^{2+}\) sensitivity; and the regulation mechanism of BK channels is more complex, involving both Ca\(^{2+}\) and voltage (Horrigan and Aldrich, 2002). Meanwhile, some authors have proposed a role of the first RCK domain in slo tetramerization (Quirk and Reinhart, 2001).

Immediately following the second RCK domain there is a highly conserved 28-amino acid region rich in aspartate residues referred to as the “Ca\(^{2+}\) bowl” (Wei et al., 1994). Mutations in this region have large effects on the Ca\(^{2+}\) sensitivity of slo channels (Wei et al., 1994; Schreiber and Salkoff, 1997; Schreiber et al., 1999; Bao et al., 2002), although other sites in the RCK1 domain appear to also participate in high affinity

Abbreviations used in this paper: CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein.

Correspondence to Fred J. Sigworth: fred.sigworth@yale.edu
Ca$^{2+}$ sensitivity (Bao et al., 2002; Xia et al., 2002) as well as in low affinity Ca$^{2+}$ and Mg$^{2+}$ sensitivity (Shi and Cui, 2001; Zhang et al., 2001; Xia et al., 2002).

A sequence near the COOH terminus is similar to that of the superfamily of serine proteinase (Ser-P) enzymes (Moss et al., 1996; Favre et al., 2000). The functional relevance of this domain is not clear. It has been demonstrated that Ser-P inhibitors bind to slo channels producing discrete subconductance events (Moss et al., 1996; Favre and Moczydlowski, 1999; Favre et al., 2000), suggesting a role of this domain in slo gating as well as modulation by other cytoplasmic or membrane-associated regulatory components (Moss et al., 1996).

The yellow and cyan variants of the green fluorescent protein (YFP and CFP respectively) constitute an excellent pair for fluorescence resonance energy transfer (FRET), and can be used to study the conformational rearrangements of ion channels that are associated with function (Zheng and Zagotta, 2003). The engineering of suitable GFP fusion proteins is however difficult. GFP insertion sites must be found that fulfill two requirements: (1) the fusion proteins should generate functional channels with properties similar to the wild-type channel; (2) the insertion sites should lie in appropriate locations of domains involved in the conformational rearrangements. Attempts to predict such sites have been unsuccessful, even in cases where the native protein structure is known. Given these uncertainties and the time-consuming process of generating individual fusion proteins using conventional molecular biology techniques, we decided to use transposable elements to randomly insert YFP and CFP into different domains of the human slo a-subunit (hslo). We have expressed these mutants in mammalian cells and studied their ability to form functional BK channels. Out of 55 constructs tested, 19 were expressed at the plasma membrane and form functional channels. Most of them appear to be comparable to wild-type channels in terms of voltage and Ca$^{2+}$ sensitivity. Only two constructs with insertions within the Ca$^{2+}$ bowl and a construct with an insertion in the RCK2 domain showed altered functional properties. Therefore, we have been able to generate rapidly a library of fluorescent hslo fusion proteins that promises to be a useful tool for future FRET studies of conformational changes of BK channels associated with function.

**Materials and Methods**

**Target Plasmids, Transposons, and Transposition Reactions**

The full-length human slo cDNA isolated from uterine smooth muscle (hslo; GenBank/EMBL/DDBJ accession no. U11058; Wallner et al., 1995) was modified by Dr. A. Tinker in the 5′ end to carry a six-histidine tag followed by the FLAG epitope DYKD- DDDK. This construct was provided to us by G. Moss (University College London, London, UK). A HindIII/NotI fragment carrying this modified hslo cDNA was cloned into a pCMVSport/β-gal-derivated vector. The CFP transposon <TcPT1> was as previously reported (Sheridan et al., 2002). The transposon encoding YFP <TvPT0> was made by replacing the AscI-flanked EGF fragment in <TgPT0> (Sheridan et al., 2002) with the corresponding YFP fragment (Venus variant; Nagai et al., 2002). In view of the low probability of transposition in an in vitro reaction, a selectable marker was introduced into each transposon in the form of a Kanamycin resistance cassette (Kanr) flanked by Srl restriction sites. Immediately before the Kanr there is a stop codon; therefore, each clone containing an in-frame insertion should encode a truncated protein with YFP or CFP at the COOH terminus.

The transposon can be inserted anywhere in the target plasmid; in our case, the probability that the insertion occurs into the hslo cDNA, in the correct orientation and reading frame, is expected to be ~9%. Thus our screening strategy consisted of several steps. First, we performed two separate in vitro transposition reactions, one for each transposon, following the protocol described by Sheridan et al. (2002). The resulting DNA was transformed into E. coli. Single colonies resistant to ampicillin (transformed clones) and kanamycin (marker for transposition) were picked and grown in 96-well plates. Transposed plasmids were then isolated in a 96-well format with Eppendorf PerfectPREP.96 Vac Direct Bind miniprep kits (Eppendorf) on a PerkinElmer MultiPROBE II HT liquid handling robot. Approximately 10,000 transposed clones were obtained; we purified DNA from 672 clones of each reaction (1,344 clones in total). We identified correctly oriented in-frame insertions by transiently transfecting each truncated construct into HEK293 cells, which were screened for fluorescence. The probability of transposition is not uniform (Goryshin et al., 1998), so we expected some clones to have identical insertion sites. The 5′ boundaries of the insertions were determined for all fluorescent clones by DNA sequencing (HHMI Biopolymer/Keck Foundation Biotechnology Laboratory, Yale University School of Medicine) to identify unique insertions and localize the exact insertion site. These clones were digested with SrlI to remove the Kanr cassette and obtain the full-length fusion constructs.

**Screening of Transposed Clones**

HEK-293 cells were seeded in 96-well glass bottom tissue culture plates (NalgeNUNC) and grown in DMEM (GIBCO BRL) supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO$_2$. Cells were transfected with plasmid DNA from individual CFP- and YFP-transposed clones and Lipofectamine 2000 (GIBCO BRL) following standard protocols. The cells were screened for YFP and CFP fluorescence ~24 h after transfection with a 20× objective on a Carl Zeiss MicroImaging, Inc. inverted fluorescence microscope.

**Cell-surface Immunostaining**

CHO cells were plated in Falcon culture slides (Becton Dickinson Labware) and grown in α-MEM (GIBCO BRL) with 10% FBS at 37°C in 5% CO$_2$. Cells were transfected 18–24 h before experiments with plasmid DNA of each full-length fusion protein and Lipofectamine 2000 (GIBCO BRL). All staining procedures were performed at 4°C. Cells were washed twice with PBS$^+$ (PBS with 2 mM CaCl$_2$ and 0.5 mM MgCl$_2$), blocked with 1% BSA and 10% goat serum for 1 h and further incubated with 5 μg/ml mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) for 45 min. Cells were then fixed with 2% formaldehyde in PBS$^+$ for 10 min, washed extensively with PBS and incubated with 10 μg/ml of an Alexa Fluor 594-conjugated goat anti-mouse IgG antibody (Molecular Probes) for 45 min. After several washes with PBS, the
cells were mounted with Aquamount (Lerner Laboratories). Images of the slides were collected with a Carl Zeiss MicroImaging, Inc. laser scanning microscope equipped with a 40× oil-immersion objective. YFP and CFP were excited using the Argon laser 488-nm line, whereas Alexa 594 was excited using the He-Ne laser 568-nm line. The emission signals were filtered with a Carl Zeiss MicroImaging, Inc. 515–565-nm filter (YFP/CFP emission) or with a 590–640 filter (Alexa 594 signal).

Triton X-100 Solubilization and Western Blot Analysis
A protocol modified from Zarei et al. (2004) was used. Transfected CHO cells were permeabilized in lysis buffer (150 mM NaCl, protease inhibitors, 1 μg/ml DNaseI, 20 mM Tris-HCl, pH 7.4) containing various concentrations of Triton X-100 (0, 0.1, 0.4, 1, 2%). After a 10-min incubation on ice, lysates were centrifuged for 5 min at 15,000 g. Solubilized fractions were set aside while insoluble fractions were washed twice with PBS and resuspended in equal volume as soluble fractions (60 μL). SDS loading buffer was added to both soluble and insoluble fractions and 30–μl aliquots were separated in 7.5% SDS-PAGE gels. We did not boil the samples, since that consistently produced aggregates of hslo subunits that would run as high molecular weight complexes. Proteins were transferred to Immun-blot PVDF membranes (Bio-Rad Laboratories), and hslo was detected by Western blotting with 10 μg/ml anti-FLAG monoclonal antibodies (Sigma-Aldrich). Anti–mouse IgG secondary antibodies conjugated to peroxidase (Sigma-Aldrich) were used at 1:10,000 dilution and the signal was developed with the ECL+ system (Amersham Pharmacia Biotech).

Electrophysiological Recordings
CHO cells were grown on 12-mm glass coverslips (Fisherbrand) in α-MEM (GIBCO BRL) with 10% FBS and transfected with plasmid DNA and Lipofectamine 2000 (GIBCO BRL). 18–24 h after transfection, fluorescent cells expressing hslo-YFP or hslo-CFP were assayed for hslo function. Recordings were done in the cell-attached or inside-out patch clamp configurations (Hamill et al., 1981) at 22–24°C. Patch pipettes were made of borosilicate glass (Kimax) and had resistances of 1–3 MΩ. Data were acquired using an EPC-9 amplifier and Pulse acquisition software (HEKA Electronik). Records were digitized at 50-μs intervals. Capacitance and leak currents were subtracted using a P/5 leak subtraction protocol. Conductance–voltage (G-V) curves were obtained by measuring the amplitude of tail currents 500 μs after repolarization to −70 mV from the various test voltages. Current levels were highly variable between patches; however, for all functional constructs, the maximal currents at 120 mV were consistently in the range of 500 pA to 60 nA, ~40% of the amplitude obtained with wild-type hslo channels. Currents obtained at all concentrations within a patch were normalized to the maximum peak current at 100 μM Ca2+ and G-V curves were fitted to a Boltzmann function using Igor Pro software (Wavemetrix Inc.). Values in the text are given as mean ± SEM.

Recording solutions contained (in mM): pipette, 80 KMeSO4, 60 N-methylglucamine-MeSO4, 20 HEPES, 2 KCl, 2 MgCl2 (pH 7.4); bath solution, 80 KMeSO4, 60 N-methylglucamine-MeSO4, 20 HEPES, 2 KCl, 1 HEDTA, and CaCl2 to give the appropriate free Ca2+ concentration (pH 7.4). No Ca2+ chelator was used in solutions containing 100 μM free Ca2+ or higher. To prevent Ba2+ block at high voltages (Diaz et al., 1996), 50 μM (+)-18-crown-6-tetracarboxylic acid (18C6TA) was added to all bath solutions. The amount of total CaCl2 needed to obtain the desired free Ca2+ concentration was calculated using the program Max Chelator (Bers et al., 1994), which was downloaded from www.stanford.edu/~patton/maxcalc.html. Free Ca2+ was measured with a Ca2+-sensitive electrode (Orion electrode, Thermo Lab-systems). Solutions were exchanged using the DAD-VC superfusion system from ALA Scientific Instruments.

RESULTS AND DISCUSSION
Our aim was to generate a library of fluorescent functional hslo constructs as a tool for future use in spectroscopic studies of BK channel conformational rearrangements during gating. Obtaining a large number of constructs in a short period of time was possible by randomly inserting fluorescent protein domains into various sites in the hslo sequence. To this end, we used a newly developed technique based on a modified Tn5 transposon (Reznikoff et al., 1999) that carries the sequence of either the yellow fluorescent protein (YFP) or the cyan fluorescent protein (CFP; Sheridan et al., 2002). Transposons consist of any sequence delimited by the mosaic ends (MEs), which are two 19-base pair repeats. In an in vitro reaction, a recombinant Tn5 transposase binds these MEs and catalyzes the random insertion of the transposon into the plasmid carrying the hslo sequence. We created two different transposons, <TvPT-0> and <TvPT-1>. When these transposons are inserted into the sequence, in the correct orientation and reading frame, they produce a fluorescent fusion protein.

We transfected an initial set of 1,344 YFP and CFP insertion clones into HEK293 cells and screened for fluorescence (see MATERIALS AND METHODS). In our system a random transposition would be expected to result in an in-frame, correctly oriented insertion into the hslo coding region with a probability of 9%. Indeed, from the 1,344 random insertion clones, we obtained 101 constructs that produced fluorescent fusion protein (61 YFP and 40 CFP fluorescent insertions). Thus, consistent with earlier studies (Sheridan et al., 2002), it appears that nearly all fusion constructs yield correctly folded GFP domains. To verify that the sequence of the fluorescent proteins had been inserted into unique sites in the hslo sequence, the constructs were digested with restriction enzymes Ascl (whose site flanks the YFP or CFP sequence in the transposons) and EcoRI (a unique site in the hslo sequence). The resulting gels revealed a common band (728 base pairs) in every lane, corresponding to the YFP or CFP insert, and two more bands of different sizes in concordance with the different sites of insertion of the transposons. A representative gel is shown in Fig. 1 A. Sequencing the 101 clones revealed 55 unique insertions (33 YFP and 22 CFP; Fig. 1 and Table I). These 55 clones were digested with SrfI and religated to remove the Kan' gene and stop codon (see MATERIALS AND METHODS), yielding the full-length constructs. All of the initial clones were still fluorescent as full-length constructs. All constructs showed similar high levels of expression in HEK293 cells, with a large proportion of perinuclear YFP or CFP fluorescence consistent with localization to the ER, probably due to overexpression of the proteins (Fig. 1 B).

Inspection of Fig. 1 C and Table I shows that even though insertions were obtained over most of the coding
Table 1

Membrane Expression and Function of Fluorescent hslo Fusion Proteins

| Insertion site (Construct #) | Fluorescent protein inserted | hslo domain | Membrane expressiona | $V_{1/2}$b | $V_{1/2}$b |
|-----------------------------|------------------------------|-------------|-----------------------|-------------|-------------|
|                             |                              |             |                       | 100 μM Ca$^{2+}$ | 1 μM Ca$^{2+}$ |
|                             |                              |             |                       | mV          | mV          |
| Wild-type hslo              | –                            | –           | Y                     | $-3 \pm 4$ (8) | $115 \pm 5$ (6) |
| 13                          | YFP                          | NH$_2$ terminus | N                     |             |             |
| 65                          | YFP                          | S0-S1 loop  | L                     |             |             |
| 182                         | YFP                          | S3 helix    | N                     |             |             |
| 195                         | YFP                          | S3 helix    | N                     |             |             |
| 305                         | YFP                          | S6 helix    | N                     |             |             |
| 336                         | YFP                          | end of S6   | N                     |             |             |
| 337                         | CFP                          | end of S6   | L                     |             |             |
| 389                         | CFP                          | RCK1        | N                     |             |             |
| 416                         | CFP                          | RCK1        | N                     |             |             |
| 471                         | YFP                          | RCK1        | N                     |             |             |
| 497                         | YFP                          | RCK1        | N                     |             |             |
| 499                         | CFP                          | RCK1        | N                     |             |             |
| 502                         | YFP                          | RCK1        | N                     |             |             |
| 512                         | CFP                          | RCK1        | L                     |             |             |
| 541                         | YFP                          | RCK1        | N                     |             |             |
| 544                         | CFP                          | RCK1        | N                     |             |             |
| 551                         | CFP                          | RCK1        | N                     |             |             |
| 571                         | YFP                          | RCK1        | N                     |             |             |
| 572                         | YFP                          | RCK1        | N                     |             |             |
| 640                         | CFP                          | linker      | Y                     | $7 \pm 5$ (4) | $112 \pm 2$ (4) |
| 667                         | CFP                          | linker      | Y                     | $8 \pm 3$ (12) | $113 \pm 4$ (7) |
| 678                         | CFP                          | linker      | Y                     | $15 \pm 8$ (4) | $119 \pm 4$ (4) |
| 712                         | CFP                          | RCK2        | N                     |             |             |
| 720                         | YFP                          | RCK2        | L                     |             |             |
| 731                         | YFP                          | RCK2        | N                     |             |             |
| 772                         | YFP                          | RCK2        | N                     |             |             |
| 780                         | YFP                          | RCK2        | N                     |             |             |
| 790                         | YFP                          | RCK2        | N                     |             |             |
| 802                         | YFP                          | RCK2        | L                     |             |             |
| 821                         | YFP                          | RCK2        | N                     |             |             |
| 829                         | CFP                          | RCK2        | Y                     | $53 \pm 7$ (4)c | $131 \pm 2$ (4)c |
| 834                         | YFP                          | RCK2        | N                     |             |             |
| 854                         | YFP                          | RCK2        | Y                     | $20 \pm 10$ (4) | $118 \pm 15$ (3) |
| 860                         | CFP                          | RCK2        | Y                     | $11 \pm 10$ (3) | $118 \pm 9$ (4) |
| 866                         | YFP                          | RCK2        | Y                     | $-14 \pm 5$ (7) | $98 \pm 5$ (8) |
| 901                         | CFP                          | Ca bowl     | Y                     | $117 \pm 5$ (5)c | $201 \pm 2$ (3)c |
| 904                         | YFP                          | Ca bowl     | Y                     | $100 \pm 4$ (4)c | $203 \pm 5$ (3)c |
| 921                         | YFP                          | Ser-P like  | N                     |             |             |
| 939                         | YFP                          | Ser-P like  | L                     |             |             |
| 964                         | YFP                          | Ser-P like  | Y                     | $6 \pm 4$ (3) | $115 \pm 5$ (3) |
| 965                         | CFP                          | Ser-P like  | Y                     | $27$ (1) | $120$ (1) |
| 972                         | CFP                          | Ser-P like  | Y                     | $19 \pm 6$ (4) | $122 \pm 5$ (3) |
| 975                         | YFP                          | Ser-P like  | L                     |             |             |
| 991                         | YFP                          | Ser-P like  | Y                     | $9$ (1) | – |
| 1002                        | CFP                          | Ser-P like  | N                     |             |             |
| 1019                        | CFP                          | Ser-P like  | Y                     | $15 \pm 6$ (2) | $118 \pm 5$ (2) |
| 1024                        | CFP                          | Ser-P like  | Y                     | $33 \pm 7$ (3) | $125 \pm 12$ (3) |
| 1040                        | YFP                          | Ser-P like  | N                     |             |             |
| 1042                        | YFP                          | Ser-P like  | N                     |             |             |
| 1049                        | YFP                          | Ser-P like  | N                     |             |             |
sequence, a majority of them cover the COOH-terminal domain, with fewer insertions in the transmembrane domain. This apparent nonrandom distribution can be explained by two reasons. First, the Tn5 transposon behavior is not completely sequence independent (Goryshin et al., 1998). Second, and more importantly, if the insertion results on an aberrant protein that is improperly folded and consequently removed by cell degradation systems, it will not be recovered as a positive insertion in our screening process, which is based on cell expression of COOH-terminal fusions of the fluorescent protein. We speculate that many insertions in the transmembrane domain may result in fusion proteins that are degraded by the cell. Nevertheless, we recovered 55 insertions of the fluorescent proteins that cover all regions of interest (Fig. 1 C and Table I). Seven constructs were obtained with insertions between the NH2 terminus and the start of the RCK1 domain, including three in helices 3 and 6 and four in loops or regions at the end of the helices; 12 constructs had insertions in the RCK1 domain; three constructs hold insertions in the linker between RCK domains; 13 constructs in the RCK2 domain; two of them showed insertions in the Ca2+-bowl domain; finally, 15 of the constructs had insertions in the Ser-P–like domain, whereas the remaining three construct insertions were in the COOH terminus of the hslo sequence.

The complete sequence of a representative insertion is shown in Fig. 2. It should be kept in mind that the transposition process involves a duplication of the nine base pairs flanking the transposon insertion site (Fig. 2; see also Sheridan et al., 2002; Reznikoff, 2003). This generates a duplication of three amino acids of the hslo sequence in addition to the linker flanking the fluorescent protein (Sheridan et al., 2002). We identify the site of an insertion by the number of the residue preceding the duplicated triplet, using the native hslo numbering (GenBank/EMBL/DDBJ accession no. U11058).

We next sought to determine which of the 55 unique constructs were able to form functional channels at the plasma membrane. All constructs showed high levels of fluorescence, but much of the protein was localized intracellularly (Fig. 1 B). Therefore, it was difficult to determine whether any of the hslo fluorescent protein was transiently transfected with the fusion constructs and imaged for fluorescence after 24 h. (C) Successful insertions of YFP or CFP into the different regions of the hslo subunit. White circles, YFP; gray circles, CFP. Approximate insertion sites are shown. Refer to Table I for the exact position in the hslo sequence where the insertion occurred.

### Table I

| Insertion site (Construct #) | Fluorescent protein inserted | hslo domain | Membrane expressiona | V1/2b |
|-----------------------------|-----------------------------|-------------|----------------------|-------|
| 1062 | YFP | Ser-P like | Y | 8 ± 8 (8) |
| 1069 | CFP | Ser-P like | Y | 25 (1) |
| 1082 | CFP | COOH terminus | Y | 16 ± 3 (3) |
| 1085 | YFP | COOH terminus | L | |
| 1088 | YFP | COOH terminus | Y | −6 ± 3 (3) |

a Determined with surface immunostaining. Y, very clear surface labeling; N, no surface labeling; L, very low surface labeling, probably not expressed at the plasma membrane.

b Mean ± SEM (number of cells). Parameters obtained from Boltzmann fits $G/G_{max} = (1 + e^{-zF1/2}/RT - 1)$ at indicated [Ca2+]i. Values of z were in the range 0.93 ± 0.05 to 1.1 ± 0.04 at 1 μM [Ca2+]i and 0.92 ± 0.01 to 0.97 ± 0.02 at 100 μM [Ca2+]i. Constructs 965, 991, and 1069 were not completely characterized at all Ca2+ concentrations, but data obtained from single cells suggest that there are no significant differences with the wild type.

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at the plasma membrane. Since screening of all 55 constructs by patch-clamp would have been time-consuming, we developed an immunoassay to detect hslo protein surface expression. Because the NH2 terminus of the hslo channel is exoplasmic (Wallner et al., 1996), and our constructs had a FLAG epitope at the NH2 terminus, we were able to test plasma membrane expression of the 55 constructs of the library by surface immunostaining. For these experiments, and for functional analysis of hslo fusion proteins, we used CHO cells because they do not have endogenous potassium channels (Yu and Kerchner, 1998). Cells were transiently transfected with the fusion constructs, and protein expressed at the plasma membrane was immunolabeled with anti-FLAG primary antibody and a secondary antibody conjugated to a red fluorophore (Alexa 594). To ensure that the immunodetected channels were exclusively at the extracellular membrane, primary antibody was added before fixation of nonpermeabilized cells and all experiments were performed at 4°C to avoid membrane internalization processes. Secondary antibody was then added to the cells as described in MATERIALS AND METHODS. As shown in Fig. 3 A, nontransposed wild-type hslo channels showed strong surface expression. All 55 constructs of the library showed YFP or CFP fluorescence in the cell, but only 19 of them showed clear membrane expression. The approximate location of the 19 insertions that are expressed at the plasma membrane is shown in Fig. 3 B (for the exact location of insertions, refer to Table I).
disrupting secondary structural elements (α helices or β strands) in the Ser-P domain. However, when the insertions are mapped onto the secondary structure, no obvious pattern emerges (unpublished data). A similar analysis was performed with insertions in the RCK domains, which also show an α/β protein structure, but again no obvious pattern is seen. These results are reminiscent of those of Sheridan et al. (2002), who transposed the G-protein subunit ε, and surprisingly obtained the most functional fusion protein with the insertion of GFP into the middle of an α-helix. The hslo COOH-terminal region tolerated two insertions out of three. This is in concordance with results by other authors, who observed that deleting the last segment of hslo leads to functional channels very similar to wild type (Quirk and Reinhart, 2001).

We then tested the function of the 19 constructs expressed at the plasma membrane. Electrophysiological properties of the fluorescent hslo fusion proteins were investigated in transiently transfected CHO cells and compared with wild-type BK channels transfected into the same cell line. A low level of single channel activity was detected in cell-attached patches. When a patch was excised to a medium containing 100 μM Ca2+, currents are shown from 100-ms depolarizations from −70 mV to +60 mV. The same patches shown in A were excised to a medium containing 100 μM Ca2+. Currents are shown from 100-ms depolarizations from −70 mV to +60 mV. The same patches shown in A were excised to a medium containing 100 μM Ca2+.

Figure 4. Electrophysiological properties of hslo fluorescent fusion proteins. (A) Hslo currents were recorded with depolarizing pulses to +60 mV from a holding potential of −70 mV. Single channel recordings of wild-type hslo (top traces) and a representative hslo-YFP construct (1062-YFP; bottom) obtained in cell-attached patches at +60 mV. The arrows indicate the closed current level. (B) The same patches shown in A were excised to a medium containing 100 μM Ca2+. Currents are shown from 100-ms depolarizations from −70 mV to +60 mV. (C) Representative current families recorded from CHO inside-out patches in 100 μM Ca2+, from a holding potential of −70 mV. WT and 1062-YFP currents are shown at voltages between −70 and +120 mV in steps of 10 mV, whereas 901-CFP currents were obtained at +10 to +200 mV. (D) Normalized G-V curves are shown from wild-type hslo (WT; no insertions; black curve and symbols) and seven representative insertions. Each set of points was fitted to a Boltzmann function. Error bars represent SEM with n values as listed in Table I.
represent SEM.

Data are shown from (A) wild type hslo, (B) an insertion in the RCK2 domain that also shifted the G-V curve of \( \sim 50 \) mV to more positive voltages. In both this and Reinhart, 1997). The two insertions in the calcium bowl (901-CFP and 904-YFP) showed a strong shift in the G-V curve at 100 \( \mu \)M Ca\(^{2+}\), \( \sim 100 \) mV in the depolarizing direction (Fig. 4 D and Table I). Interestingly, an insertion in the RCK2 domain, 829-CFP, also showed a shift in the G-V curve of \( \sim 50 \) mV to more positive voltages.

To characterize the Ca\(^{2+}\) sensitivity of the various constructs, we studied the G-V relationships at Ca\(^{2+}\) concentrations between 1 \( \mu \)M and 100 \( \mu \)M. As shown in Table I and Fig. 5, \( V_{1/2} \) values at the different Ca\(^{2+}\) concentrations for 16 of the 19 constructs were not significantly different from the wild-type hslo channel (Fig. 5 A), indicating that the insertion of the fluorescent proteins did not affect the ability of Ca\(^{2+}\) to regulate the channel. Nevertheless, one insertion in the Ca\(^{2+}\) bowl (construct 901-CFP) reduced the ability of Ca\(^{2+}\) to shift the G-V curve to more negative voltages. In both this construct and 904-YFP the mutants’ curves were \( \sim 100 \) mV right-shifted when compared with the corresponding wild-type curves between 1 and 100 \( \mu \)M Ca\(^{2+}\) (Table II; Fig. 4 D; Fig. 5, C, D, and F). These results are reminiscent of findings by Schreiber and Salkoff (1997) and Bao et al. (2002), who observed large effects on G-V curves from deletions or point mutations in the Ca-bowl region. Our results, obtained after a random mutagenesis of the hslo channel, give additional unbiased support to the conclusion that the Ca\(^{2+}\) bowl is a sensitive region for regulatory effects on the channel.

When studied over the 1–100 \( \mu \)M Ca\(^{2+}\) range, the 829-CFP construct also showed a significant shift of the G-V curve (Table II; Fig. 4 D; Fig. 5, E and F). The S829 residue is situated in the COOH terminus of the RCK2 domain that showed little change, (C and D) insertions in the RCK2 domain that also shifted the G-V curves between 1 and 100 \( \mu \)M Ca\(^{2+}\)) used to obtain the G-V curves at 100 mV right-shifted when compared with the corresponding wild-type curves between 1 and 100 \( \mu \)M Ca\(^{2+}\) for the constructs shown in A–D. The points plotted are average parameter values determined from experiments fitted individually with a Boltzmann function. The wild-type values are connected with lines. Error bars are smaller than the symbols. (G) Apparent gating valence \( z \) vs. \([\text{Ca}^{2+}]\). Symbols represent channel constructs as in E. Values of \( V_{1/2} \) and \( z \) are listed in Table II. Error bars represent SEM.

**Figure 5.** Insertions 829, 901, and 904 show altered G-V curves. Data are shown from (A) wild type hslo, (B) an insertion in the RCK2 domain whose G-V curves were significantly shifted, and (E) an insertion in the RCK2 domain that also shifted the G-V curve. Ca\(^{2+}\) concentrations are as indicated in the figure. On the left are representative current families (with 100 \( \mu \)M Ca\(^{2+}\)) used to obtain the G-V curves on the right. Holding potential was \( \sim 70 \) mV. Cells were depolarized to between \( \sim 70 \) and \( \sim 120 \) mV (WT, 866-YFP, and 829-YFP), +10 and +200 (901-CFP), or +10 and +190 (904-YFP). All repolarizations were to \( \sim 70 \) mV. Note the different time scale in B. (F) Half maximal activation voltage \( V_{1/2} \) vs. \([\text{Ca}^{2+}]\) for the constructs shown in A–D. The points plotted are average parameter values determined from experiments fitted individually with a Boltzmann function. The wild-type values are connected with lines. Error bars are smaller than the symbols. (G) Apparent gating valence \( z \) vs. \([\text{Ca}^{2+}]\). Symbols represent channel constructs as in E. Values of \( V_{1/2} \) and \( z \) are listed in Table II. Error bars represent SEM.

**Table II**

| [Ca\(^{2+}\)] | Construct | \( V_{1/2} \) | \( z \) | \( n \) |
|----------------|------------|----------------|---|---|
| 1 \( \mu \)M | Wild type | 113 ± 5 | 0.99 ± 0.01 | 6 |
| 1 \( \mu \)M | 866-YFP | 98 ± 8 | 0.98 ± 0.03 | 8 |
| 1 \( \mu \)M | 901-CFP | 201 ± 3 | 1.11 ± 0.04 | 3 |
| 1 \( \mu \)M | 904-YFP | 203 ± 5 | 1.05 ± 0.03 | 3 |
| 1 \( \mu \)M | 829-YFP | 131 ± 2 | 1 ± 0.02 | 3 |

Values are given as mean ± SEM. Parameters were obtained from fits to 

\[ G/G_{\text{max}} = \frac{1}{1 + e^{(V - V_{1/2})/z}} \]

at the indicated [Ca\(^{2+}\)].
domain, 54 residues away from the Ca$^{2+}$ bowl. Interestingly, the other three insertions obtained in the RCK2 region (854-YFP, 860-CFP, and 866-YFP) showed no significant differences in the G-V curves with the wild type, although they are closer in the amino acid sequence to the Ca$^{2+}$ bowl region.

Finally, we speculated that the reasons why many of the insertions were not expressed at the membrane could be one or a combination of the following: a total misfolding and aggregation of hslo subunits; a defect in protein tetramerization; or defects in channel trafficking. We used a biochemical approach to address the first possibility. The fusion proteins were expressed in CHO cells and tested for solubility in buffers containing different concentrations of the nonionic detergent Triton X-100. Insolubility in this detergent is an indicator of protein misfolding and aggregation (Manganas et al., 2001; Zarei et al., 2004). We performed these experiments with five representative constructs that are expressed at the plasma membrane (insertions 667 in the linker, 854 and 866 in the RCK2 domain, 901 in the Ca$^{2+}$ bowl, and 1062 in the Ser-P domain) and another four representative intracellularly retained insertions (insertions 13 at the NH$_2$ terminus, 336 at the end of S6, 471 in the RCK1 domain, and 780 in the RCK2). Every construct tested has a solubilization profile indistinguishable from the wild-type hslo protein (representative examples are shown in Fig. 6). A substantial part of the protein was solubilized with 0.1% of Triton X-100 and solubilization was complete with 1% of Triton X-100. This rules out the possibility that some mutant proteins are grossly misfolded and aggregated.

The results presented in Fig. 6 suggest that the insertion of the GFP domain in certain regions of the channel could interfere more subtly with hslo folding, causing defects that impair protein tetramerization or interfere with trafficking of the channel. Quirk and Reinhart (2001) have demonstrated the existence of several regions required for functional channel tetramerization and expression. The most important comprises the first half of the RCK1 domain, and six of our constructs present insertions in that area; none of them result in surface-expressed protein (Table I). These authors also showed that more distal domains (amino acids 500–528) could contribute to tetramerization of the channel. Two of the fluorescent fusion hslo proteins of our library contain insertions in this region and are also unable to go to the membrane (Table I).

Random insertions of GFP domains have previously been performed in three other systems, where the frequency of obtaining functional fusion proteins has been in the range of 10–20%. Biondi et al. (1998) used a strategy involving random nicks to introduce the GFP sequence into the *Dictyostelium* protein kinase A regulatory subunit gene. Of the 14 full-length fusion proteins that were characterized, three showed cAMP-dependent regulatory activity. Sheridan et al. (2002) used transposons to create GFP fusions with the G-protein $\alpha$ subunit and with the glutamate receptor subunit GluR1. From 12 $G\alpha$ constructs they found one with clear functional activity; out of 29 GluR1 fusion constructs they reported glutamate-activated currents in six. In view of these results, our 35% success rate (19 high-expressing functional fusion proteins of our library contain insertions in this region) is quite high, and illustrates a remarkable ability of the BK channel to tolerate insertions in its tail domain. The battery of functional constructs obtained here promise to be useful in future spectroscopic studies of channel conformational changes.

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