

**Interactions with filamin A stimulate surface
expression of large conductance Ca^{2+} -activated K^{+}
channels in the absence of direct actin binding**

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Non-standard abbreviations

ABD	Actin-binding domain of filamin A
BK _{Ca} channels	Large-conductance Ca ²⁺ -activated K ⁺ channels
FLN	filamin A
GFP	green fluorescence protein
GST	glutathione-S-transferase
HA	hemagglutinin epitope
PI3K	phosphoinositide 3-kinase
PMSF	phenylmethylsulphonyl fluoride
RCK	Regulator of conductance of K ⁺ channels domain
Slo1	Pore-forming subunit of BK _{Ca} channels

Abstract

Large conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels play an important role in the regulation of cell physiology in a wide variety of excitable and non-excitable tissues. Filamin A is a conserved and ubiquitous actin-binding protein that forms perpendicular actin cross-links, and contributes to changes in cell shape, stiffness, and motility. A variety of membrane proteins bind to filamin A, which regulates their trafficking in and out of the plasma membrane. Filamin A is therefore thought to couple membrane dynamics with those of the underlying cytoskeleton. Filamin A was identified in a yeast two-hybrid screen of a neuronal transcriptome using a subunit of BK_{Ca} channels as bait, and the interaction was confirmed by a variety of biochemical assays in native neuronal cells and in HEK293T cells expressing BK_{Ca} channels. BK_{Ca} channels do not traffic to the plasma membrane in M2 melanoma cells which lack filamin A, but normal trafficking is seen in A7 cells which express filamin A, or in M2 cells transiently transfected with filamin A. Importantly, stimulation of plasma membrane expression of BK_{Ca} channels also occurs when M2 cells are transfected with filamin A constructs that lack the actin binding domain and that do not bind actin *in vivo* or *in vitro*. Filamin A is necessary for normal trafficking of BK_{Ca} channels to the plasma membrane, but this effect does not require interactions with actin microfilaments, and it is possible that other actions of the filamin family of scaffolding proteins are independent of effects on actin.

Introduction

Large conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels are expressed in a wide variety of tissues including nerve, muscle, secretory cells, and epithelia, where they play key roles in regulation of cell physiology. The gating of BK_{Ca} channels is controlled by membrane potential and by localized changes in intracellular free Ca^{2+} (Lu et al., 2006). The principal subunits of BK_{Ca} channels, encoded by the Slo1 gene, have seven putative transmembrane-spanning segments that form the pore and at least a portion of the voltage sensor. They also have a large intracellular C-terminus that is necessary for Ca^{2+} -induced changes in gating (Lu et al., 2006). These regions contain domains that are thought to form Ca^{2+} -binding sites required for activation under physiological conditions, including the regulator of conductance of K^+ (RCK) (Schreiber and Salkoff, 1997) and calcium bowl domains (Bao et al., 2004).

The cytoplasmic C-terminal of Slo1 also contains motifs that regulate surface expression in the plasma membrane (Kwon and Guggino, 2004) some of which are subjected to alternative splicing (Kim et al., 2007a, 2007b; Ma et al, 2007). Given this, it is not surprising that a number of proteins associate with portions of the cytoplasmic C-terminal regions of Slo1 (Lu et al., 2006). These include BK_{Ca} β -subunits, which regulate the gating (Toro et al., 2006) and trafficking (Kim et al., 2007a) of BK_{Ca} channels, as well as cytoskeletal elements (Park et al., 2004; Brainard et al., 2005; Tian et al., 2006; Ma et al. 2007), protein phosphatases (Ma et al., 2007), proteases (Jo et al., 2005) and adapter proteins such as β -catenin (Lesage et al., 2004). In the present study, we show that an actin-binding scaffolding protein, filamin A, also interacts with BK_{Ca} channels in part by binding to domains in the C-terminals of Slo1 subunits.

Filamins are a highly conserved class of actin-binding phosphoproteins that crosslink actin filaments and thereby increase the rigidity of the submembrane F-actin cortex (Stossel et al., 2001). Native mammalian filamin A is a homodimer of 280 kDa containing an N-terminal actin-binding domain (ABD), 24 repeats of a ~96 residue motif, two flexible hinge regions, and a C-terminal self-dimerization region (Stossel et al., 2001; Feng and Walsh, 2004; Popowicz et al., 2006). To date, more than 45 different proteins have been shown to interact with filamin A. These include transmembrane receptors (Sharma et al., 1995; Loo et al., 1998; Lin et al., 2001), ion channels (Petrecca et al., 2000; Sampson et al., 2003; Gravant et al., 2004), and a variety of signaling molecules (Ohta et al., 1999; Tigges et al., 2003). By forming a direct interaction with the underlying actin cytoskeleton, filamins regulate the stability of the cell surface, the trafficking of membrane proteins, and the integration of cell signals with cell shape and the organization of the actin cytoskeleton (Stossel et al., 2001).

We have previously shown that trafficking of BK_{Ca} channels to the plasma membrane of native neurons is a dynamic process that is positively and negatively regulated by a variety of growth factors (Dryer et al., 2003). In order to understand these processes in more detail, we carried out a yeast-two hybrid screen to identify BK_{Ca} channel-interacting proteins that could contribute to regulation of trafficking. Filamin A was identified in this screen, and interactions with multiple BK_{Ca} subunits were confirmed by independent methods, as described further below. Importantly, we show that filamin plays a role in regulation of BK_{Ca} channel trafficking to the plasma membrane and that direct actin interactions are not necessary for this effect, as they persist in filamin A constructs unable to bind to actin.

Materials and Methods

Yeast two-hybrid screen. Yeast two-hybrid analysis was carried out using the Matchmaker System™ (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. We constructed a cDNA library of the embryonic day 9 (E9) chick ciliary ganglion transcriptome using random primers that allow for homologous recombination into the pGADT7-rec plasmid. The resulting library, which encodes fusion proteins comprised of expressed motifs and an attached N-terminal GAL4-activation domain, was transformed into the AH109 yeast strain, and selected on a SD/Leu- single dropout medium. The yeast library concentration was adjusted to 2×10^7 cfu/ml. We obtained filamin A using two different baits: The first bait was a construct encoding amino acids 175-200 of the chick $\beta 1$ subunit of BK_{Ca} channels (Kim et al., 2007a), cloned in-frame into the pGBKT7 bait vector in the Matchmaker™ system. The second bait was a construct comprised of residues 1076-1137 of the QEDRL isoform of avian Slo1 (Kim et al., 2007b). The first 51 residues in this bait are also present in the VEDEC isoform of Slo1. These bait vectors encode fusion proteins with an N-terminal DNA-binding domain of the yeast GAL4 protein (residues 1-147). The bait vectors were transformed into the Y187 strain, which were selected on SD/Trp- single dropout medium. The transformed AH109 cDNA library cells were then mated with the transformed Y187 bait cells. Positive colonies expressing putative interacting proteins were selected by *MEL1* expression. This gene encodes α -galactosidase and is driven by an upstream GAL4-UAS. Its expression was determined by blue-white selection carried out on a quadruple dropout medium (SD/Ade-/His-/Trp-/Leu-) supplemented with the chromogenic substrate

5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside, which yields a blue product after cleavage by α -galactosidase. After selection, the pGADT7 plasmids encoding fragments of putative interacting proteins were isolated by standard methods, transformed into *E. coli* Top Ten™ cells (Invitrogen, Carlsbad, CA), sequenced, and subjected to BLAST search analysis.

Plasmid constructs. Expression plasmids encoding N-terminal Myc-tagged Slo1_{VEDEC} and Slo1_{QEERL} isoforms of Slo1 were kindly provided by Dr. Min Li of the Department of Neuroscience at Johns Hopkins University (Kim et al., 2007b; Ma et al., 2007). Constructs encoding β 1-green fluorescent protein (GFP) fusion proteins, and glutathione S-transferase (GST)- β 1 fusion proteins, including GST- β 1N (residues 1-22), GST- β 1L (residues 40-155), and GST- β 1C (residues 175-200), were described previously (Kim et al., 2007a). Plasmids encoding GST-Slo1_{VEDEC}, GST-Slo1_{QEERL} were generated by PCR from full-length chick Slo1 and cloned into pGEX-KG expression vector (Amersham Biosciences, Piscataway, NJ). A plasmid encoding full-length filamin A in pREP4 vector (Invitrogen) was provided by Dr. J. Hartwig (Harvard Medical School, Boston, MA). Constructs encoding N-terminal GFP-and hemagglutinin epitope (HA)-tagged truncated filamin A proteins were generated by subcloning various PCR products into pcDNA3.1/NT-GFP-TOPO vector (Invitrogen) and pCMV-HA vector (Clontech Laboratories, Inc, Mountain View, CA), respectively. Filamin fusion protein constructs included one containing a portion of the N-terminal actin-binding domain (ABD), as well as separate proteins comprised of filamin A repeats 14-15 (FLN₁₄₋₁₅), FLN₁₆₋₁₇, and FLN₁₈₋₁₉. We also prepared GST-filamin A fusion proteins by subcloning repeats 14-15,

16-17, and 18-19 into the pGEX-6P-1 expression vector (Amersham Biosciences, Piscataway, NJ). The fidelity of all constructs was confirmed by sequencing.

Cell culture and transfection. A human melanoma cell line lacking filamin A (M2 cells) and an isogenic cell line stably transfected with a full-length filamin A cDNA (A7 cells), were provided by Dr. J. Hartwig. Cells were grown in α -minimal essential medium (MEM) (Invitrogen) supplemented with 8% newborn calf serum and 2% fetal bovine serum. HEK293T (human embryonic kidney) cells were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10 % heat-inactivated fetal bovine serum at 37°C in a 5 % CO₂ incubator. In most experiments, M2, A7 and HEK293T cells were transiently transfected in 6-well plates (for biochemistry) or 24-well plates (for electrophysiology) using Lipofectamine-2000™ (Invitrogen) in serum-reduced medium (Opti-MEM, Invitrogen) following the manufacturer's instructions. The DNA concentration in the transfection medium was 1 μ g/ml of each plasmid. Cells were used for physiology or biochemistry 24-48 h after transfection. Embryonic day 9 (E9) or E13 ciliary ganglia were dissected from eggs obtained from Spafas, Peoria, IL, USA.

Electrophysiology. In experiments with M2 and A7 cells, plasmids encoding GFP or GFP-fusion proteins were co-transfected with either VEDEC or QEERL expression vectors, and whole cell recordings were made from green fluorescent cells using standard methods similar to those described previously (Kim et al., 2007a, 2007b). The bathing solution contained (in mM): NaCl 150, KCl 0.08, MgCl₂ 0.8, CaCl₂ 5.4, glucose 10, HEPES 10, and the pH was adjusted to 7.4 with NaOH. The pipette solution contained

(in mM): NaCl 145, KCl 2, MgCl₂ .6.2, CaCl₂ 5 μM, pH 7.2. M2 and A7 cells do not express endogenous voltage-activated Ca²⁺ currents, and these ionic conditions were chosen to provide sufficient intracellular Ca²⁺ (5 μM) for activation of BK_{Ca} channels by depolarizing step pulses, while keeping the resulting macroscopic currents small enough to avoid saturation of the patch clamp amplifier or significant series resistance errors. The later were achieved by reducing the concentration of permeant ions by ~60-fold while still maintaining a physiological E_K of -80 mV. Recording electrodes were made from thin borosilicate glass and fire-polished. They had resistances of 3-4 MΩ when filled with pipette saline and it was possible to compensate up to 85% of this without introducing oscillations into the current output of the patch clamp amplifier (Axopatch 1D, Axon Instruments). All physiological experiments were conducted at room temperature. Currents were evoked by a series of steps from a holding potential of -60 mV, digitized, and analyzed off-line using PClamp software (Axon Instruments). We have previously shown that all of the macroscopic outward current detected by this protocol is carried by BK_{Ca} channels (Kim et al., 2007a).

Cell-surface biotinylation assays. M2 and A7 cells were grown on 6-well plates for 24-48 h after transfection. Cell-surface biotinylation was carried out as described in detail previously (Kim et al., 2007a, 2007b). Briefly, intact cells were treated with a membrane impermeable biotinylation reagent, sulfo-*N*-hydroxy-succinimidobiotin (Pierce Biotechnology, Rockford, IL) (1 mg/ml in PBS buffer) for 1 h on ice with gentle shaking. The reaction was stopped, cells were lysed, and biotinylated proteins from the cell surface were recovered from lysates by incubation with immobilized streptavidin-agarose

beads (Pierce Biotechnology). A sample of the initial cell lysate was also retained for analysis of total proteins. These samples were separated on SDS-PAGE, and proteins were quantified by immunoblot analysis. These and all subsequent biochemical experiments were repeated at least three times.

Co-immunoprecipitation and immunoblot analysis. For co-immunoprecipitation, GFP- β 1 and N-terminal Myc-tagged VEDEC were expressed in M2 and A7 cells. Cells were lysed in 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, and protease inhibitor mixture (Sigma, St. Louis, MO). Cell extracts (500-700 μ g of protein) were incubated in the presence of primary antibodies anti-GFP (Invitrogen), anti-Myc (Cell signaling) or IgG (1-2 μ g) for 4 h at 4 °C, followed by the addition of 20 μ l of protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 12 h. Pellets were washed four times, boiled for 5 min in SDS sample buffer, and subjected to SDS-PAGE on 10% gels. Cell extracted protein (50-100 μ g) was used as control in each experiment. Blots were blocked with 5% nonfat dried milk dissolved in TBST buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, washed three times with TBST buffer, incubated with the primary antibody overnight at 4 °C, washed again with TBST, and the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The proteins were visualized using a chemiluminescent substrate (Pierce Biotechnology). E9 and E13 CG were lysed and the soluble cell extracts (300 μ g of protein) were incubated in the presence of primary antibodies anti-Slo1_{VEDEC} (Kim et al., 2007b), anti- β 1 (Alomone Labs Ltd, Jerusalem, Israel) or IgG (1-2

μg) for 4 h at 4 °C, followed by the addition of 20 μl of protein A/G agarose for 12 h at 4° C with gentle rotation. Pellets were washed and boiled in sample buffer and separated on 10% SDS-PAGE and analyzed by immunoblot as described above.

GST pull-down assay. GST, GST-FLN₁₄₋₁₅, GST-FLN₁₆₋₁₇, GST-FLN₁₈₋₁₉, GST-Slo1_{VEDEC}, GST-Slo1_{QEERL}, GST-β1N, GST-β1L, or GST-β1C fusion proteins were expressed and extracted from *E.coli* strain BL21 and 100-200 μg of each were bound to glutathione-Sepharose 4B beads according to manufacturer's instructions (Amersham Biosciences). Transfected HEK293T cells with Myc-tagged Slo1_{VEDEC} or GFP-tagged β1, M2 and A7 cells were lysed, and the soluble cell extracts (300-500 μg of protein) were added to the beads and incubated overnight at 4° C with gentle rotation. Beads were washed three times with PBS containing 0.1-0.5% Triton X-100 before the bound proteins were eluted with glutathione elution buffer. Eluates were incubated in sample buffer and separated on 10% SDS-PAGE gels. For immunoblot analyses of filamin-A expression in M2 and A7 cells, cells were lysed and incubated with the primary anti-filamin A (Chemicon, Temecula, CA) and anti-actin (Chemicon). Cells were washed in ice-cold PBS and lysed in Laemmli buffer, and samples were boiled for 5 min at 95°C and separated by SDS-PAGE on 9% gels and analyzed by immunoblot as described above.

Confocal microscopy. For immunofluorescent labeling, A7 cells were transfected with GFP-FLN₁₄₋₁₅, GFP-FLN₁₆₋₁₇, GFP-FLN₁₈₋₁₉, or GFP-FLN_{ABD}. These cell, along with nontransfected M2 and A7 control cells, were fixed in 4% paraformaldehyde, blocked,

and permeabilized in PBST, and exposed to the anti-GFP (Invitrogen) and anti-filamin A (Chemicon) and incubated with Alexa Flour-568 phalloidin (Molecular probes, Eugene, OR) for F-actin staining. To examine the role of filamin in surface expression of BK_{Ca} channels, M2 and A7 cells were transiently transfected with N-terminal myc-tagged Slo1_{VEDEC} or Slo1_{QEERL}, either alone or together with HA-tagged truncated filamin constructs described above. Cells were subsequently exposed to fluorescein-conjugated goat anti-myc (Abcam, Cambridge, MA) (1:500) in Opti-MEMTM medium for 1 hr at 37° to label surface Slo1 channels. Cells were then washed in PBS, fixed by 30 min exposure to 4% paraformaldehyde in PBS, rinsed in PBS, blocked with 10% normal goat serum, and then permeabilized in PBS containing 0.5% Triton X-100. They were then incubated with mouse anti-myc antibody (1:1000) for 1 h (antibody 9B11) and then exposed to Alexa-568-conjugated anti-mouse IgG (Molecular Probes) (1:1000) for 1h to label intracellular Slo1 channels. The cells were then rinsed in PBS and mounted using Vectashield (Vector Laboratories, Burlingame, CA). All images were collected on an Olympus FV-1000 inverted stage confocal microscope using a Plan Apo N 60X 1.42NA oil-immersion objective. Green fluorescence (from FITC) was evoked using an excitation wavelength of 495 nm while monitoring emission at 519 nm. Red fluorescence (from Alexa-568) was evoked by excitation at 580 nm and emission was monitored at 620 nm.

Statistics. All quantitative data are presented as mean \pm S.E.M. The data in bar graphs were compiled from 9-25 cells in each group. Data were analyzed by one-way ANOVA followed by *post hoc* analysis using Tukey's honest significant difference test for unequal sample size, with $p < 0.05$ regarded as significant.

Results

Filamin A binds to the C-terminus of BK_{Ca} channels. We initially obtained filamin A in a screen for interacting proteins of BK_{Ca} channels using the C-terminus of the avian β 1 subunit (175-200 amino acids) as a bait in a yeast two-hybrid screen of an E9 chick ciliary ganglion neuron cDNA library. We used the β 1 C-terminus as a bait because we previously presented evidence that the avian β 1 subunit can stimulate the surface expression of at least some isoforms of BK_{Ca} channels in native ciliary ganglion neurons as well as in heterologous expression systems (Kim et al., 2007a). We found ~20 putative interacting partners, and this study focuses on one of them, filamin A, which we obtained eight times. The partial filamin A cDNA fragments that were isolated in this assay are shown schematically in Fig. 1A. Similar fragments of filamin A also appeared several times in a subsequent yeast-two hybrid screen carried out using the C-terminal domains of Slo1 as a bait. The filamin A clones insolated in these screens began at variable starting points and were all located close to and on either side of a hinge region in the molecule that separates filamin repeats 15 and 16. To examine whether these fragments directly bind to BK_{Ca} channels, we prepared a series of GST-filamin (FLN) fusion proteins, depicted in Fig. 1B, and used them in a pull-down assay on extracts of HEK293T cells expressing myc-tagged Slo1 channels or GFP-tagged avian β 1 subunit of BK_{Ca} channels (Fig. 1B). We observed that GST-FLN₁₄₋₁₅, GST-FLN₁₆₋₁₇ and GST-FLN₁₈₋₁₉ were able to interact with both classes of BK_{Ca} channel subunits, whereas GST did not. In addition, we were able to show that filamin A was present in immunoprecipates prepared from E9 and E13 chick ciliary ganglia using antibodies prepared against Slo1 channels or β 1-subunits (Fig. 1C). Our subsequent analyses have

been facilitated by the availability of a human melanoma-derived cell line that lacks filamin A (M2 cells), as well as an isogenic melanoma cell line stably transfected with filamin A (A7 cells) (Cunningham et al., 1992). We obtained these cell lines from Dr. J. Hartwig of Harvard Medical School. As expected, confocal immunofluorescence and immunoblot analysis confirmed that filamin A was highly expressed throughout A7 cells but not in M2 cells (Fig. 2A). Actin was present in both cell lines, and the cortical F-actin layer subjacent to the plasma membrane showed a similar appearance in A7 and M2 cells (Fig. 2A). Therefore, we transiently transfected both cell lines with myc-tagged Slo1 channels or GFP-tagged β 1 subunits, and then carried out immunoprecipitations using antibodies against the tags. We observed that filamin A was present in the immunoprecipitates prepared from A7 cells but not from M2 cells (Fig. 2B). In addition, we used these cell lines to analyze interactions of BK_{Ca} subunits with filamin A using channel subunit GST fusion proteins described previously (Kim et al., 2007a, 2007b). We observed that a GST fusion protein comprised of the last 25 residues of the C-terminus of β 1 (GST- β 1C) was able to interact with filamin in a GST pull-down assay carried out on extracts of A7 cells. However, GST fusion proteins composed of the first 22 residues of N-terminus (GST- β 1N) or the two membrane spanning regions and the entire extracellular loop domain (GST- β 1L) were not able to pull down filamin, and none of the channel constructs could pull filamin out of M2 cells (Fig. 2C). Finally, we have previously shown that chick ciliary ganglion neurons express transcripts and proteins of two Slo1 splice variants that differ at the extreme C-terminal (Kim et al., 2007b; Ma et al., 2007). We refer to these variants as Slo1_{VEDEC} and Slo1_{QEERL} after the five last residues in each isoform. We transiently transfected A7 cells with GST-fusion proteins comprised

of the C-terminals of both of these Slo1 variants and carried out GST pull-down assays. We observed that both Slo1 variants can bind to filamin A (Fig. 2D), suggesting that the interaction occurs in the large C-terminal cytoplasmic domains, possibly upstream from where these splice variants diverge. In summary, we have confirmed and extended the results of the yeast two-hybrid screen, and have shown that the filamin A can bind to multiple BK_{Ca} subunits, including the cytoplasmic C-terminals of two different splice variants of Slo1, as well as the cytoplasmic C-terminus of avian β 1 subunits. It is worth noting that there is no obvious sequence homology in those domains.

Filamin A regulates the surface expression of BK_{Ca} channels. M2 melanoma cells that lack filamin A have been reported to have a reduced surface expression of many receptors and ion channels compared to otherwise isogenic cells that express filamin A (Petrecca et al., 2000; Lin et al., 2001; Onoprishvili et al., 2003; Thelin et al., 2007). To test if filamin A had a similar effect on the trafficking of BK_{Ca} channels, we expressed full-length myc-tagged BK_{Ca} channel variants (Slo1_{VEDEC} or Slo1_{QEERL}) in A7 cells and M2 cells. Fig. 3A shows confocal images of surface expression of the Slo1 channels labeled with fluorescein-conjugated anti-myc antibody. The cells were then fixed, blocked, permeabilized, and labeled with a non-conjugated anti-myc raised in a different species to obtain signal from intracellular channels. We observed significant amounts of Slo1_{QEERL} channels at the cell surface (*green fluorescence*) and in intracellular pools (*red fluorescence*) in A7 cells. However, surface signal for Slo1_{VEDEC} was below the threshold of detection by this method in either cell line, although intracellular signal was comparable to that seen with Slo1_{QEERL}. Note that we used identical processing

procedures, myc antibodies and laser excitation intensities to determine the distribution of both Slo1 isoforms. The difference in constitutive surface expression of Slo1_{VEDEC} and Slo1_{QEERL} in A7 cells is consistent with results in several other cell types (Kim et al., 2007a, 2007b; Ma et al., 2007). However, the failure to detect surface expression of Slo1_{QEERL} channels in M2 cells suggests an essential role for filamin A in allowing for significant expression of BK_{Ca} channels on the cell surface. A similar pattern was obtained using cell-surface biotinylation assays in which channel detection in immunoblots was carried out using anti-myc (Fig. 3B). We observed that steady-state surface expression of Slo1_{QEERL} channels was greater in A7 cells than in M2 cells, suggesting an essential role for filamin A in channel trafficking. Consistent with our previous experiments in other cell lines (Kim et al., 2007a, 2007b) we also observed that surface expression of Slo1_{QEERL} was greater than Slo1_{VEDEC} channels in A7 cells. In all of these experiments, total Slo1 protein levels were present at comparable levels in both cell lines and with both constructs. Electrophysiological data from A7 and M2 cells provide an independent line of evidence supporting a role for filamin A in regulating the surface expression of BK_{Ca} channels. In these experiments, we examined whole-cell currents using methods described previously for other cell lines (Kim et al., 2007a, 2007b). The recording pipettes contained 5 μ M Ca²⁺ in order to allow activation of BK_{Ca} channels by step pulses from a holding potential of -60mV. Currents evoked by steps to +80 mV were used for quantification of surface expression. We could not detect currents in non-transfected cells subjected to these same voltage-clamp protocols (data not shown). However, robust whole-cell currents were recorded from A7 cells expressing Slo1_{QEERL} channels, but mean currents were very much smaller in M2 cells expressing the same

Slo1_{QEERL} construct (Fig. 4A, 4B). Moreover, co-expression of full-length filamin A with Slo1_{QEERL} in M2 cells allowed for robust macroscopic currents not significantly different from those seen in A7 cells (Fig. 4B). In other words, it is possible to rescue Slo1_{QEERL} currents by restoring filamin A expression. We observed that macroscopic currents in cells expressing Slo1_{VEDEC} were always smaller than in cells expressing Slo1_{QEERL}, but we could still detect a statistically significant effect of filamin A (Fig. 4B). Given that filamin A binds to both the Slo1 and β 1 subunits of BK_{Ca} channels, it also bears noting that co-expression of β 1 with the Slo1 subunits did not affect the general pattern – that is, currents were substantially reduced in M2 cells compared to A7 cells expressing the same constructs (Fig. 4C), even though β 1 did tend to increase currents, as we have seen in other cell types (Kim et al., 2007a).

Effects of filamin A on surface expression of BK_{Ca} channels does not require direct interactions with actin. Previous studies have shown that Slo1 channels bind directly to actin (Brainard et al., 2005; Ma et al., 2007), and therefore we found functionally significant interactions of Slo1 channels with an actin-binding protein to be somewhat surprising (but see Tian et al., 2006). The structure of filamin A lends itself to formation of a number of different kinds of larger complexes besides those with actin, and therefore we asked whether the effect of filamin A on Slo1 trafficking actually requires filamin A-actin interactions. To do this, we prepared a series of GFP-tagged filamin A fragments (GFP-FLN_{ABD}, GFP-FLN₁₄₋₁₅, GFP-FLN₁₆₋₁₇ and GFP-FLN₁₈₋₁₉) shown schematically in Fig. 5A, and examined their effects on trafficking of Slo1_{QEERL} subunits expressed in M2 cells. Recall that these are the portions of the filamin molecule that appeared in our yeast

two-hybrid screens. We observed that GFP-FLN₁₄₋₁₅, GFP-FLN₁₆₋₁₇ and GFP-FLN₁₈₋₁₉ by themselves were able to stimulate cell surface expression of Slo1_{QEERL} channels as accessed by cell-surface biotinylation assays (Fig. 5B) and by whole-cell recordings (Fig. 5C), whereas GFP-FLN_{ABD} and GFP were ineffective. Indeed, fusion proteins comprised of two filamin repeats were approximately as effective as full-length filamin-A at stimulating whole-cell currents. This pattern was also seen using confocal imaging of surface Slo1_{QEERL} channels labeled using fluorescein-conjugated antibodies against the myc tags (Fig. 6). In these experiments, the filamin A fragments were expressed as N-terminal HA-tagged fusion proteins to avoid interference with the fluorescence assay. Specifically, we observed that all of the constructs made of filamin repeats stimulated surface expression of Slo1_{QEERL}, as does full-length filamin A, but that the HA-tagged construct made from the ABD was ineffective. We confirmed by two different procedures that filamin A fragments lacking the ABD fail to interact with actin (Fig. 7). First, we observed with a pull-down assay that GST-FLN_{ABD} exhibited robust binding to actin in A7 cell extracts, whereas GST-FLN₁₄₋₁₅, GST-FLN₁₆₋₁₇, GST-FLN₁₈₋₁₉, and GST do not (Fig. 7A). Moreover confocal microscopy in transfected A7 cells showed extensive co-localization of GFP-FLN_{ABD} with cortical F-actin revealed by staining with Alexa-568-conjugated phalloidin (Fig. 7B). By contrast, GFP-FLN₁₄₋₁₅, GFP-FLN₁₆₋₁₇, GFP-FLN₁₈₋₁₉ do not show significant co-localization with F-actin and appear to be excluded from the periphery of the cell. Collectively, these data indicate that actin interactions are not required for filamin A to stimulate surface expression of BK_{Ca} channels.

Discussion

BK_{Ca} channels encoded by Slo1 are structurally and functionally diverse and exhibit differences in gating, modulation, and trafficking that arise from alternative splicing, posttranslational modification, and co-expression of different modulatory subunits (Lu et al., 2006). We and others have previously reported that different C-terminal splice variants of Slo1 show different trafficking patterns in developing neurons and in transformed cell lines (Kim et al., 2007a, 2007b; Ma et al., 2007), and that co-expression of β 1 subunits can modify these patterns (Kim et al., 2007a). Using fragments of the chick β 1 and Slo1 subunits as baits in yeast two-hybrid screens, we identified filamin A as a BK_{Ca}-interacting protein, and confirmed these interactions using several independent biochemical procedures. The two most important observations of this study are that filamin A is necessary for robust surface expression of BK_{Ca}, and that filamin A does not need to interact with actin to produce this effect. We also observed that filamin A can stimulate surface expression of Slo1 in either the presence or absence of the β 1 subunit.

A variety of membrane proteins have been reported to interact with filamin A, including ion channels (Petrecca et al., 2000; Sampson et al., 2003; Gravante et al., 2004), G-protein coupled receptors (Li et al., 2000, Enz, 2002, Onoprishvili et al., 2003; Seck et al., 2003), and signaling molecules (Marti et al., 1997 ; Ohta et al., 1999). Filamin A interactions with these proteins have a variety of functional consequences, including regulation of surface expression (Binda et al., 2002; Sampson et al., 2003; Thelin et al., 2007), that have been attributed to actin interactions (Lin et al., 2001; Vadlamudi et al., 2002). In other words, filamin A is generally considered to provide a mechanism to

couple membrane and cytoskeletal dynamics. The data in the present study raise the possibility that filamin A may have additional roles that do not require direct binding to actin.

Interestingly, recent studies on nuclear proteins have reached a similar conclusion. For example, a naturally occurring fragment of filamin A comprised of repeats 16-23 has been shown to translocate to the nucleus, binds to androgen receptors and thereby prevent transactivation of target genes in prostate cancer cell lines (Loy et al., 2003; Wang et al., 2007). That filamin fragment also lacks the ABD and therefore produces effects that are not directly related to actin binding. In this regard, it bears noting that the filamin A antibodies that we used are able to detect proteins less than 240 kD in immunoblots prepared from chick ciliary ganglion and A7 cells (Fig. 1, 2), raising the possibility that filamin A fragmentation may be a widespread phenomenon. On the other hand, the filamin immunoreactivity that interacts with BK_{Ca} channels appears to be full length (280 kD), and we have been unable to detect a change in filamin A fragmentation in response to growth factor treatments that stimulate BK_{Ca} expression on the cell surface (data not shown). Filamin A can interact with a variety of proteins, including actin-binding proteins such as the focal adhesion-associated proteins (Nikki et al., 2002). This raises the possibility that indirect interactions with actin, mediated by a bridge protein, could allow stimulation of BK_{Ca} trafficking by filamin fragments that lack the canonical actin-binding domain. While we cannot entirely exclude this possibility, it bears noting that the filamin fragments expressed in HEK293T cells do not immunoprecipitate or co-localize with actin (Fig. 7). Therefore, any such bridge would have to be transient and/or unstable.

While filamin A regulation of the surface expression of BK_{Ca} channels can occur in the absence of direct interactions with actin, we cannot exclude that filamin-mediated interactions with cortical actin may play an important physiological role. For example, Tian et al. (2006) recently showed that a different actin-binding adapter protein known as cortactin can bind to different regions of the cytoplasmic C-terminals of BK_{Ca} channels. That interaction does not affect trafficking of BK_{Ca} channels, but instead affects the gating properties of channels that are already in the plasma membrane, conferring mechanical sensitivity onto the channels that can be eliminated by agents that depolymerize F-actin. It is possible that filamin A plays a similar role for BK_{Ca} channels that have reached the plasma membrane, but this idea is very difficult to test because of the effect of filamin A on trafficking. Alternatively, filamin A may bring other important signaling enzymes into close physical proximity to BK_{Ca} channels, thereby insuring more rapid modulation owing to reduced diffusion times, etc. The observation that filamin A can interact with β 1 subunits as well as pore-forming subunits is interesting in this regard, as there are at least some neuromodulators that act preferentially on BK_{Ca} channels in the presence of β -subunits (Lu et al., 2006).

Previous studies from our laboratory have shown that the steady-state surface expression of BK_{Ca} channels is stimulated by growth factors in developing vertebrate neurons (Cameron et al., 1998, 2001; Chae et al., 2005) and transformed cell lines (Kim et al., 2007a, 2007b). Importantly, these effects require activation of phosphoinositide 3-kinase (PI3K) and Akt pathways (Lhuillier and Dryer, 2002; Chae et al., 2005). In this regard, filamin A is a substrate for several proteins that are downstream effectors of PI3K and Akt, including p21-activated protein kinase (Vadlamudi et al., 2002) and ribosomal

S6 kinase (Woo et al., 2004). This raises the possibility that filamin A plays a role in the regulated trafficking of BK_{Ca} channels, perhaps through changes in its phosphorylation state. In the present study, we observed that filamin A affects trafficking of both of the Slo1 isoforms. In previous studies in other cell lines, we have observed that the VEDEC isoform exhibits low levels of constitutive expression on the cell surface, but that this is can be stimulated by growth factor treatment (Kim et al., 2007b) or by co-expression of the β 1 subunit (Kim et al., 2007a). By contrast, the QEERL exhibits greater constitutive surface expression in all of the cell lines that we have examined previously. Those qualitative patterns were also observed in the melanoma cell lines examined here. One difference is that growth factor treatment (TGF β 1) can increase surface expression of the QEERL isoform as well as the VEDEC isoform of Slo1 in A7 melanoma cells, but it has no effect in M2 cells (unpublished data).

The steady-state surface expression of BK_{Ca} channels, as with other membrane proteins, is the net product of forward (insertion or recycling) and reverse (endocytosis and degradation) trafficking processes. Filamin A could be acting on one or both of these processes (Sek et al., 2003; Li et al., 2000). The current data do not allow us to pinpoint which of these processes are controlled by filamin A interactions with BK_{Ca} channels.

In summary, we have shown that the scaffolding protein interacts with multiple BK_{Ca} channel subunits and that this interaction is necessary for the normal surface expression of these channels. However, this effect does not require filamin A interactions with actin.

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Footnotes

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Figure legends

Fig. 1. Identification of cDNA clones encoding filamin A by yeast two-hybrid screen and confirmation of binding of filamin A to BK_{Ca} channel subunits. A, Schematic diagram of filamin A structure and four filamin A encoding cDNA clones obtained in yeast two-hybrid screen. The numbers in brackets indicate the amino acid residues that correspond to each fragment and the number of times each clone was isolated in the screen. B, Three different GST-filamin (FLN) fusion proteins, named according to the repeats of filamin A comprised in the constructs, are shown schematically (*top*). These fusion proteins could pull down N-terminal myc-tagged Slo1 channels and GFP-tagged avian β 1 subunits of BK_{Ca} channels from extracts of HEK293T cells expressing the tagged channel subunits. The the proteins isolated in pull-down assay were probed with antibodies directed against the myc and GFP tags. C, The interaction between filamin A and native BK_{Ca} subunits was confirmed by co-immunoprecipitation from extracts of E9 and E13 chick ciliary ganglia. Immunoprecipitation was carried out with antibodies against Slo1 (*top*) and β 1 (*bottom*), and the precipitates were analyzed by immunoblot using antibodies against filamin A.

Fig. 2. Expression of filamin A and interactions with BK_{Ca} subunits in human melanoma cell lines. A, Confocal immunofluorescence and immunoblot analysis of A7 and M2 melanoma cell lines (*left*). Cells were labeled with anti-filamin A antibody (*green*) and with Alexa Flour-568 phalloidin, a probe for F-actin (*red*). Note lack of filamin A signal in M2 cells. F-actin signal appears similar in both cell lines. Immunoblot analysis to the right confirms expression of filamin A in A7 cells but not in M2 cells. Actin signals were

detected from both cell lines. B, Lysates of A7 and M2 cells expressing myc-tagged Slo1 or GFP-tagged β 1 were immunoprecipitated with antibodies against the tags. Filamin A could be detected in the immunoprecipitates prepared from A7 cells but not from M2 cells. Filamin A was also present in the original lysates (input) from A7 but not M2 cells. C, A GST- β 1 fusion protein that includes the cytoplasmic C-terminal domains of β 1 (GST- β 1C) was able to pull down filamin A from lysates of A7 cells but not M2 cells. However, GST fusion proteins containing only the cytoplasmic N-terminus (GST- β 1N) or extracellular loop domain (GST- β 1L) were not able to interact with filamin A. D, GST-fusion proteins containing the C-terminals of two different Slo1 splice variants (Slo1_{VEDEC} and Slo1_{QEERL}) can bind to filamin A in lysates of A7 cells.

Fig. 3. Differences in surface expression of Slo1_{VEDEC} and Slo1_{QEERL} isoforms of BK_{Ca} channels expressed in A7 and M2 cells. A, Confocal immunofluorescence using antibodies against myc tags of Slo1 channels in A7 and M2 cells. Cell surface channels in cells expressing myc-tagged Slo1_{VEDEC} or Slo1_{QEERL} were labeled with an FITC-conjugated goat anti-myc applied to intact cells (*green*). Intracellular channels were stained using a mouse anti-myc revealed using Alexa-568-conjugated anti-mouse IgG (*red*). The same laser excitation intensities were used for image collection from cells expressing both Slo1 isoforms. Note that Slo1_{QEERL} channels are highly expressed on the surface and in intracellular pools in A7 cells, but Slo1_{VEDEC} channels are below the level of detection on the cell surface. Surface expression of both Slo1 variants was below the threshold of detection in M2 cells. B, Cell-surface biotinylation assays shows a similar pattern. Steady-state surface expression of Slo1_{QEERL} channels was greater in A7 cells

than in M2 cells. However, surface expression of Slo1_{QEERL} was rescued in M2 cells transiently co-expressing full-length filamin A (FLN). Surface expression of Slo1_{VEDEC} channels was low in A7 cells and M2 cells. Co-expression of full-length filamin A with Slo1_{VEDEC} channels in M2 cells did not cause a substantial increase in constitutive surface expression.

Fig. 4. Filamin A expression stimulates whole-cell currents carried through BKCa channels in melanoma cells. A, Examples of typical families of whole-cell currents evoked by a series of depolarizing voltage steps from a holding potential of -80 mV in A7 and M2 cells expressing Slo1_{QEERL} channels. Recording pipettes contained 5 μ M CaCl₂ to allow for robust activation of BK_{Ca} channels in the absence of voltage-activated Ca²⁺ channels. The concentrations of permeant K⁺ ions on both sides of the cell membrane were reduced 20-fold to maintain evoked currents in the nanoampere range. Note much larger currents in A7 cells. B, Summary of results from A7 or M2 cells expressing Slo1_{QEERL} or Slo1_{VEDEC} channels. Data are means \pm s.e.m. of currents evoked by a depolarizing step to +80 mV, and data were obtained from at least 10 cells in each group. Note that the presence of filamin A (i.e. in A7 cells or in M2 cells transiently co-expressing filamin A) leads to larger mean currents regardless of which Slo1 variant is expressed. However, mean currents are always larger in cells expressing Slo1_{QEERL} channels than those expressing Slo1_{VEDEC} channels. * indicates $P < 0.05$ compared to M2 cells expressing Slo1_{VEDEC} by itself. # indicates $P < 0.05$ compared to cells expressing Slo1_{QEERL} by itself. C, Co-expression of β 1 with Slo1 subunits did not change the overall pattern.

Fig. 5. The effects of filamin A fragments on surface expression of BK_{Ca} channels in M2 cells. A, Schematic diagram of GFP-tagged filamin A fragments. B, Cell-surface biotinylation assay showing that co-expression of GFP-FLN₁₄₋₁₅, GFP-FLN₁₆₋₁₇, or GFP-FLN₁₈₋₁₉ increases surface expression of Slo1_{QEERL} channels in M2 cells that do not express filamin A. However, GFP-FLN_{ABD} or GFP have no effect on surface expression of Slo1_{QEERL} channels in M2 cells. C, A similar pattern was obtained by monitoring mean whole-cell currents evoked by depolarizing steps in whole-cell recordings from M2 cells. * indicates $P < 0.05$ compared to cells expressing GFP.

Fig. 6. Co-expression of HA-tagged filamin A fragments increases cell-surface expression of Slo1_{QEERL} channels in M2 cells. Confocal images showing that HA-tagged filamin A repeat fragments rescue surface expression of Slo1_{QEERL} channels in M2 cells even though they lack the filamin A actin-binding domain. HA-tagged FLN_{ABD} is ineffective at stimulating Slo1 surface expression.

Fig. 7. Analysis of interactions between actin and filamin A fragments in A7 cells. A, GST pull-down assay performed on A7 cells showing that GST-FLN_{ABD} binds to actin. By contrast, actin interactions with GST-FLN₁₄₋₁₅, FLN₁₆₋₁₇, FLN₁₈₋₁₉, and GST, are undetectable. B, Confocal microscopy of A7 cells expressing GFP-tagged FLN fragments (*green*) with F-actin (*red*). Note extensive co-localization of GFP-FLN_{ABD} with F-actin. GFP-FLN₁₄₋₁₅, GFP-FLN₁₆₋₁₇, and GFP-FLN₁₈₋₁₉ do not co-localize with F-actin. Regions of co-localization appear yellow in merged images.

Figure 1

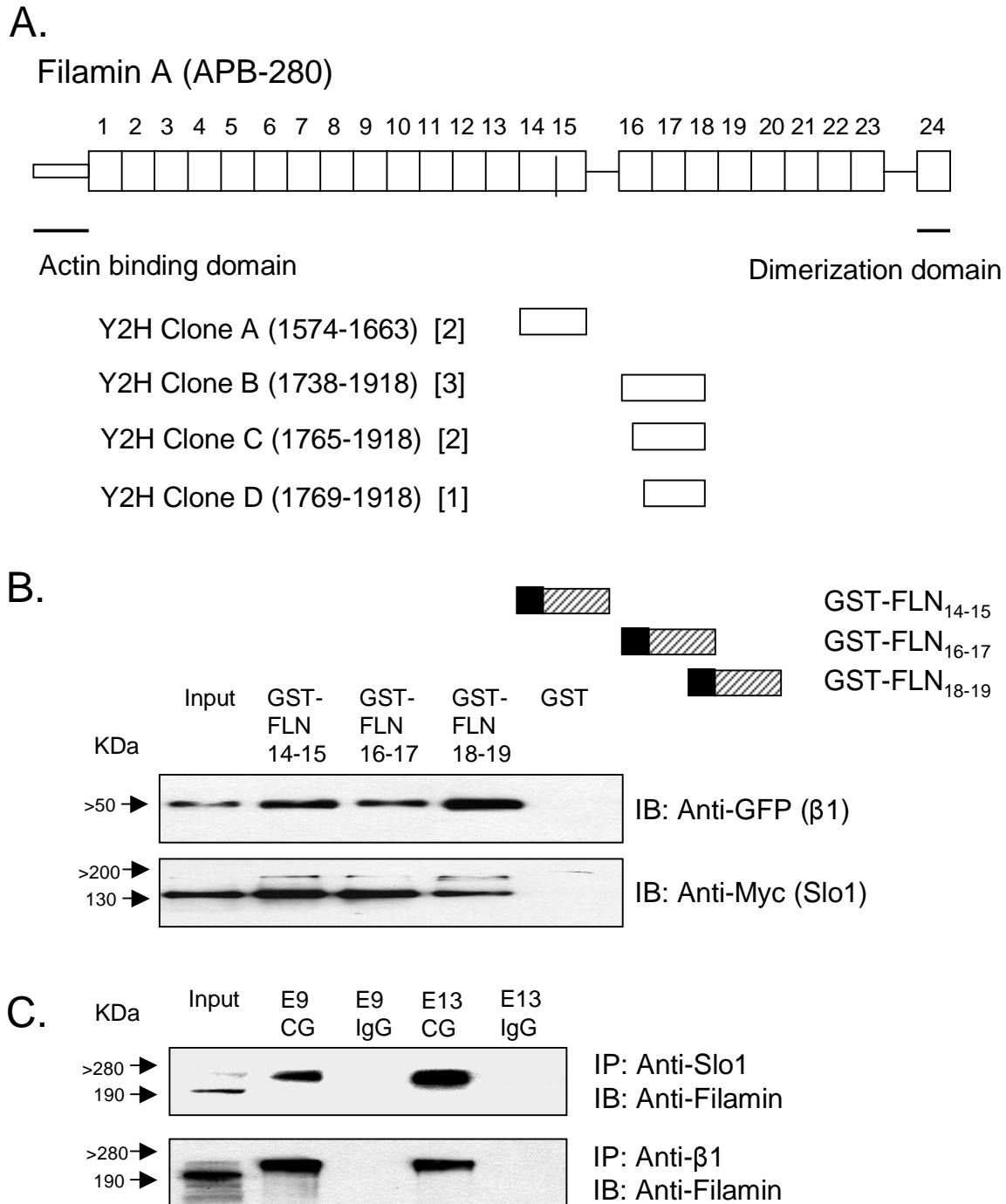


Figure 2

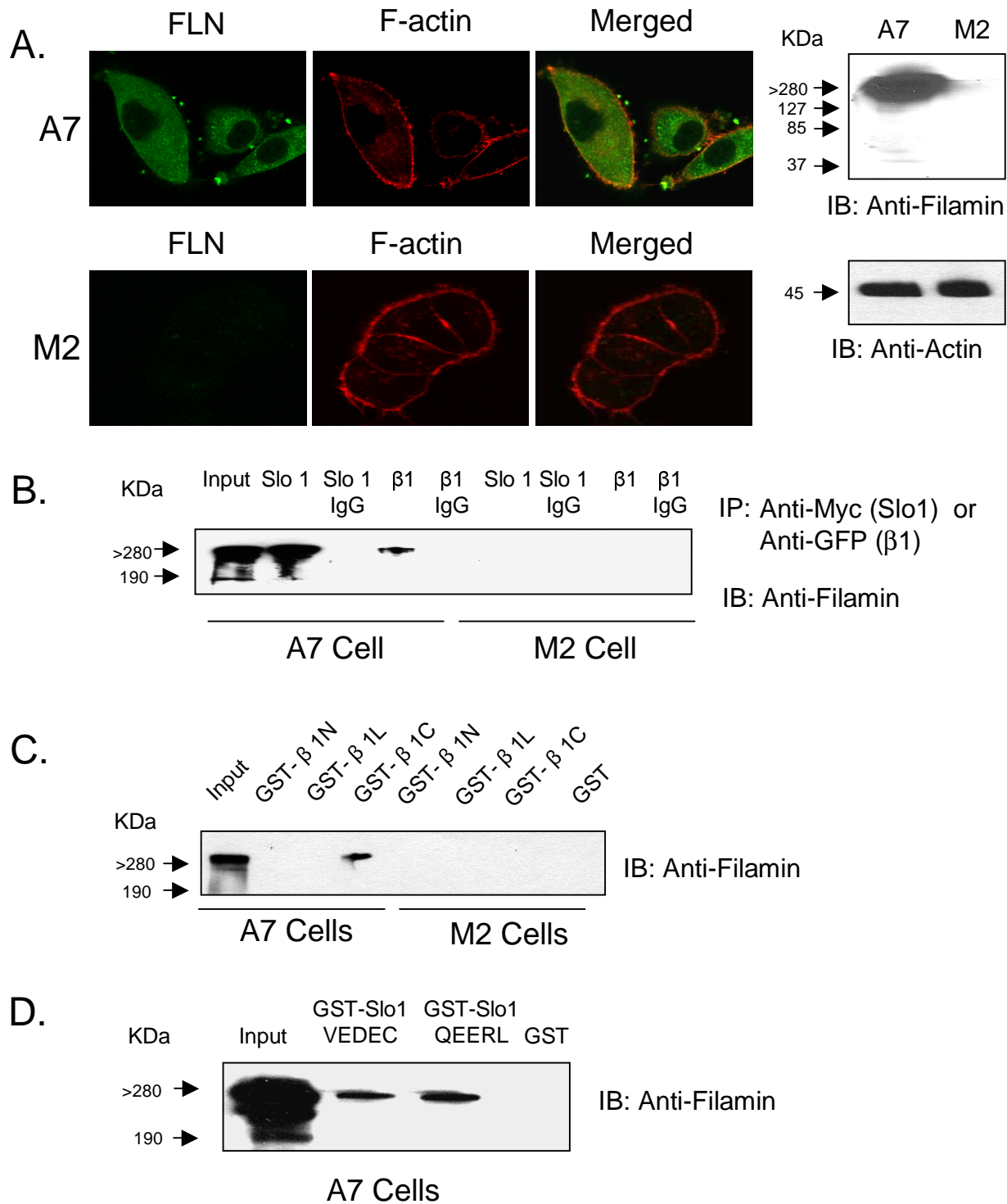


Figure 3

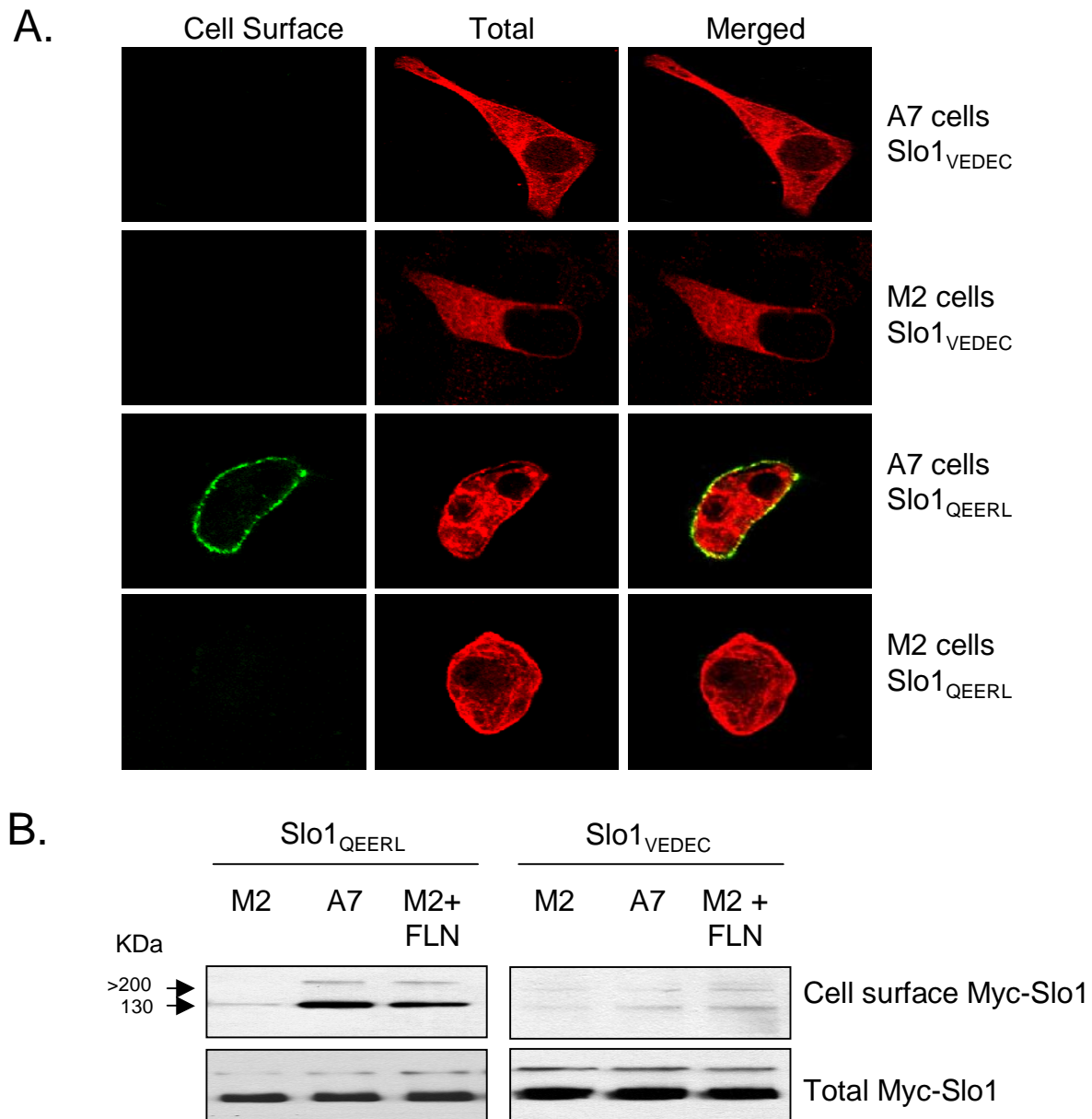


Figure 4

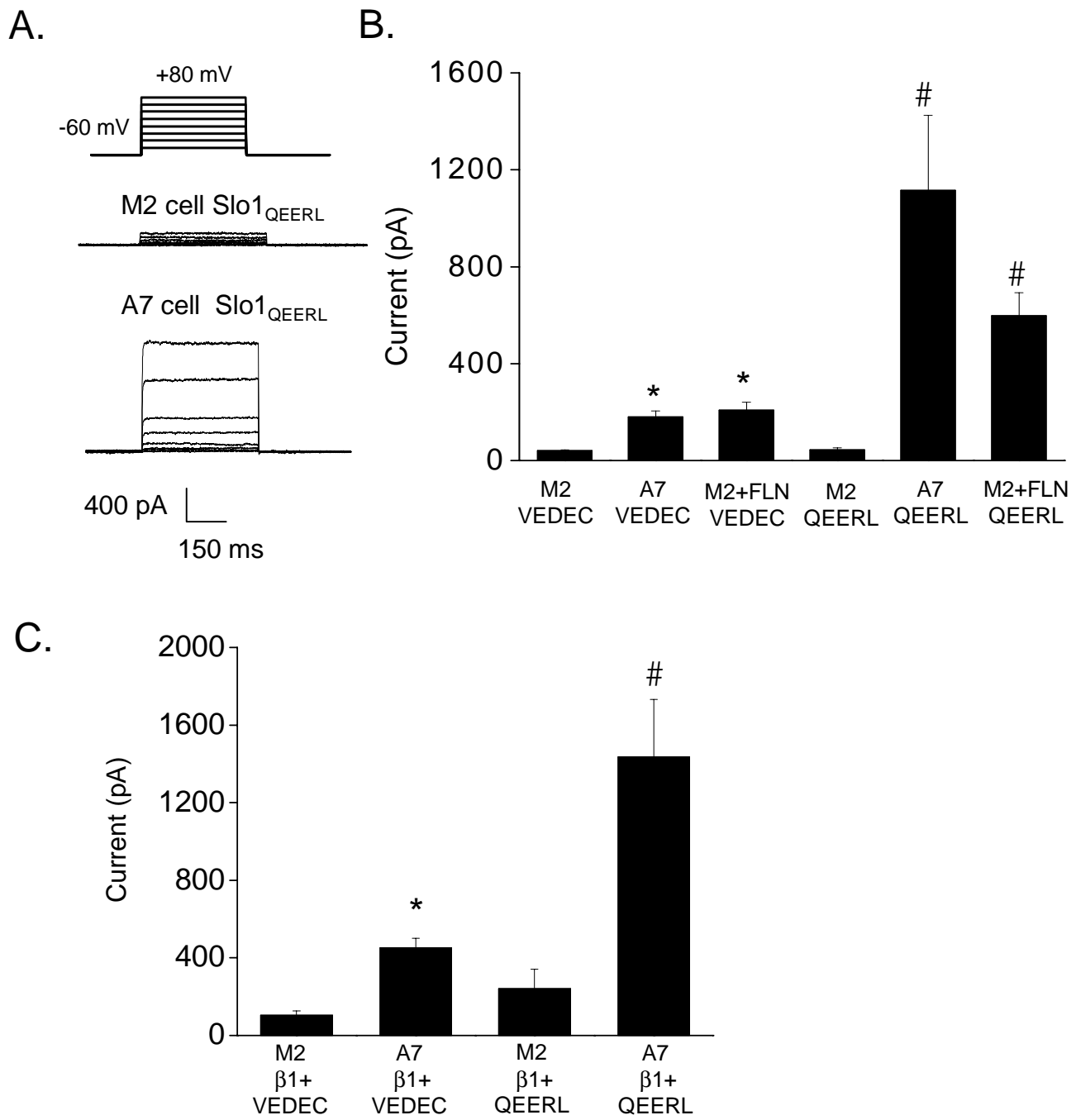


Figure 5

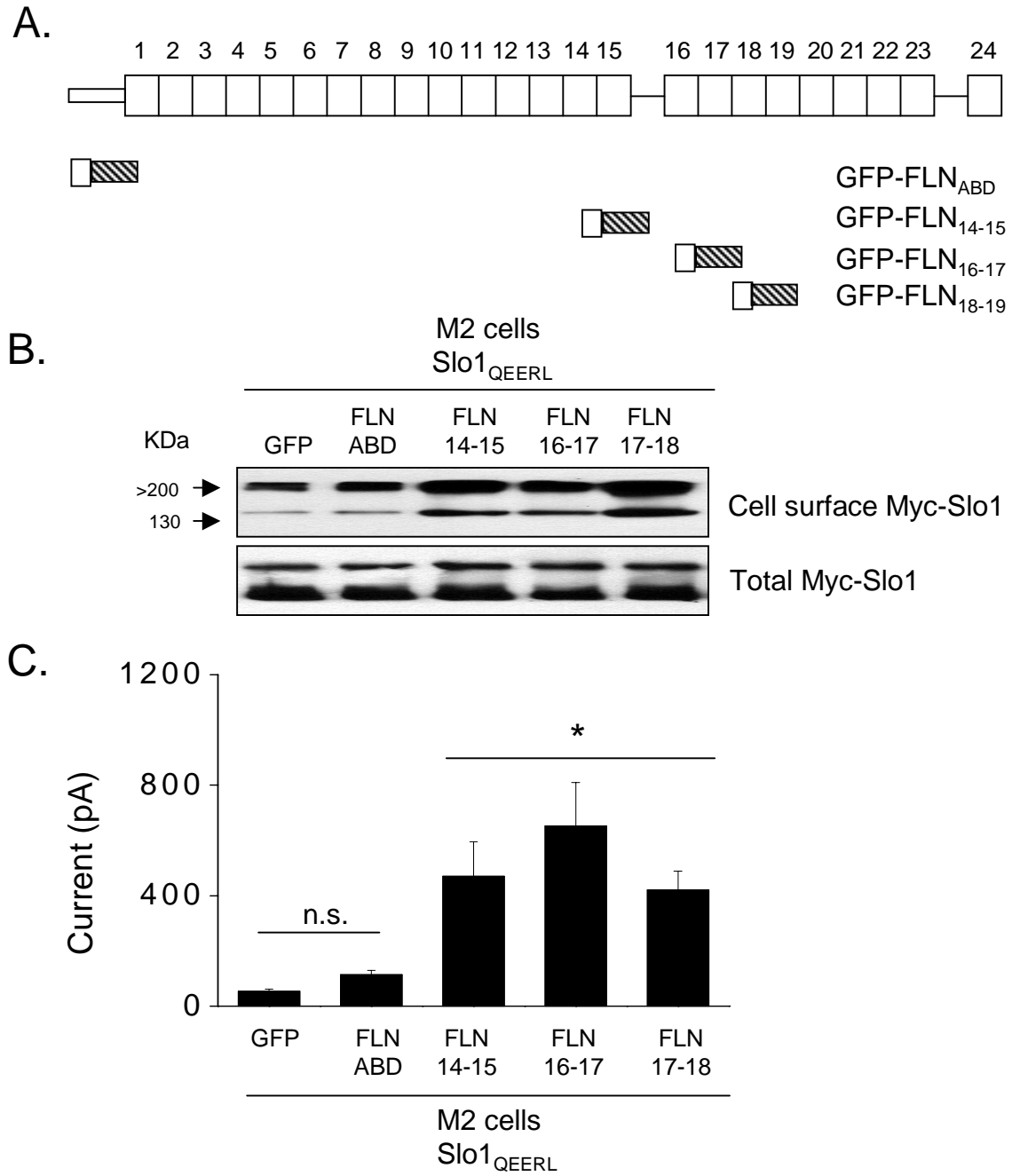


Figure 6

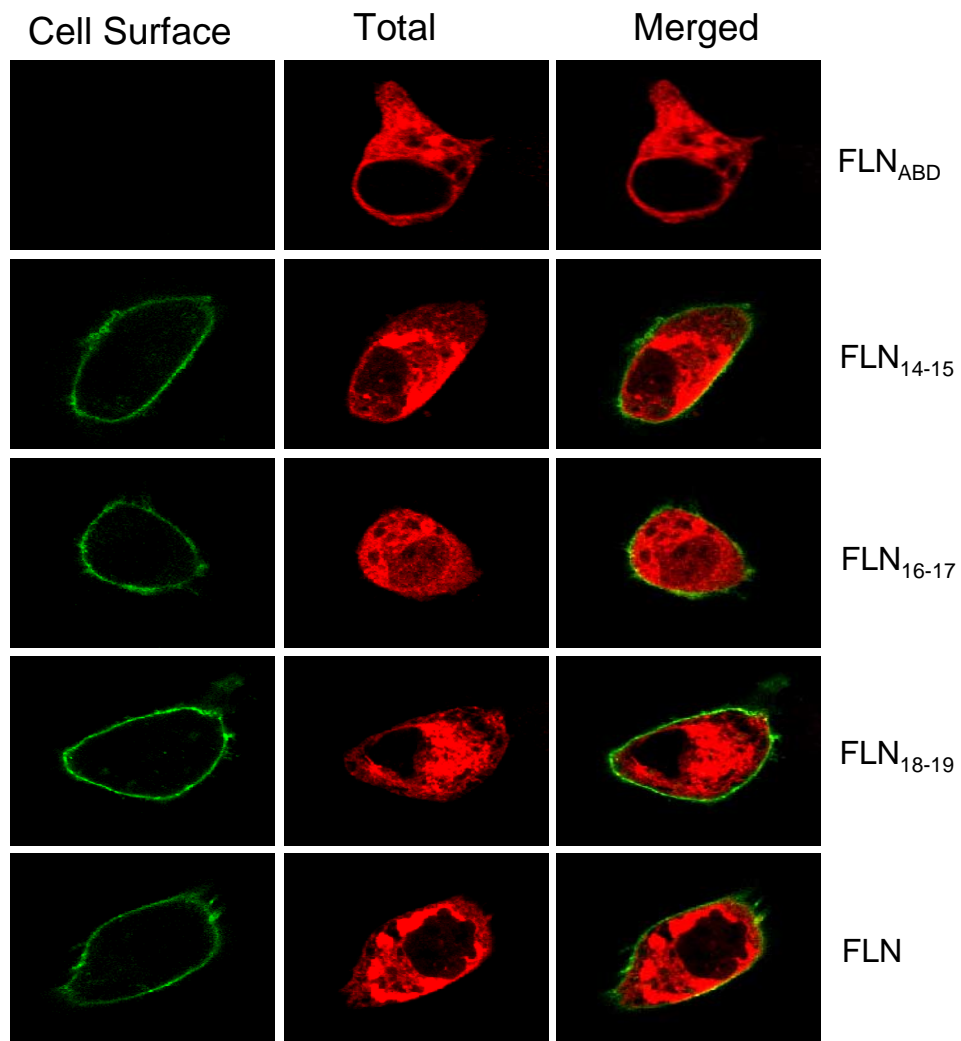


Figure 7

