

Na_v1.5 E1053K mutation causing Brugada syndrome blocks binding to ankyrin-G and expression of Na_v1.5 on the surface of cardiomyocytes

Peter J. Mohler^{*†‡}, Ilaria Rivolta^{†§}, Carlo Napolitano[§], Guy LeMaitre[¶], Stephen Lambert[¶], Silvia G. Priori[§], and Vann Bennett^{*‡}

^{*}Howard Hughes Medical Institute and Departments of Cell Biology, Biochemistry, and Neurosciences, Duke University Medical Center, Durham, NC 27710;

[§]Molecular Cardiology, Istituto di Ricovero e Cura a Carattere Scientifico Fondazione Salvatore Maugeri, University of Pavia, 27100 Pavia, Italy; and

[¶]Department of Cell Biology and Program in Neuroscience and Cell Dynamics, University of Massachusetts Medical School, Worcester, MA 01605

Edited by William A. Catterall, University of Washington School of Medicine, Seattle, WA, and approved September 23, 2004 (received for review May 25, 2004)

We identify a human mutation (E1053K) in the ankyrin-binding motif of Na_v1.5 that is associated with Brugada syndrome, a fatal cardiac arrhythmia caused by altered function of Na_v1.5. The E1053K mutation abolishes binding of Na_v1.5 to ankyrin-G, and also prevents accumulation of Na_v1.5 at cell surface sites in ventricular cardiomyocytes. Ankyrin-G and Na_v1.5 are both localized at intercalated disc and T-tubule membranes in cardiomyocytes, and Na_v1.5 coimmunoprecipitates with 190-kDa ankyrin-G from detergent-soluble lysates from rat heart. These data suggest that Na_v1.5 associates with ankyrin-G and that ankyrin-G is required for Na_v1.5 localization at excitable membranes in cardiomyocytes. Together with previous work in neurons, these results in cardiomyocytes suggest that ankyrin-G participates in a common pathway for localization of voltage-gated Na_v channels at sites of function in multiple excitable cell types.

arrhythmia | *SCN5A* | targeting | trafficking | sudden cardiac death

Voltage-gated Na (Na_v) channels initiate rapid and highly coordinated waves of depolarization that are essential for rhythmic beating of the heart (1). Mutations in Na_v1.5 (encoded by *SCN5A*), the major voltage-gated Na⁺ channel isoform in adult heart, can cause a dominantly inherited cardiac arrhythmia known as Brugada syndrome (2). Brugada syndrome is characterized by incomplete right bundle branch block and increased risk of sudden cardiac death as a result of ventricular fibrillation (2).

Ability of Na_v1.5 to generate physiologically effective action potentials is determined by its cellular localization as well as its channel properties. Na_v1.5 is concentrated at intercalated discs, which are specialized sites of contact between cardiomyocytes and are enriched in gap junctions. Proximity of Na_v1.5 to gap junctions, which provide rapid ion exchange between adjacent cells, allows efficient transcellular propagation of the action potential. In addition, Na_v1.5 is localized in cardiomyocyte T-tubules adjacent to voltage-gated Ca²⁺ channels, and thus is well positioned to initiate the calcium-induced calcium release that triggers contraction (3, 4).

Although little is known about the mechanism for strategic targeting of Na_v1.5 in cardiomyocytes, clustering of voltage-gated Na channels in the nervous system at axon initial segments requires association with ankyrin-G. Targeted knockout of ankyrin-G in the mouse cerebellum abolishes restriction of Na_v1.6 and 1.2 to axon initial segments of Purkinje and granule cell neurons (5, 6). Ankyrin-G associates with the pore-forming α -subunit of Na_v1.2 through a 9-aa motif on loop 2 (VPIALGESD; refs. 7 and 8). This sequence is required for association between ankyrin-G and Na_v1.2, and for targeting of Na_v1.2 to axon initial segments of cultured hippocampal neurons (7, 8). Na_v1.5 contains a nearly identical sequence in loop 2 (see Fig. 2A; *VPIAVAESD*), suggesting that Na_v1.5 also could associate with ankyrin-G. Here we report the interaction and colocaliza-

tion of Na_v1.5 and 190-kDa ankyrin-G in heart, and present evidence that a human Na_v1.5 mutation associated with Brugada syndrome that blocks ankyrin-G binding, also disrupts Na_v1.5 surface expression in cardiomyocytes.

Materials and Methods

Antibodies. Affinity-purified ankyrin-G polyclonal antibody was generated against recombinant 190-kDa ankyrin-G death domain/regulatory domain. The protein was injected into rabbits and sequentially adsorbed by (i) GST-affinity column, (ii) ankyrin-B C-terminal affinity column, and (iii) affinity isolation by using an ankyrin-G C-terminal affinity column. Ankyrin-G Ig was used at 0.5 μ g/ml (IF) and 0.25 μ g/ml (IB). Other antibodies include mouse anti-connexin 43 (2 μ g/ml, Chemicon), goat anti-desmoplakin (2 μ g/ml, Santa Cruz Biotechnology), mouse anti-ZO-1 (1 μ g/ml, Zymed), goat and rabbit anti-N-cadherin (2 μ g/ml, Santa Cruz Biotechnology), goat anti- β -catenin (2 μ g/ml, Santa Cruz Biotechnology), Na_v1.5-specific antibodies SH1 (IB, 1 μ g/ml; IF, 2 μ g/ml, a generous gift from William Catterall, University of Washington, Seattle; ref. 9) and hH1 (2 μ g/ml, Alomone), and affinity-purified hemagglutinin (HA) monoclonal antibody (2 μ g/ml). Na_v1.5 antibodies were tested by using WT HEK293 cells or HEK293 cells expressing Na_v1.5. For both Na_v1.5 antibodies (SH1, hH1) we observed intercalated disk and T-tubule staining.

Lentiviral Expression. HA Na_v1.5 and HA Na_v1.5 E1053K were TOPO-cloned into pLenti6 V5-D for expression in cardiomyocytes by using Virapower Expression system (Invitrogen). pLenti6 HA/V5 Na_v1.5 and HA/V5 Na_v1.5 E1053K were cotransfected with pLP1, pLP2, and pLP/VSVG (packaging plasmids) into 293FT cells (5 \times 10⁶ cells) by using Lipofectamine. After 24 h, the medium was replaced. Seventy-two hours after transfection, 293FT cell supernatants were harvested, centrifuged to remove cell debris, and titered by using serial-dilutions of the virus on NIH 3T3 cells to determine the number of Blasticidin-resistant colonies. Because of the large size of Na_v1.5, viral titers were low. Equal titers of WT or mutant viral supernatants were added to freshly isolated adult rat ventricular cardiomyocytes for 18 h. Three days after transduction (4-day-old cells), cardiomyocytes were lysed to determine relative expression of HA/V5 Na_v1.5 and HA/V5 Na_v1.5 E1053K. Three days after transduction (4-day-old cells), cells

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Na_v, voltage-gated Na; HA, hemagglutinin; NF, neurofascin; MBD, membrane-binding domain.

[†]P.J.M. and I.R. contributed equally to this work.

[‡]To whom correspondence may be addressed. E-mail: v.bennett@cellbio.duke.edu or peter.j.mohler@vanderbilt.edu.

© 2004 by The National Academy of Sciences of the USA

were detached from the plates and protein localization was determined by immunofluorescence.

Expression of Recombinant Na⁺ Channels. Na channels were expressed in HEK293 cells as described (10). Briefly, transient transfection was carried out with equal amounts of Na⁺ channel α -subunit cDNA (WT or mutant, EK), and h β ₁ cDNA subcloned into the pcDNA3.1 vector (total cDNA, 2.5 μ g). In addition the same amount of CD8 cDNA was cotransfected as reporter gene. Expression of channels was studied by using patch clamp procedures 48 h after transfection. Targeting assays were performed as above by using HA-tagged Na_v channel constructs.

Adult Rat Cardiomyocyte Isolation. Animal care was in accordance with institutional guidelines. Adult cardiomyocytes were prepared as described (11). Cells were cultured in the presence of 1 μ g/ml insulin, 0.55 μ g/ml transferrin, 0.5 ng/ml selenium, and 10 mM 2,3-butanedione monoxime (Sigma; ref. 12).

Electrophysiology. Membrane currents were measured by using whole cell patch-clamp, with Axopatch 200B amplifiers (Axon Instruments). All measurements were obtained at room temperature (22°C). Macroscopic whole cell Na⁺ current was recorded by using published solutions and protocols (10). PCLAMP8 (Axon Instruments, Union City, CA), EXCEL (Microsoft), and ORIGIN 6.1 (Microcal Software, Northampton, MA) were used for data acquisition and analysis. Data are represented as mean \pm SEM. Two-tailed Student's *t* test was used to compare means; *P* < 0.05 was considered statistically significant.

Immunoprecipitations. See *Supporting Text*, which is published as supporting information on the PNAS web site.

Immunostaining. Freshly isolated or cultured adult rat cardiomyocytes were fixed in cold ethanol (for permeabilized protocol) or 2% paraformaldehyde (for nonpermeabilized protocol) for 20 min. Cells were centrifuged 2 min at 200 \times g and washed three times with PBS (pH 7.4). Cells were blocked for 3 h in PBS with 0.025% Triton X-100/3% fish oil gelatin. Cells were stained overnight in primary antibody (in blocking buffer) at 4°C on a rotating platform. Cells were washed as above in blocking buffer. Cells were incubated in secondary antibody (Alexa Fluor 488, 568, and 633) for 12 h at 4°C in blocking buffer. After washes, buffer was removed and the pellet was resuspended in Vectashield and plated onto Mattek 35-mm plates. For the nonpermeabilized cell staining protocol, staining was performed at 4°C and detergent was not included in any buffer. We also performed permeabilized cell staining (HA antibody) in parallel with nonpermeabilized staining to confirm that cells were expressing the lentiviral-expressed WT and mutant HA-Na_v1.5. Images were collected on Zeiss 510 Meta confocal microscope (see details on image collection and processing in *Supporting Text*).

Na_v1.5 Constructs and Mutagenesis. Full-length human Na_v1.5 cDNA was subcloned into pcDNA3.1 and sequenced to verify that there were no mutations. DNA coding for an HA epitope (YPYDVPDYA) was engineered in-frame into the extracellular S5–S6 loop of Na_v1.5 domain 1 by using QuikChange mutagenesis. This construct was used to generate Na_v1.5 with human Brugada mutation E1053K by QuikChange mutagenesis. WT and mutant HA-tagged cDNAs were PCR-amplified and Topo-cloned into pLenti6/V5 (Invitrogen), and the complete cDNAs (\approx 12 kb) were sequenced to confirm that no mutations were introduced by PCR. NF/Na_v1.5 chimeras were created as described in ref. 7 by using loop 2 from Na_v1.5 instead of Na_v1.2. The WT chimera was then altered by mutagenesis to create HA NF-Na_v1.5 Δ and HA NF-Na_v1.5 E1053K.

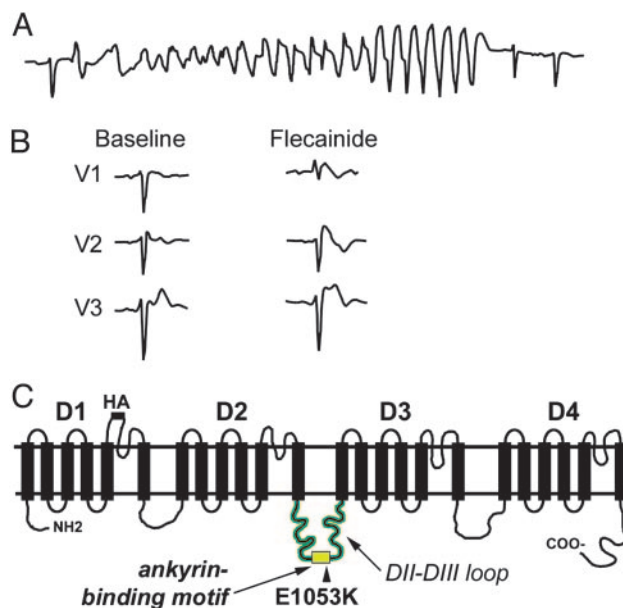


Fig. 1. Brugada syndrome associated with *SCN5A* E1053K mutation. (A) ECG monitor strip of the index patient carrier of the E1053K mutation showing self-terminating polymorphic ventricular tachycardia recorded at the arrival to the emergency room. (B) ECG recordings in leads V1–V3 of the index patient carrier of the E1053K mutation at baseline and during i.v. flecainide administration (2 mg/kg in 10 min). (C) Human Na_v1.5 proposed secondary structure. Potential ankyrin-binding motif is located in loop 2. Human Brugada mutation E1053K is located within these nine amino acids.

Results

Brugada Syndrome Associated with Human *SCN5A* E1053K Mutation.

We identified a *de novo* *SCN5A* mutation in a 47-year-old female with a history of palpitations with an unexplained syncopal spell occurring at rest. Runs of polymorphic ventricular tachycardia were observed at the arrival to the emergency room (Fig. 1A). Baseline ECG was within normal limits with the exception of a 1-mm ST segment elevation in lead V1 and incomplete right bundle branch block (Fig. 1B). No cardiac abnormalities were observed by echocardiogram, and programmed electrical stimulation did not induce any clinically significant arrhythmias. The diagnosis of Brugada syndrome was established by an i.v. flecainide test, which revealed an abnormal response with the induction of a 3-mm ST segment elevation in leads V1–V2 and a typical coved type morphology (2). An implantable cardioverter defibrillator (ICD) was positioned. No ICD shock has been delivered at 3-year follow up, and no ventricular arrhythmias were detected.

All exons of *SCN5A* in this individual were analyzed by DHPLC and direct DNA sequencing. A single G-to-A nucleotide transition (G3157A) was identified leading to substitution of a highly conserved glutamic acid with lysine (E1053K). Interestingly, this mutation is localized in the predicted 9-aa ankyrin-binding motif in the DII–III loop of Na_v1.5 (VPLAVX[E → K]SD) (Figs. 1C and 2A). Given that the E1053K mutation is associated with Brugada syndrome and Brugada syndrome is caused by altered or aberrant function of Na_v1.5, the E1053K mutation also causes aberrant Na_v1.5 function *in vivo*. However, the E1053 residue is not located near predicted channel pore-forming regions or voltage-sensor, and is not found in the III–IV linker implicated in fast inactivation (13). Nevertheless, the DII–III loop may directly affect the Na_v1.5 channel activation voltage, as suggested by a recent report studying Na_v1.4/Na_v1.5 channel chimeras (14).

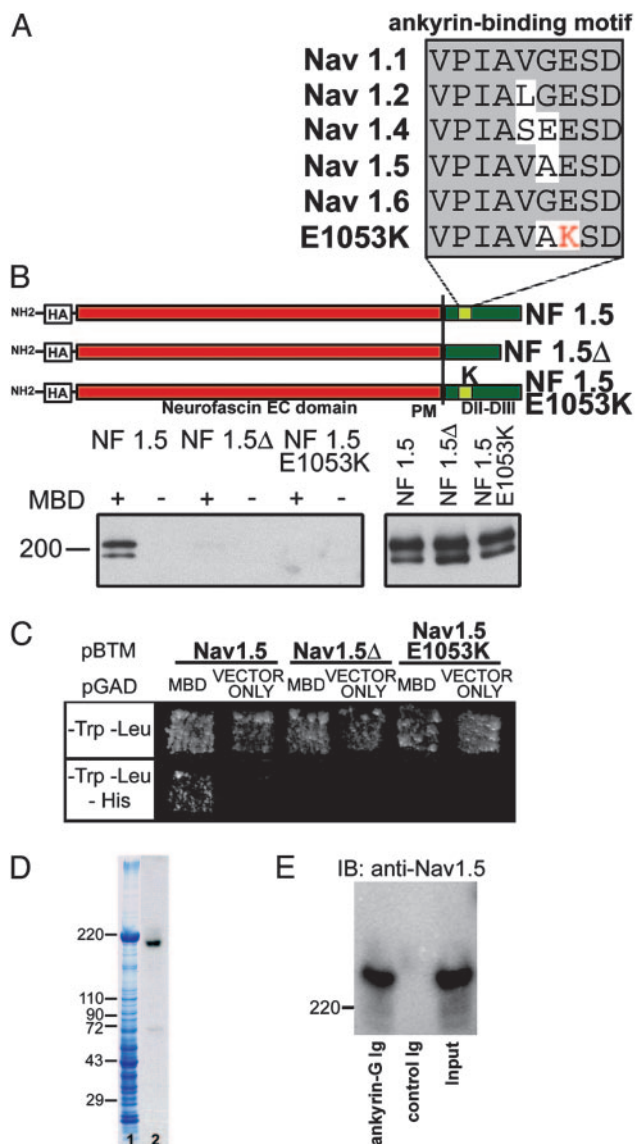


Fig. 2. Human *SCN5A* mutation E1053K eliminates interaction with ankyrin-G. (A) Alignment of ankyrin-binding motif from $Na_v1.2$ with other Na_v isoforms. Bottom is $Na_v1.5$ amino acid alignment from human with Brugada syndrome (E1053K). (B) NF/ $Na_v1.5$ chimeras. A diagram of NF/ $Na_v1.5$ chimeras using loop 2 sequence from $Na_v1.5$, $Na_v1.5\Delta$ (deletion of VPIAVAESD), or $Na_v1.5$ E1053K mutant is shown. Constructs were expressed in baby hamster kidney cells and lysates incubated with GST-AnkG MBD(+/-) or GST(-/-)-conjugated beads. Bound protein (Left) or 6% of total lysate used (Right) was analyzed by immunoblotting with HA-specific antibodies. The doublet observed in immunoblots represents both glycosylated and nonglycosylated forms of NF/ $Na_v1.5$ chimeras in total cell lysates (23). (C) *Saccharomyces cerevisiae* was cotransformed with pBTM116- $Na_v1.5$, pBTM116- $Na_v1.5\Delta$, or pBTM116- $Na_v1.5$ E1053K, and pGAD424 alone, and pGAD424-MBD. Cotransformants were selected on -LT media and then tested for HIS3 reporter gene activation. (D) 190-kDa ankyrin-G associates with $Na_v1.5$ *in vivo*. Lane 1, Coomassie blue stained gel of rat heart lysate. Lane 2, immunoblot analysis of lane 1 by using ankyrin-G polyclonal Ig. (E) Adult rat ventricle detergent-soluble lysate was incubated with affinity-purified ankyrin-G Ig conjugated to beads or control Ig alone. Bound protein was eluted and analyzed by immunoblot analysis using $Na_v1.5$ -specific antibody (SH1). Input, 20% of soluble protein.

Human *SCN5A* Mutation E1053K Eliminates $Na_v1.5$ Interaction with Ankyrin-G. We performed *in vitro* binding assays to determine whether ankyrin-G associates with $Na_v1.5$. A HA-tagged chi-

mera of the extracellular and transmembrane domain of neurofascin (NF) fused with loop 2 of $Na_v1.5$ (see Fig. 2B) was expressed in baby hamster kidney cells. Detergent-soluble lysates from these cells were incubated with immobilized GST-ankyrin-G membrane-binding domain (MBD) or GST alone. Bound protein was examined by immunoblotting using HA antibody. Like HA NF- $Na_v1.2$ loop 2 (7), HA NF- $Na_v1.5$ loop 2 associates with GST-MBD but not GST alone (Fig. 2B).

We next determined whether the VPIAVAESD motif in loop 2 (Fig. 2B) is necessary for ankyrin-G/ $Na_v1.5$ binding. A HA-NF- $Na_v1.5$ mutant chimera was created that lacked the VPIAVAESD motif (NF- $Na_v1.5$ loop 2Δ; Fig. 2B). Although it is expressed at similar levels as the WT chimera, NF- $Na_v1.5$ loop 2Δ did not associate with ankyrin-G MBD (Fig. 2B). Additionally, ankyrin-G MBD associates with $Na_v1.5$ loop 2 but not $Na_v1.5$ loop 2Δ in yeast two-hybrid assays (Fig. 2C). Therefore, similar to results with $Na_v1.2$ in neurons (7), interaction of $Na_v1.5$ with ankyrin-G membrane-binding domain requires the VPI-AXXESD motif in loop 2.

We introduced the human E1053K mutation into HA NF- $Na_v1.5$ to evaluate the effect of this mutation on ankyrin-G binding. HA NF- $Na_v1.5$ loop 2 E1053K did not associate with ankyrin-G MBD even though the mutant chimera was expressed at similar levels as the WT chimera (Fig. 2B). Similar results were observed when ankyrin-G MBD and $Na_v1.5$ loop 2 E1053K were used in yeast two-hybrid assays (Fig. 2C). Therefore, the mutation E1053K associated with Brugada syndrome also abolishes interaction between $Na_v1.5$ and ankyrin-G.

Ankyrin-G Associates with $Na_v1.5$ in Heart Tissue. We determined the ankyrin-G isoform(s) expressed in heart by using an affinity-purified antibody (see *Materials and Methods*). Immunoblot analysis using this Ig revealed that 190-kDa ankyrin-G is the predominant ankyrin-G isoform in adult rat heart (Fig. 2D) as well as adult mouse heart (data not shown). In contrast, 107-kDa ankyrin-G, which is the predominant ankyrin-G isoform in skeletal muscle (15, 16), was present at low levels.

The ability of 190-kDa ankyrin-G and $Na_v1.5$ to associate in heart was evaluated by coimmunoprecipitation experiments. Detergent-soluble lysates from adult rat heart were incubated with affinity-purified ankyrin-G Ig coupled to protein G Sepharose. After extensive washes, protein specifically bound to the beads was eluted and analyzed by SDS/PAGE and immunoblotting using affinity-purified $Na_v1.5$ antibody (SH1). Ankyrin-G coimmunoprecipitates a significant fraction ($\approx 10\%$) of total soluble $Na_v1.5$ (Fig. 2E). Therefore, ankyrin-G associates with the α -subunit of the voltage-gated Na_v channels in heart ($Na_v1.5$, Fig. 2E) as well as brain ($Na_v1.2$ and $Na_v1.6$; refs. 5, 6, and 8).

Ankyrin-G and $Na_v1.5$ Are Localized at Intercalated Disc and T-Tubule Membranes. $Na_v1.5$ is concentrated primarily at intercalated disc and T-tubule membranes in ventricular cardiomyocyte (refs. 9 and 17, see also Fig. 3B). Ankyrin-G localization in adult ventricle was assessed by using confocal imaging of adult rat isolated ventricular cardiomyocytes using ankyrin-G affinity-purified polyclonal Ig. Like $Na_v1.5$, ankyrin-G is highly expressed at ventricular intercalated disc and T-tubule membranes (Fig. 3A). Therefore, the localization of 190-kDa ankyrin-G and $Na_v1.5$ at intercalated discs and in T-tubules in ventricular cardiomyocytes is consistent with an *in vivo* interaction between these proteins.

$Na_v1.5$ E1053K Is Not Expressed at the Membrane Surface of Cardiomyocytes. The effect of the E1053K mutation on cellular localization of $Na_v1.5$ was assessed by expression of WT $Na_v1.5$ and $Na_v1.5$ E1053K in isolated adult rat cardiomyocytes. Full-length human $Na_v1.5$ cDNA was engineered to contain an extracellular HA epitope (S5-S6 linker) at a site previously

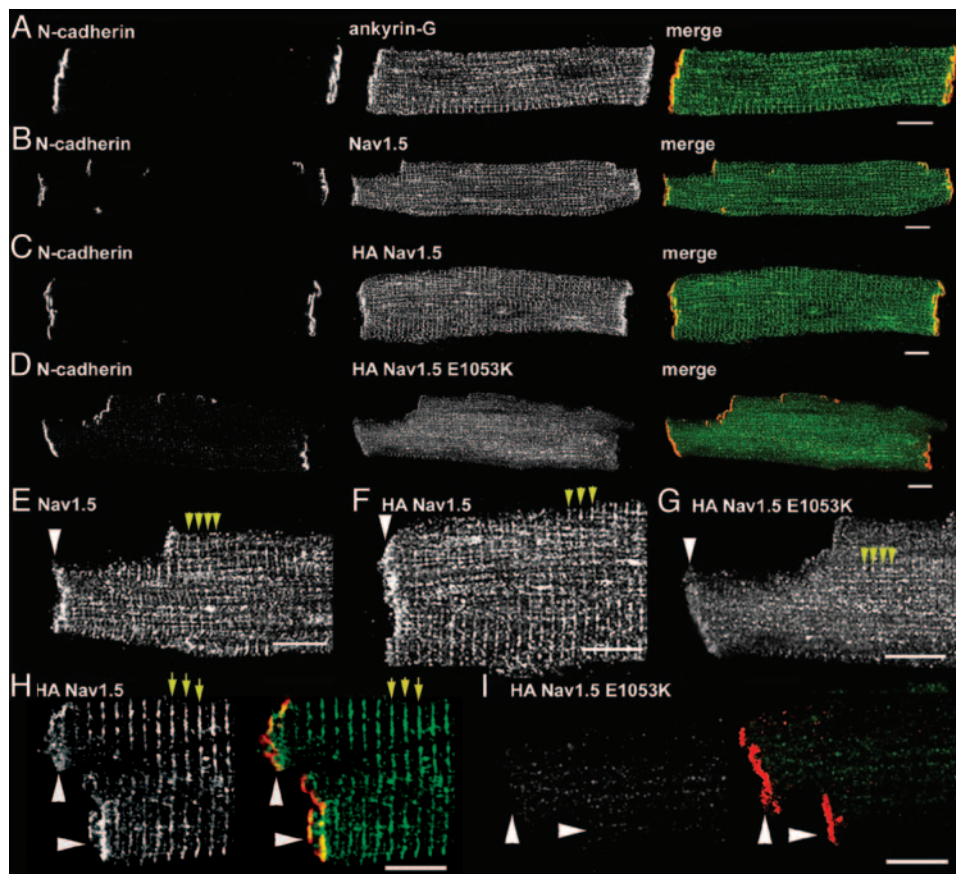


Fig. 3. One hundred ninety-kilodalton ankyrin-G is coexpressed with $\text{Na}_v1.5$ at intercalated disc and T-tubule membranes and $\text{Na}_v1.5$ E1053K mutant is mislocalized from intercalated disc/T-tubule membrane of cultured adult rat cardiomyocytes. (A) Ankyrin-G localization (green) at intercalated disc and T-tubule membranes of adult rat cardiomyocytes colabeled with N-cadherin (red). (Scale bar, 10 μm .) (B) $\text{Na}_v1.5$ localization (green, SH1 antibody) at intercalated disc and T-tubule membranes of adult rat cardiomyocytes colabeled with N-cadherin (red). (Scale bar, 10 μm .) (C) Localization of N-cadherin (red) and lentiviral-expressed WT HA- $\text{Na}_v1.5$ (green, HA antibody) in 4-day cultured adult rat cardiomyocytes. (D) Localization of N-cadherin (red) and lentiviral-expressed WT HA- $\text{Na}_v1.5$ E1053K (green, HA antibody) in 4-day-old adult rat cardiomyocytes. (E–G) High-magnification image of intercalated disc region of above images. Intercalated disc marked by arrowhead. T-tubules are denoted by yellow arrows. Note small puncta near T-tubules in HA $\text{Na}_v1.5$ E1053K-transduced cell. (Scale bar, 10 μm .) (H) Localization of N-cadherin (red) and lentiviral-expressed WT HA- $\text{Na}_v1.5$ (green, HA antibody) in nonpermeabilized 4-day cultured adult rat cardiomyocytes. Intercalated disc is marked by arrowhead. T-tubules are denoted by yellow arrows. (I) Localization of N-cadherin (red) and lentiviral-expressed WT HA- $\text{Na}_v1.5$ E1053K in nonpermeabilized 4-day cultured adult rat cardiomyocytes. (Scale bar for H and I, 10 μm .)

shown to not affect channel localization or gating (HA $\text{Na}_v1.5$, see Fig. 1C) (18). HA- $\text{Na}_v1.5$ E1053K was also created by using site-directed mutagenesis. Each plasmid was completely sequenced (≈ 12 kb) to ensure that no additional mutations were introduced. Both cDNAs were cloned into a lentiviral expression plasmid, and lentivirus corresponding to both cDNAs was generated and titered. Ventricular cardiomyocytes were isolated from adult rat hearts and transduced with virus containing either HA- $\text{Na}_v1.5$ or HA- $\text{Na}_v1.5$ E1053K. At 2 days after transduction (3-day-old cells), both proteins are made with the correct molecular weight and at equivalent levels (data not shown). Localization of each protein in adult ventricular cardiomyocytes was determined in 4-day-old cardiomyocytes by using affinity-purified HA antibody. Isolated 4-day cultured adult cardiomyocytes transduced with lentivirus maintain viability based on the following criteria: First, transduced and nontransduced adult cultured cardiomyocytes can “round up” after 3–4 days in culture. Therefore, we only analyzed cardiomyocytes with normal rod-shaped morphology. Second, transduced cardiomyocytes normally target intercalated disc, T-tubule, sarcoplasmic reticulum, and other cardiomyocyte proteins including desmoplakin, ZO-1, β -catenin, N-cadherin, ryanodine receptor 2, SERCA2, α -actinin, and endogenous

$\text{Na}_v1.5$ (Fig. 3B and Fig. 6, which is published as supporting information on the PNAS web site). Third, we observed no significant differences in endogenous $\text{Na}_v1.5$ expression by immunoblot or immunofluorescence after 1–4 days in culture (data not shown).

Virally expressed HA- $\text{Na}_v1.5$, like endogenous $\text{Na}_v1.5$, is expressed on the membrane surface of nonpermeabilized cardiomyocytes (Fig. 3H) and is targeted to both intercalated disc and T-tubule domains (Fig. 3B, C, E, and F). In contrast, HA- $\text{Na}_v1.5$ E1053K is minimally expressed on the surface membrane of nonpermeabilized cardiomyocytes (Fig. 3I), and correspondingly is nearly absent from intercalated disc and T-tubule membranes (Fig. 3D and G). Instead, HA- $\text{Na}_v1.5$ E1053K staining of permeabilized cells is limited to small longitudinally oriented puncta throughout the cardiomyocyte (Fig. 3G). These puncta are not evident in nonpermeabilized cells and, therefore, may represent intermediates between the endoplasmic reticulum and the Golgi apparatus, or potentially post-Golgi intermediates normally destined for intercalated disc or T-tubule membranes. Therefore, the $\text{Na}_v1.5$ E1053K mutation associated with Brugada syndrome eliminates ankyrin-G binding, and abolishes normal accumulation of the channel at the cell surface of transfected cardiomyocytes.

sion and ability to generate voltage-sensitive current in HEK293 cells and thus does not grossly perturb $\text{Na}_v1.5$ folding, processing through the Golgi, or function. However, the E1053K mutation does abolish binding of $\text{Na}_v1.5$ to ankyrin-G, and also prevents accumulation of $\text{Na}_v1.5$ at cell surface sites in ventricular cardiomyocytes. Moreover, we also report that ankyrin-G and $\text{Na}_v1.5$ are both localized intercalated discs and T-tubule membranes in cardiomyocytes, and that $\text{Na}_v1.5$ coimmunoprecipitates with 190-kDa ankyrin-G from detergent-soluble lysates from rat heart. It is possible that the E1053K mutation affects activities of the channel in addition to ankyrin-G binding. However, the most parsimonious interpretation of the data considered together is that $\text{Na}_v1.5$ associates with ankyrin-G and that ankyrin-G is required for cell surface expression of $\text{Na}_v1.5$ in cardiomyocytes. The ankyrin-G-binding motif (VPIAXX-ESD) is conserved among $\text{Na}_v1.1$, 1.2, 1.4, 1.5, and 1.6, and also is required for Na_v channel targeting in both neurons (7, 8). Therefore, an ankyrin-G-based mechanism for Na_v channel targeting appears a conserved feature of both cardiomyocytes and neurons. Ankyrin-G may also be required for targeting of $\text{Na}_v1.1$ to specialized sites in neurons and of $\text{Na}_v1.4$ to the neuromuscular junction in skeletal muscle. It is of interest in this regard that ankyrin-G is located at the neuromuscular junction in the postsynaptic domain enriched in Na_v channels (16, 19, 20).

The pathway for restricting/targeting of $\text{Na}_v1.5$ or other Na_v channels to the cell surface apparently operates in a cell-specific manner. As noted above, the E1053K mutation, or deletion of the 9-aa ankyrin-binding motif (Fig. 2A) does not block targeting of $\text{Na}_v1.5$ to the cell surface of HEK293 cells (Fig. 7, which is published as supporting information on the PNAS web site). Moreover, delivery of $\text{Na}_v1.2$ to unmyelinated axons of granule cell neurons is unaffected by the absence of ankyrin-G (5), and $\text{Na}_v1.2$ lacking the ankyrin-binding motif is also efficiently targeted to the plasma membrane of HEK293 cells (Fig. 7). An additional targeting motif responsible for endocytosis from dendritic membranes has been identified in $\text{Na}_v1.2$ (21). The distinct trafficking profile of $\text{Na}_v1.5$ in cardiac myocytes compared to HEK293 cells suggests the presence of highly special-

ized protein targeting machinery in heart. In the future, it will be important to use differentiated cardiomyocytes and neurons to evaluate the molecular mechanisms for Na_v channel targeting to specialized domains such as intercalated discs and axon initial segments.

The lack of surface expression of $\text{Na}_v1.5$ E1053K in rat ventricular cardiomyocytes suggests that patients heterozygous for the *SCN5A* G3157A (E1053K) mutation would not display a full complement of $\text{Na}_v1.5$. Reduction in the number of $\text{Na}_v1.5$ channels could result in Brugada syndrome, which occurs in patients heterozygous for mutations causing loss of $\text{Na}_v1.5$ activity. Our functional data demonstrate that the $\text{Na}_v1.5$ E1053K mutation also affects $\text{Na}_v1.5$ channel properties (Fig. 5). Therefore, although the majority of $\text{Na}_v1.5$ E1053K does not reach the membrane surface of cardiomyocytes, we cannot exclude the possibility that residual $\text{Na}_v1.5$ E1053K may reach the cell surface. Enhanced inactivation of these residual channels, as demonstrated by our findings, could further contribute to the Brugada phenotype because of an additional reduction in sodium current.

In summary, this study provides evidence consistent with the possibility that ankyrin-G participates in an unsuspected common pathway for restricting/targeting Na_v channels to plasma membrane sites in cardiomyocytes as well as initial segments of nerve axons. An unresolved question for the future is whether ankyrin-G acts solely as a localized scaffolding protein that stabilizes $\text{Na}_v1.5$ at specialized sites once it is delivered to the plasma membrane, or whether ankyrin-G also has a role in sorting and trafficking of these channels. The recent finding that ankyrin-G is required for biogenesis of the lateral membrane domain of epithelial cells (22) suggests functions for ankyrin-G that go beyond simple scaffolding.

This work was supported by the Howard Hughes Medical Institute and a focused giving grant from Johnson and Johnson (to V.B. and P.J.M.), Telethon Grant P0227/01, "Ricerca Finalizzata" DG-RSVE-RF2001-1862, and Cariplo Foundation Grant 2001.3009/10.9079 (to C.N., I.R., and S.G.P.), and National Institutes of Health Grant R01NS36637 (to S.L.).

- Papadatos, G. A., Wallerstein, P. M., Head, C. E., Ratcliff, R., Brady, P. A., Benndorf, K., Saumarez, R. C., Trezise, A. E., Huang, C. L., Vandenberg, J. I., *et al.* (2002) *Proc. Natl. Acad. Sci. USA* **99**, 6210–6215.
- Priori, S. G., Napolitano, C., Gasparini, M., Pappone, C., Della Bella, P., Giordano, U., Bloise, R., Giustetto, C., De Nardis, R., Grillo, M., *et al.* (2002) *Circulation* **105**, 1342–1347.
- Cohen, S. A. (1996) *Circulation* **94**, 3083–3086.
- Scriven, D. R., Dan, P. & Moore, E. D. (2000) *Biophys. J.* **79**, 2682–2691.
- Zhou, D., Lambert, S., Malen, P. L., Carpenter, S., Boland, L. M. & Bennett, V. (1998) *J. Cell Biol.* **143**, 1295–1304.
- Jenkins, S. M. & Bennett, V. (2001) *J. Cell Biol.* **155**, 739–746.
- Lemaitre, G., Walker, B. & Lambert, S. (2003) *J. Biol. Chem.* **278**, 27333–27339.
- Garrido, J. J., Giraud, P., Carlier, E., Fernandes, F., Moussif, A., Fache, M. P., Debanne, D. & Dargent, B. (2003) *Science* **300**, 2091–2094.
- Maier, S. K., Westenbroek, R. E., Schenkman, K. A., Feigl, E. O., Scheuer, T. & Catterall, W. A. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 4073–4078.
- Rivolta, I., Abriel, H., Tateyama, M., Liu, H., Memmi, M., Vardas, P., Napolitano, C., Priori, S. G. & Kass, R. S. (2001) *J. Biol. Chem.* **276**, 30623–30630.
- Mohler, P. J., Schott, J. J., Gramolini, A. O., Dilly, K. W., Guatimosim, S., duBell, W. H., Song, L. S., Haurogne, K., Kyndt, F., Ali, M. E., *et al.* (2003) *Nature* **421**, 634–639.
- Sambrano, G. R., Fraser, I., Han, H., Ni, Y., O'Connell, T., Yan, Z. & Stull, J. T. (2002) *Nature* **420**, 712–714.
- Balsler, J. R. (2001) *J. Mol. Cell Cardiol.* **33**, 599–613.
- Bennett, E. S. (2004) *J. Membr. Biol.* **197**, 155–168.
- Gagelin, C., Constantin, B., Deprette, C., Ludosky, M. A., Recouvreur, M., Cartaud, J., Cognard, C., Raymond, G. & Kordeli, E. (2002) *J. Biol. Chem.* **277**, 12978–12987.
- Kordeli, E., Ludosky, M. A., Deprette, C., Frappier, T. & Cartaud, J. (1998) *J. Cell Sci.* **111**, 2197–2207.
- Kucera, J. P., Rohr, S. & Rudy, Y. (2002) *Circ. Res.* **91**, 1176–1182.
- Baroudi, G., Acharfi, S., Larouche, C. & Chahine, M. (2002) *Circ. Res.* **90**, E11–E16.
- Bailey, S. J., Stocksley, M. A., Buckel, A., Young, C. & Slater, C. R. (2003) *J. Neurosci.* **23**, 2102–2111.
- Flucher, B. E. & Daniels, M. P. (1989) *Neuron* **3**, 163–175.
- Garrido, J. J., Fernandes, F., Moussif, A., Fache, M. P., Giraud, P. & Dargent, B. (2003) *Biol. Cell* **95**, 437–445.
- Kizhatil, K. & Bennett, V. (2004) *J. Biol. Chem.* **279**, 16706–16714.
- Ren, Q. & Bennett, V. (1998) *J. Neurochem.* **70**, 1839–1849.