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A Novel Strategy Using Cardiac Sodium Channel Polymorphic Fragments to Rescue Trafficking Deficient *SCN5A* Mutations

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Abstract

Background—Brugada Syndrome (BrS) is associated with mutations in the cardiac sodium channel ($Na_v1.5$). We previously reported that the function of a trafficking-deficient BrS $Na_v1.5$ mutation, R282H, could be restored by co-expression with the sodium channel polymorphism H558R. Here, we tested the hypothesis that peptide fragments from $Na_v1.5$, spanning the H558R-polymorphism, can be used to restore trafficking of trafficking-deficient BrS sodium channel mutations.

Methods and Results—Whole-cell patch-clamping revealed that co-transfection in HEK293 cells of the R282H channel with either the 40 or 20 amino acid cDNA fragments of $Na_v1.5$ containing the H558R polymorphism restored trafficking of this mutant channel. Fluorescence Resonance Energy Transfer (FRET) suggested that the trafficking-deficient R282H channel was misfolded and this was corrected upon co-expression with R558-containing peptides which restored trafficking of the R282H channel. Importantly, we also expressed the peptide spanning the H558R-polymorphism with 8 additional BrS $Na_v1.5$ mutations with reduced currents, and demonstrated that the peptide was able to restore significant sodium currents in 4 of them.

Conclusions—In the present study, we demonstrated that small peptides, spanning the H558R-polymorphism, are sufficient to restore trafficking defect of BrS-associated $Na_v1.5$ mutations. Our findings suggest that it might be possible to use short-cDNA constructs as a novel strategy that is tailored to specific disease-causing mutants of BrS.

Keywords

arrhythmia; electrophysiology; gene therapy; ion channels; *SCN5A*

Brugada Syndrome (BrS) is associated with an increased risk of sudden cardiac death resulting from episodes of polymorphic ventricular tachycardia.^{1,2} Mutations in the α -subunit of the cardiac sodium channel ($Na_v1.5$), encoded by the *SCN5A* gene, have been

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found in 15 to 30% of BrS patients, currently representing the most common BrS genotype.^{3–5} To date, more than 100 *SCN5A* mutations have been linked to BrS.⁶ Most of those are missense mutations leading to loss-of-function phenotypes either by decreasing sarcolemmal expression of Na_v1.5 channels^{7,8}, producing non-functional channels⁹, or altered biophysical properties^{10–13}. Current treatment options for BrS include implantable cardioverter-defibrillator (ICD) and drug therapy mostly with isoproterenol.¹⁴ However, none of these treatment options directly addresses the channel dysfunction that is the underlying source of BrS associated arrhythmias.

Failure of sarcolemmal expression has been identified as one important cellular mechanism of ion channel dysfunction in inherited arrhythmias, including BrS.^{7,8} For example, we have demonstrated that the BrS-causing mutation R282H, produces a trafficking-deficient sodium channel.⁷ Interestingly, we have also identified a healthy proband despite carrying R282H. We demonstrated that this individual did not develop BrS because R282H trafficking was restored in presence of the common sodium channel H558R-polymorphism as second allele.⁷ Here, we test a new strategy using peptide fragments of the cardiac sodium channel spanning the H558R-polymorphism, to restore trafficking and function of BrS mutations, by directly aiding in channel folding. This is not without precedent, since peptide fragments have already shown potential for treating channelopathies. For example, the most common mutation of the cystic fibrosis transmembrane conductance regulator, ΔF508-CFTR, which inhibits CFTR processing and trafficking, was rescued by co-expression of specific peptide or protein fragments derived from wild-type CFTR.^{15,16} In addition, it has been shown that trafficking of a hERG mutant channel could be restored by peptide fragments spanning an ER retention signals exposed on the C-terminus of the channel protein.¹⁷

We show that co-expression of BrS mutant channels with peptide fragments containing the H558R-polymorphism similarly rescues protein trafficking, and restores functional sodium channels to the cell surface. Finally, we employ FRET to analyze the mechanism of current restoration and show that peptide fragments containing the H558R-polymorphism initiate the productive folding of originally processing-defective BrS mutants in the ER.

Methods

Mutagenesis

Nine BrS trafficking-deficient mutations located in the pore of domain 1 of the cardiac sodium channel were studied. The R282H, G292S, V294M, K317N, L325R, G351V, D356N, R367H and R376H mutations and H558R-polymorphism were created on the Na_v1.5 background (PubMed Accession No. NM198056) expressed in the GFP-IRES vector (BD Biosciences Clontech, San Jose, CA) using the Stratagene QuikChange XL Site Directed Mutagenesis Kit.

Creation of Channel Fragments

For FRET experiments, channel fragments were created by amplifying small pieces of either the wild-type or the H558R-polymorphic channel (Figure 1). Primers were designed to amplify 40 and 20 amino acids fragments from Na_v1.5. Forward primers contained a Kozak sequence (5'-GCC GCC ACC-3') before the start codon (5'-ATG G-3') to facilitate efficient translation initiation; reverse primers for the peptides expressed in pcDNA3 contained a stop codon. In addition, for the electrophysiological experiments, peptides fused to Enhanced Cyan Fluorescence Protein (ECFP) in pECFP-N3 were also generated. Sequences for peptide fragments are: wild-type 20 amino acid- NSTAGESESHHTSLLVPWPL, Polymorphic 40 amino acid-

GSEADFADDENSTAGESESHRTSLLVPWPLRRRTSAQGQPS and polymorphic 20 amino acid- NSTAGESESHRTSLLVPWPL.

Expression of Na_v1.5 and Fragments in HEK293 Cells

Transient transfection of full-length Na_v1.5 cDNA and Na_v1.5 fragments cDNA into human embryonic kidney (HEK293) cells was accomplished using the Polyfect transfection kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. When channels were expressed alone, 1 μg of full-length Na_v1.5 cDNA was used. For co-transfections, 1 μg of full-length Na_v1.5 cDNA and 1 μg of Na_v1.5 fragment cDNA were combined leading to an excess of peptide in relation to full-length channel. Assuming similar transfection levels for both full-length channels and the fragments, this would result in an estimated 90:1 or 45:1 molar ratio (peptide fragment : full length sodium channel), due to their difference in sizes, for the 20 amino acid and 40 amino acid fragments, respectively. For direct application of the R558-containing peptide to the cell interior, a synthetic 20 amino acid peptide ([H]-NSTAGESESHRTSLLVPWPL-[OH]) was synthesized by Princeton BioMolecules which was added to the internal pipette solution at 10 μM final concentration. Purity of the peptide was ≥98%.

Biochemical Analysis of Na_v1.5 Channels

HEK293 cells from similar confluent cultures were used to isolate proteins. Western blotting experiments were performed as previously described.¹⁸ To determine the expression level of the Na_v1.5 protein in total protein lysates, proteins were extracted from HEK293 cells, transiently transfected with full-length Na_v1.5 cDNA or co-transfections of full-length Na_v1.5 cDNA with Na_v1.5 fragment cDNA. Non-transfected cells were used as the negative control. Equal amounts of protein extracts were separated on 3–8% Tris-Acetate gel. Sodium channel proteins were detected using a rabbit polyclonal sodium channel antibody (Upstate).

Surface Biotinylation experiments were performed according to the manufacturer's protocol. Briefly, before cell lysis, surface proteins were labeled at 4°C with 0.25mg/ml Sulfo-NHS-SS-Biotin (Pierce). After cell lysis, biotinylated proteins were isolated through a NeutrAvidin Agarose resin column (Pierce) and after elution, Western Blot experiments were performed.

Patch-Clamp Experiments

Macroscopic sodium currents from transfected cells were recorded using the whole-cell configuration of the patch-clamp technique as previously described.¹⁹ Cells that emitted green fluorescence (indicating the presence of the sodium channel) and cyan fluorescence (indicating the presence of the peptide) were considered successfully transfected. Low resistance electrodes (<2 MΩ) were used, and a routine series resistance compensation of an Axopatch 200A was performed to values >80% to minimize voltage clamp errors. The uncompensated R_{series} was therefore less than 2 MΩ. Voltage clamp command pulses were generated by a microcomputer using PCLAMP software version 9.02 (Axon Instruments, Foster City, CA). Membrane currents were filtered at 5 kHz and digitized with 12-bit resolution. The internal solution contained (in mmol/L) NaCl 35, CsF 105, EGTA 10, and Cs-HEPES 10 adjusted to pH 7.4. The bath solution contained (in mmol/L) NaCl 140, KCl 5, MgCl₂ 1, CaCl₂ 2, HEPES 10, and glucose 10 adjusted to pH 7.4. Experiments were performed at room temperature (22°C to 23°C).

Whole-cell sodium current densities, cell capacitance, current-voltage relationship, conductance, time course of recovery from inactivation, steady-state inactivation and persistent current were measured as previously described.^{7,19}

Constructs for FRET

The FRET construct was created using a transposon approach, which randomly inserts Enhanced Yellow Fluorescent Protein (EYFP) from the transposon construct into the Na_v1.5 construct. The p(AmpR)R6Kyori transposon (Epicenter Technologies) was altered to contain EYFP upstream of the Ampicillin gene. Through mutagenesis, restriction sites were created on each end of the Amp gene to facilitate removal. The newly formed EYFP-transposon was then transposed into the pre-existing Na_v1.5 vector with Kanamycin resistance and with ECFP fused to the C-terminal region of the sodium channel. The double antibiotic resistance, Amp/Kan, was used to select for transposed clones. Diagnostic digests followed by sequencing were used to ensure that the EYFP inserted correctly in the sodium channel gene. Removal of the Amp gene from correctly transposed clones was accomplished by digestion with engineered restriction sites. The construct containing EYFP inserted at position 1022 was used because it produced significant FRET measurement and functional sodium currents. Mutagenesis was done on this construct to insert the R282H BrS mutation.

As a positive control for the FRET experiments we used a CFP-YFP dimer, in which CFP and YFP were linked by a short amino acid chain. As a negative FRET control we used CFP and YFP co-expressed in HEK293 cells from separate vectors, pECFP-C1 and pEYFP-N1 (BD Biosciences Clontech, San Jose, CA), respectively.

Image Analysis and Calculation of FRET Ratios

Images were acquired with an Olympus IX71 fluorescent microscope equipped with a Hamamatsu ORCA-ER charge coupled device (12 bit) and controlled by SLIDEBOOK software (Intelligent Imaging Innovations, Denver, CO). Filter cube specifications for the fluorescent channels were as follow for excitation and emission, respectively: ECFP, 430±25 and 470±30 nm; EYFP, 500±20 and 535±30 nm, and FRET, 430±25 and 535±30 nm.

Image analysis involved three basic operations: subtraction of background autofluorescence and blurred light, quantification of fluorescence intensity, and calculation of a corrected FRET (FRET_c) using the following equation:

$$FRET_c = (I_{DA} - aI_{AA} - dI_{DD}) / I_{DD}$$

Where I_{DA} is the fluorescence intensity of the cell measured from the FRET filter set and I_{DD} and I_{AA} are the fluorescent intensities of the same cell measured from the ECFP (donor) and EYFP (acceptor) filter sets, respectively. The cross-talk coefficients, a and d , are considered constant and a property of our microscope's filter sets, with $a=0.03$ and $d=0.63$. The corrected FRET ratio (FRET_c) includes normalization to I_{DD} to correct for variances in protein expression from cell to cell. FRET_c ratios reported, in the presence or absence of 5 µg/mL Brefeldin A, were measured from the ER in order to accurately compare between unrescued and rescued channels and avoid differences that could be due to channel processing.

Statistical Analysis

All data are reported as mean ± (standard error of the mean) SEM. The normality and equality of variance were tested using the Shapiro-Wilk test and Levene's test, respectively. Two sets of data were compared using the unpaired Student's *t*-test. One-way ANOVA with Dunnett's multiple-comparison test using the statistical program SPSS16 was used for multiple sets of data. The level of significance for all statistical tests was $P<0.05$.

Results

Peptides Containing the H558R-Polymorphism Restore Trafficking of R282H Mutant Channels

To determine if channel fragments were capable to restore trafficking of the BrS mutant channel, whole-cell sodium currents were recorded from HEK293 cells co-expressing the R282H mutant channel and CFP-fused peptide fragments of either wild-type or polymorphic cardiac sodium channels (Figure 1).

Representative current traces elicited from transfected cells are shown in Figure 2A. Cells expressing R282H alone showed a severe reduction in sodium current density, in line with previous reports that this mutation produces a trafficking deficient channel.^{7,20} Interestingly, when the mutant channel was co-transfected with a 40 amino acid channel fragment containing the H558R-polymorphism, R282H+R558-40aa, whole-cell current was increased to 30% of wild-type (WT) level. Furthermore, co-expression of a 20 amino acid fragment containing the polymorphism, R282H+R558-20aa, further increased current density (Figure 2A–B) to 75% of the WT level. However, when WT channels were co-expressed with the 40 amino acid fragment containing the polymorphism, WT+R558-40-aa, the current density was not significantly different from WT channels alone (-474.1 ± 125.9 pA/pF vs -337.7 ± 105.8 pA/pF, $P > 0.05$). These results indicate that a small R558-containing peptide is able to specifically restore trafficking of the mutant channel.

To ascertain that the polymorphism was necessary for rescue, the mutant channel was co-expressed with the corresponding 20 amino acid fragment of the WT channel, R282H +H558-20aa (Figure 2A–B). In this condition, restoration of current was not observed, indicating that the polymorphism is required to restore defective trafficking of the R282H mutant channel. To rule out any acute effects of the peptide, the 20 amino acid R558-containing peptide was synthesized and applied in the intracellular solution during whole-cell recordings. Acute expression of synthetic peptide did not alter peak current density of WT (-478.31 ± 104.4 pA/pF, $n=6$) and did not restore function of the R282H mutant channel (-23.8 ± 4.2 pA/pF, $n=7$).

Biophysical Properties of Rescued Currents

Having shown that R558-containing peptides fused to CFP were capable of restoring trafficking of the R282H mutant channel, we next compared the biophysical properties of rescued currents to WT currents. The current-voltage relationship, activation curve, voltage-dependence of steady-state inactivation, recovery from inactivation, and time constant of inactivation were measured for WT (open symbols) and rescued R282H currents (closed symbols). We found that normalized current-voltage relationships (Figure 2C) were not different between rescued and WT currents. The activation curve (Figure 3A) for rescued currents was also similar to WT. However, rescued R282H currents differed from WT currents in steady-state inactivation, which was shifted to more depolarized voltages, and recovery from inactivation was faster (Figures 3B–C and Table 1). The time course of inactivation (Figure 3D) was slower for channels rescued with the peptides, but only reached significance for channels treated with the smaller 20 amino acid peptide. Since the channel appeared to have impaired inactivation, we also measured the level of TTX-sensitive persistent current at the end of 300-ms depolarizing pulse for the R282H currents rescued by the R558-40aa peptide. These experiments demonstrated a significant increase in the level of persistent current (R282H: $0.37 \pm 0.08\%$ $n=7$ vs WT: $0.15 \pm 0.07\%$ $n=5$).

Importantly, none of these differences were observed when WT channels were treated with the R558-containing peptide, WT + R558-40aa (Figure 3). Taken together, the biophysical data suggests that the rescued R282H channels express a gain-of-function phenotype

dominated by impaired inactivation, and that the fragments do not affect biophysical properties of WT channels.

Peptides Containing the H558R-Polymorphism Increase Na_v1.5 protein Level in the Sarcolemma

We next sought to determine whether the restoration of the R282H mutation by the R558-containing peptide was due to alteration of Na_v1.5 protein expression or correction of defective channel trafficking. To test this, we first transiently expressed the R282H mutation with or without the 20 amino acid fragment containing the polymorphism (R558-20aa). Then, we evaluated the expression level of the Na_v1.5 proteins by Western blots. We first measured whole-cell lysate and noticed that the expression level of R282H was similar to that of WT Na_v1.5 proteins (Figure 4A). Figure 4A shows that the presence of the fragment containing the H558R-polymorphism, R558-20aa, did not alter the total expression level of the R282H proteins in whole-cell lysate. In fact, sodium channel protein levels were similar for WT and R282H (with or without the R558-containing peptide). Since we observed an increase in peak current density for the R282H mutant in the presence of R558-containing peptides (Figure 2A–B), we performed surface biotinylation experiments to investigate the absence or presence of the R282H mutant in the cell membrane with or without the R558-containing peptide. Figure 4B and 4C confirm the absence of the R282H mutant in the cell membrane, as expected for a trafficking-deficient mutant. Importantly though, in presence of the R558-containing peptide, the R282H mutant sodium channel is inserted into the cell membrane, in line with current recordings.

R282H Affects Channel Conformation

In order to function properly, newly synthesized ion channels need to assemble and fold correctly in the ER, and traffic properly to their respective membrane subdomain.²¹ We have previously demonstrated that the absence of current observed with the R282H mutation is due to a trafficking defect.⁷ A common cause of trafficking deficient channels is misfolding and retention by the quality control machinery in the ER.^{22–24} Thus, one possible mechanism by which the R558-containing peptide may rescue the R282H mutant is by restoring proper folding of the mutated channel to an exportable conformation. FRET was used to examine folding of the trafficking-deficient mutant channel, R282H, in the presence and absence of the R558-containing peptide. To accomplish this, a sodium channel construct containing a pair of fluorescent proteins capable of transferring energy from one to the other was created.

The cardiac sodium channel used for these experiments, YFP-1022, had ECFP fused to the C-terminus of the channel and EYFP inserted into the Domain II-III linker at amino acid position 1022 (Figure 5A). This construct produced functional channels (Figure 5B). EYFP-1022 was associated with a corrected FRET (FRET_c) value of 0.27±0.01 (Figure 5C). This value was significantly greater than the negative control (0.16±0.01, p<0.001). When the R282H mutation was created on EYFP-1022 (EYFP-1022/R282H), the FRET_c value decreased significantly to 0.18±0.01 but was still significantly different from negative control (p=0.02). The FRET decrease indicated that the 3-D conformation of the channel has changed so that the EYFP and ECFP were now further apart. This is likely due to the fact that the R282H mutant channel is improperly folded. Interestingly, when fragments containing the polymorphism were co-expressed with the YFP-1022/R282H construct, FRET_c was restored to values similar to non-mutated YFP-1022 p>0.05 (Figure 5C), which suggests that the mutated channel YFP-1022/R282H has now obtained a conformation similar to that of the functional YFP-1022 channel; this likely explains the ability of the mutant channel to traffic in presence of the R558-containing peptide. Similarly, when the YFP-1022/R282H construct was expressed in presence of the drug mexilitine which is

known to restore trafficking of the R282H mutant, FRETc values were also restored to the level of the non-mutated YFP-1022 (data not shown). This supports the hypothesis that the mutant channel, either in presence of the drug or with the R558-containing peptide is now folding properly which allows the channel to traffic to the cell membrane forming functional channels.

To exclude non-specific effects of the peptide, non-mutated EYFP-1022 channels were co-expressed with R558-containing peptide. No FRETc increase was observed in these cells (Figure 5C), confirming that the increase in FRETc seen when EYFP-1022/R282H was co-expressed with the R558-containing peptide is specific. To further verify that the polymorphism was necessary for rescuing the improperly folded EYFP-1022/R282H channel, the EYFP-1022/R282H channel was co-expressed with the corresponding 40 or 20 amino acid fragment of the WT channel. In this experiment, restoration of FRETc values were not observed (Figure 5C), indicating that the polymorphism is required to promote the proper folding of the R282H channel.

Thus, these data suggest that restoration of folding in the ER will lead to protein trafficking to the sarcolemma which will result in the increase in peak current density observed (Figure 2A–B). To separate folding effects in the ER from forward trafficking to the cell surface membrane, we used the fungal metabolite Brefeldin A (BFA) which prevents anterograde transport from the Endoplasmic Reticulum (ER) to the Golgi apparatus. BFA treatment is thought to lead to a rapid accumulation of proteins within the ER following collapse of the Golgi apparatus.^{25,26} Consequently, incubation of cells co-expressing the R282H mutation and the R558-containing peptide (R282H+R558-20aa, n=6), together with BFA did not generate functional sodium current. In marked contrast, this blocking of forward trafficking with 5µg/mL BFA applied for 24 hours did not alter FRETc values $p>0.05$ (Figure 5C). These results indicate that conformational changes induced by EYFP-1022/R282H take place in the ER and not en route to the cell surface. Taken together, our FRET data suggest that the peptide containing the polymorphism rescues the R282H mutant by restoring proper folding of the mutated channel in the ER.

The Peptide Fragment Containing the H558R-Polymorphism Can Restore Currents for Multiple BrS Mutants

To expand on our observations with R282H, we tested 8 additional sodium channel BrS mutations located in the same region as R282H (pore domain I): G292S, V294M, K317N, L325R, G351V, D356N, R367H and R376H (Figure 1); mutations were taken from the Inherited Arrhythmia Database website (<http://www.fsm.it/cardmoc/>). Similarly to R282H, all mutants expressed alone resulted in significant decreased sodium currents compared to WT (Table 2). In fact, the K317N, L325R, G351V, D356N, R367H and R376H, resulted in non-measurable currents. For the G292S and V294M mutants, small and significantly reduced currents compared to WT were measured. Importantly, when these mutants were co-expressed with the R558-20aa peptide, the current density was increased for G292S, V294M, K317N and R376H (Table 2). The biophysical properties of these rescued currents were similar to WT channels, except for K317N which displayed a shift toward depolarized voltages for steady-state inactivation (Table 2). However, even in the presence of the R558-containing peptide, there were still no measurable currents for L325R, G351V, D356N and R367H (Table 2). Thus, we now show that out of 9 BrS-associated $Na_v1.5$ mutations tested, the peptide was able to restore large sodium currents in 5 of them, confirming that this mechanism/strategy is not unique to the R282H mutation.

Discussion

Brugada Syndrome is associated with mutations in the cardiac sodium channel that decrease whole-cell sodium currents, often by reducing channel expression at the cell surface. This lack of whole-cell sodium current is believed to cause an increased risk of sudden cardiac death in patients with BrS.^{27,28} In this study, we showed that small peptides, spanning the H558R-polymorphism, are sufficient to restore trafficking defect of a subset of BrS-associated Na_v1.5 mutations. The central hypothesis of this work was first based on our previous study which provided us with enough supporting evidence of the rescue of the R282H mutant by the H558R-polymorphism (both clinical profile and cellular electrophysiology).⁷ However, we further hypothesized that this new strategy using peptide fragments of the cardiac sodium channel, spanning the H588R-polymorphism, could be used to restore trafficking and function of other BrS mutations in which reduced sodium current densities are also observed. Nine BrS trafficking-deficient mutations located in the pore of domain 1 of the cardiac sodium channel were studied. The peptide containing the H558R-polymorphism was able to increase current densities in five of these mutants. This is the first study showing that short fragments containing H558R-polymorphism can correct defective trafficking phenotype in BrS-associated Na_v1.5 mutations. The FRET experiments suggest that the R558-containing peptides may act as “chemical chaperones” that promote proper protein folding and thereby facilitating the exit of rescued mutant ion channel from the ER.

It is commonly thought that channels which fail to traffic to the cellular membrane are misfolded, but very little work has been done to test this notion. Therefore, we employed a FRET based approach to study folding of the channel. To the best of our knowledge, this FRET approach has not been used to study folding of sodium channels. When R282H was introduced in a functional channel construct, containing CFP and YFP, the folding of the channel became altered, as shown by the decrease in FRETc signal and confirmed by the severe reduction of whole-cell sodium currents produced by the R282H mutation (Figure 5). Previous studies have shown that this mutant channel is retained in the ER⁷, and it appears that altered folding may be the cause of ER retention. In the presence of either of the peptides containing the H558R-polymorphism (40 aa and 20 aa), folding of the R282H mutant channel, as indicated by the restored FRETc value, was corrected. This data suggests that in the presence of the R558-containing peptide, the mutant channel is able to fold properly. Furthermore, blocking forward protein trafficking by BFA did not alter FRETc values. These data confirm that the R558-containing peptides specifically rescue the R282H mutant by restoring proper folding of the mutated channel in the ER. This indicates that the mechanism by which the polymorphism rescues the mutant channel involves either directly assisting in channel folding or allowing another protein to interact with the channel to correct folding.

A growing body of literature exists suggesting that trafficking defect of mutant channels can be rescued by fragments of polypeptides. Fragments of the HERG and CFTR channel have been shown to restore trafficking to mutant channels.^{15,16,29} Kupersmidt et al showed that a 100 amino acid peptide was able to restore trafficking to a HERG mutation in which the C-terminal region was truncated.¹⁷ When a peptide containing the Endoplasmic Retention signal, RXR, was co-expressed with the mutant channel, the peptide acted as a decoy, allowing the mutant channel protein to bypass ER quality control system. Moreover, Owsianik et al proposed a similar mechanism for the ability of peptides to rescue the Δ F508-CFTR mutation.¹⁶ However, after observing a similar rescue of the same channel, Cormet-Boyaka et al proposed that several different mechanisms may contribute to the fragments' ability to help the mutant CFTR channel process, including that the fragments could be providing ER exit codes or burying areas on the CFTR channel that would lead to channel aggregation and destruction.¹⁵ However, none of these mechanisms seem to be occurring

with our sodium channel fragments. Three RXR endoplasmic retention signals have been identified in the Domain I-II linker³⁰ of Na_v1.5, the region from which our fragments originate, but none are present in the fragments. Thus it seems unlikely that they are acting as decoys. The FRET data also suggest that the fragments are involved in folding, rather than providing or hiding ER signals.

The rescue of sodium channel mutations with mexilitine has been well documented.^{8,31,32} The theory behind these rescues is that the drug, which is a Na_v1.5 blocker, stabilizes the pore of the channel allowing for trafficking. It is possible that the R558-containing peptides work similarly, in that they may be able to stabilize part of the channel for proper folding in the ER, and thus restore trafficking. However it appears unlikely that the fragments are acting as channel blockers. First, we did not observe a reduction of current density when WT channels were co-expressed with either the 40 amino acid fragment or in the presence of the synthesized 20 amino acid R558-containing peptide. If the fragment was acting as a blocker, we would expect to see some decrease in whole-cell current density in both transient transfection and acute effect experiments. Also, we know that the whole polymorphic channel can rescue the mutant channel⁷ and it is therefore unlikely that the polymorphic channel is blocking the mutant channel. Moreover, R558-containing peptide did not alter the expression level of the R282H proteins (Figure 4A). Thus we suspect that the mechanism of rescue with the R558-containing peptides differs from the one of sodium channel blockers. However, previous work in HERG has shown that channel blocking is not necessarily required for the rescue of trafficking-deficient channels to occur.³³ Thus, it is possible for the mechanism of rescue of such drugs and of the peptide to be similar, and yet different than the blocking effect.

When trafficking is restored to the mutant R282H and K317N channels, changes in biophysical properties are uncovered suggesting the complexity of the disease. This finding is also consistent with a growing body of literature suggesting that some *SCN5A* mutations can cause both long QT and BrS phenotypes in members of one family, or LQT in one family and BrS in another.^{34–36} Pfahnl et al recently described a similar phenomenon, involving another trafficking-deficient BrS mutation, T353I, rescued by mexilitine.³² Interestingly, overnight exposure to 0.1 mM mexilitine increased T353I channel trafficking to the membrane to near normal levels. However, this mutant channels also showed a significant increased in late current by 1.6%, a finding seen with long QT mutations.

The destabilization of inactivation observed in rescued R282H mutant channels is consistent with the biophysical changes caused by mutations associated with Long QT Syndrome Type 3. It is plausible that the presence of a small late sodium current may be masked due to the inefficient channel trafficking, giving rise to the concern that protecting a patient from BrS may cause another type of arrhythmia. However, the initial patient, identified by Poelzing et al, who carries both the polymorphism and mutation, is healthy, displaying symptoms of neither BrS nor Long QT Syndrome.⁷ The possible explanation for the discrepancy in the alteration of inactivation kinetics following the R558-containing peptide treatment might be due to the difference in sodium channel population expressed in our heterologous expression system. In the present experiment, only the R282H channel is expressed, whereas in the previous report⁷, both H558R polymorphic channel and R282H channel were expressed. Therefore in the latter experiment, the whole-cell currents measured are the summation of the currents flowing through both H558R polymorphic channel and the rescued R282H channel. Thus, we speculate that the kinetic of the currents generated from the H558R polymorphic channel might conceal the alteration in fast inactivation of the rescued R282H mutant channel.

Conclusions and Study Limitation

We have thus shown that small peptides, spanning the H558R-polymorphism, are sufficient to restore trafficking defects of some BrS-associated Na_v1.5 mutations. Due to their ability to rescue a mutant channel *in vitro*, we reasoned that it may be possible to use this R558-containing peptide as an alternative strategy aiming to rescue the trafficking defect of mutant cardiac sodium channels *in vivo*. We acknowledge the presence of limitations in our study. First, this rescuing strategy works on a subset of mutations and we are not aware yet of the exact mechanism which underlies this discrepancy between mutations. Of course, further studies are needed to test whether this rescue will occur *in vivo*. But importantly, although a future clinical application will require several further investigations, this report remains the first to present a new alternative strategy for treating the trafficking defects of BrS-associated sodium channels that for the first time addresses the underlying cause of the disease by restoring the function of the mutant channels. Additionally, this new approach has the advantage of using only a small fragment of the target gene which should reduce the problems usually seen with gene therapy when an entire gene is transferred. Therefore, polymorphic-containing peptides might represent a promising option to rescue trafficking-deficient channels.

Brugada Syndrome (BrS) is an inherited primary electrical cardiac disorder. It is characterized by ST-segment elevation in the right precordial leads (V1–V3) and increased susceptibility to sudden cardiac death due to episodes of polymorphic ventricular tachycardia. The genetic abnormalities that cause BrS have been linked to mutations in the *SCN5A* gene which encodes for the pore forming alpha-subunit of the cardiac sodium channel (Na_v1.5). A trafficking defect of mutant cardiac sodium channels has been identified as one important cellular mechanism underlying a loss-of-function of cardiac sodium channels in BrS. Current treatment options for BrS include implantable cardioverter-defibrillator (ICD) and drug therapy mostly with isoproterenol. However, none of these treatment options directly addresses the channel dysfunction at the underlying source of BrS associated arrhythmias. We previously reported that the function of a trafficking-deficient BrS Na_v1.5 mutation, R282H, could be restored by co-expression with the cardiac sodium channel containing the H558R-polymorphism. In the present study, we demonstrate that small peptides, spanning the H558R-polymorphism, are sufficient to restore trafficking defect of BrS-associated Na_v1.5 mutations. Therefore, peptides containing the H558R-polymorphism might represent a promising option to rescue trafficking-deficient BrS mutations. In addition, our findings also suggest that it might be possible to use short-cDNA constructs as a novel strategy that is tailored to specific disease-causing mutations associated with BrS.

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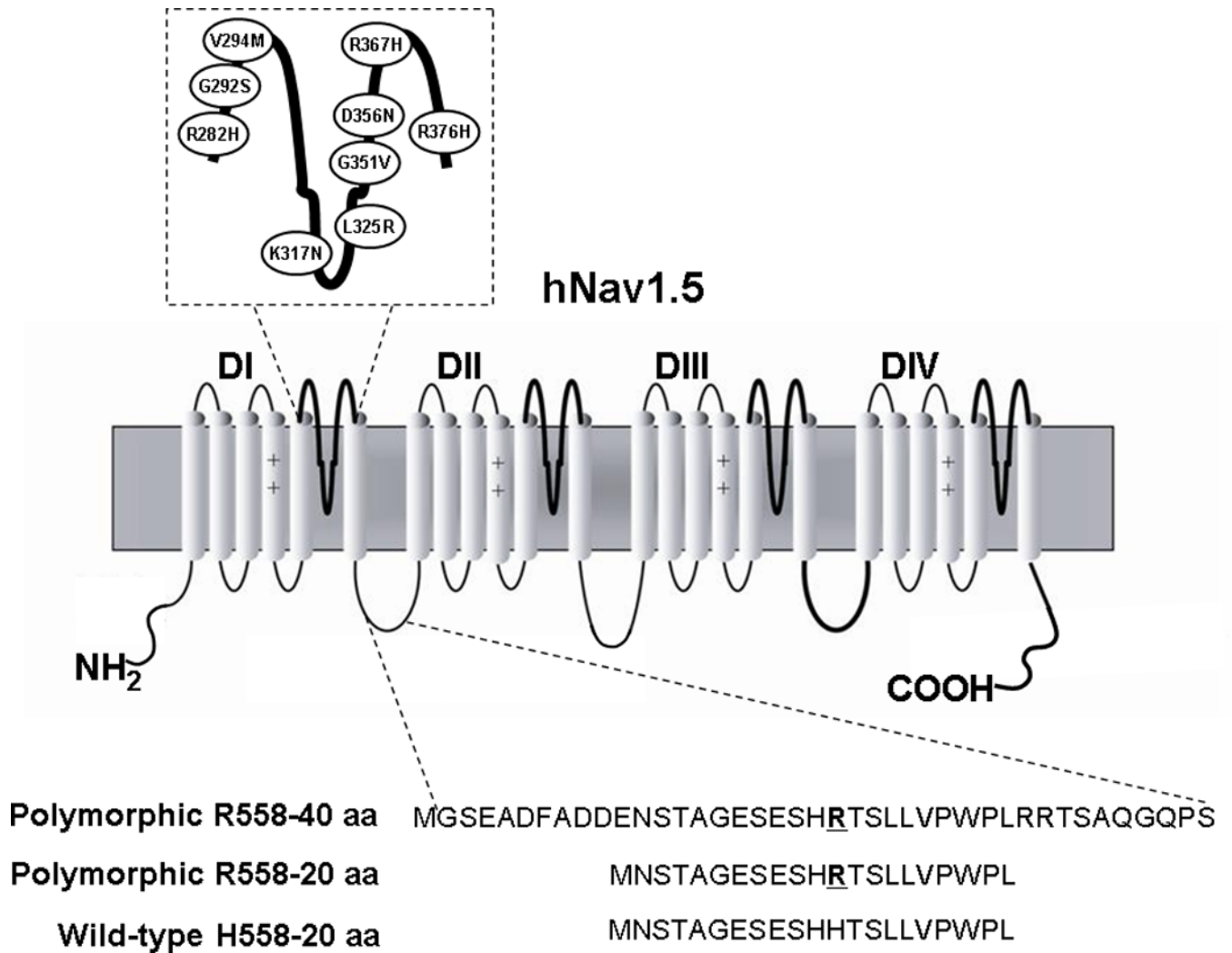


Figure 1. Schematic Representation of the α -Subunit of hNav1.5. Ovals represent the locations of the mutations characterized in this study. The lower panel shows the sequence of the fragments that were created by amplifying a piece of either the wild-type (H558) or the polymorphic channel (R558).

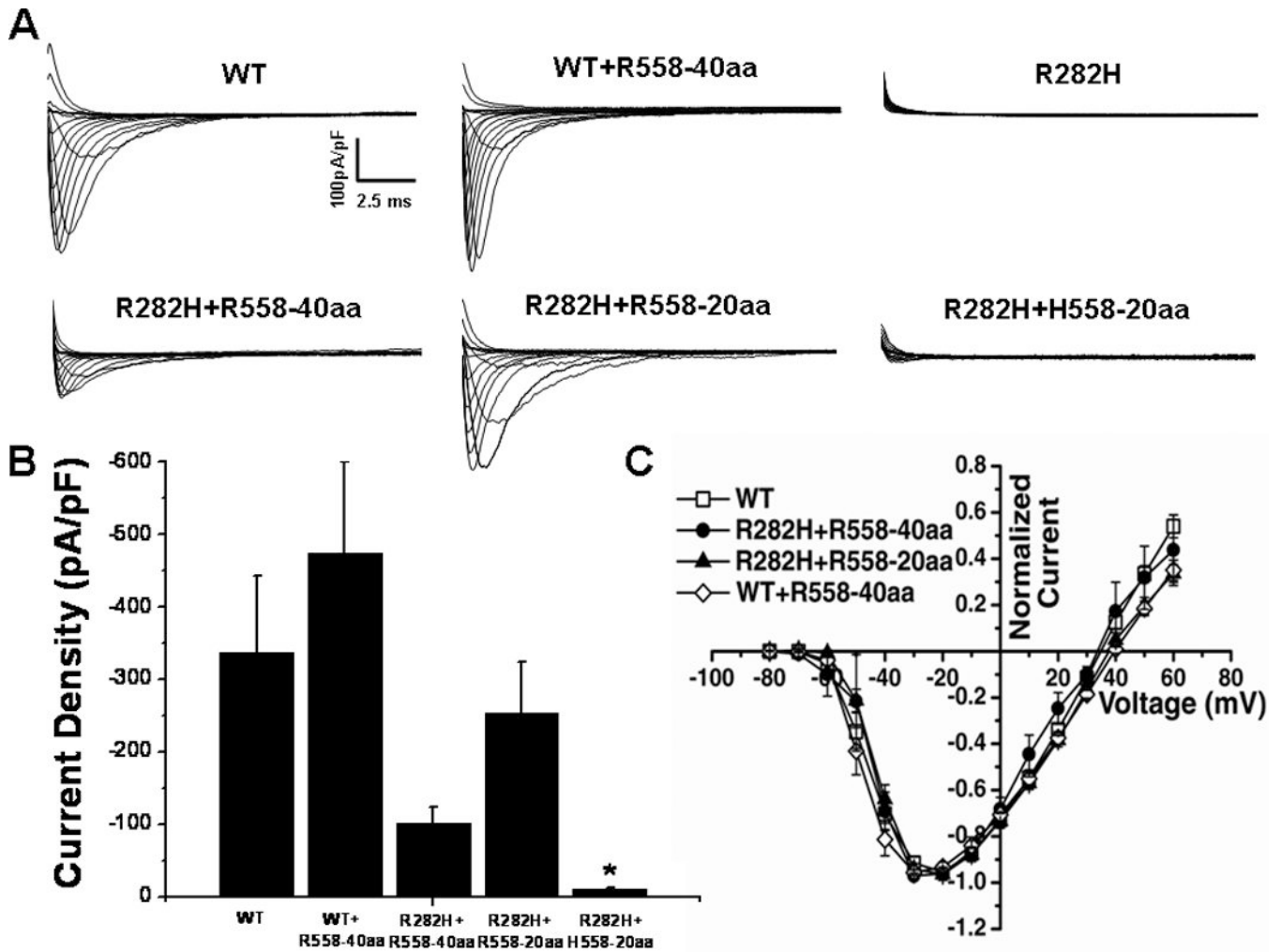


Figure 2.

Peptides Containing the H558R-Polymorphism Restore Function of the R282H BrS Mutant Channel A. Whole-cell sodium current traces recorded from transfected HEK293 cells. The R282H mutation expressed alone does not generate functional sodium current. Current was restored when the mutant was co-expressed with R558-containing peptide fused with CFP (R282H+R558-40aa and R282H+R558-20aa), but not when co-expressed with the WT peptide (R282H+H558-20aa). B. Peak sodium current densities. Current density was similar for WT (n=6), R282H+R558-40aa (n=8), and R282H+R558-20aa (n=12) and significantly lower for R282H+H558-20aa (n=11). C. Current-Voltage Relationships were similar for all conditions. * $P < 0.05$ compared to WT.

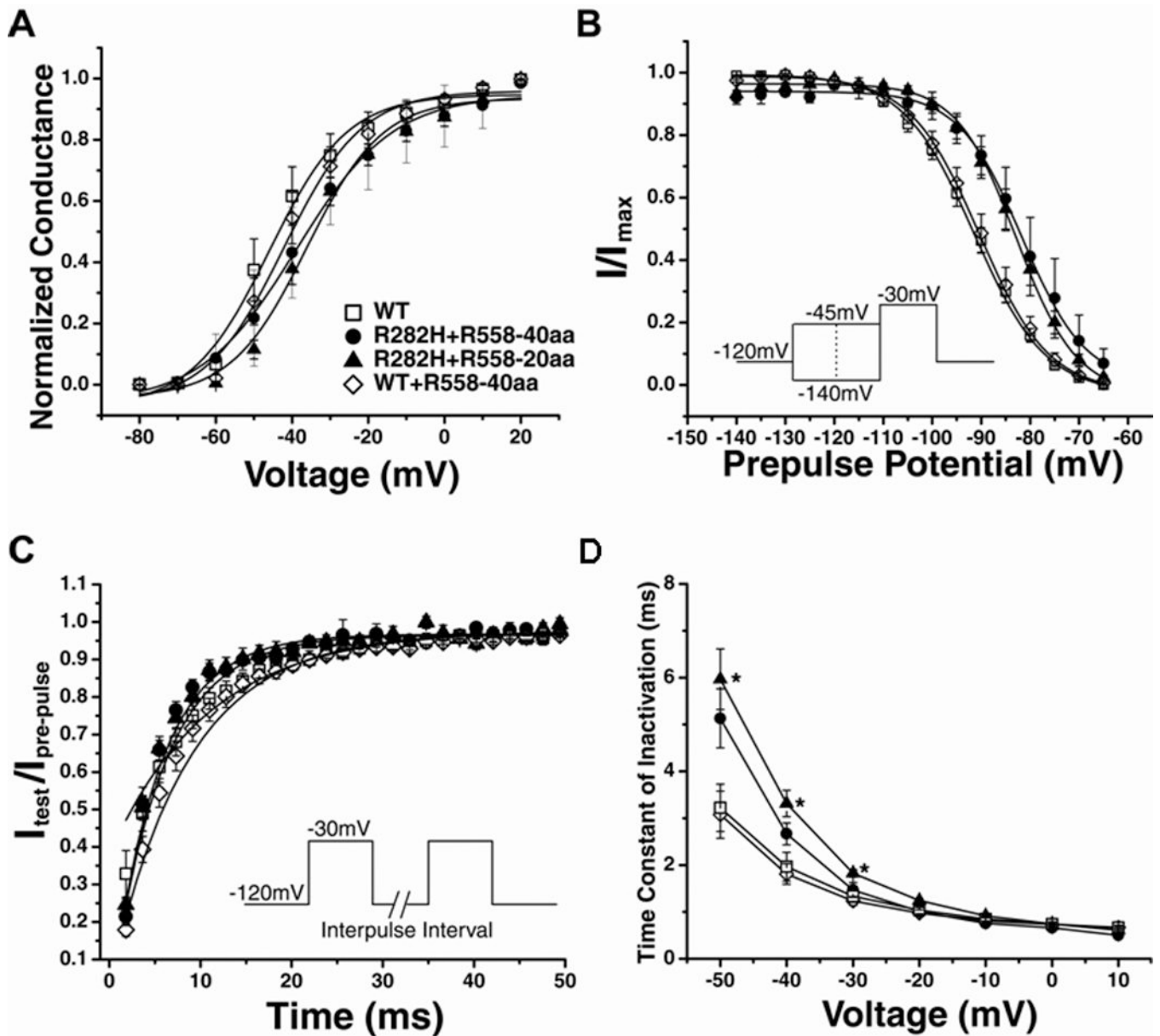


Figure 3.

Biophysical Properties of Rescued Currents are Altered A. Conductance was similar for WT, rescued channels (R282H+R558-40aa and R282H+R558-20aa), and WT channels treated with the R558-containing peptide (WT+R558-40aa). B. Steady-State Inactivation of rescued currents (closed symbols) was significantly shifted toward depolarized potentials. C. Recovery from Inactivation. The time constants of recovery from inactivation were significantly faster for rescued channels. D. Time Constant of Inactivation. Inactivation of R282H+R558-20aa channels (n=9) was significantly slower than WT channels (n=7). R282H+R558-40aa channels (n=5) and WT+R558-40aa channels (n=9) behaved similarly to WT. * $P < 0.05$ compared to WT.

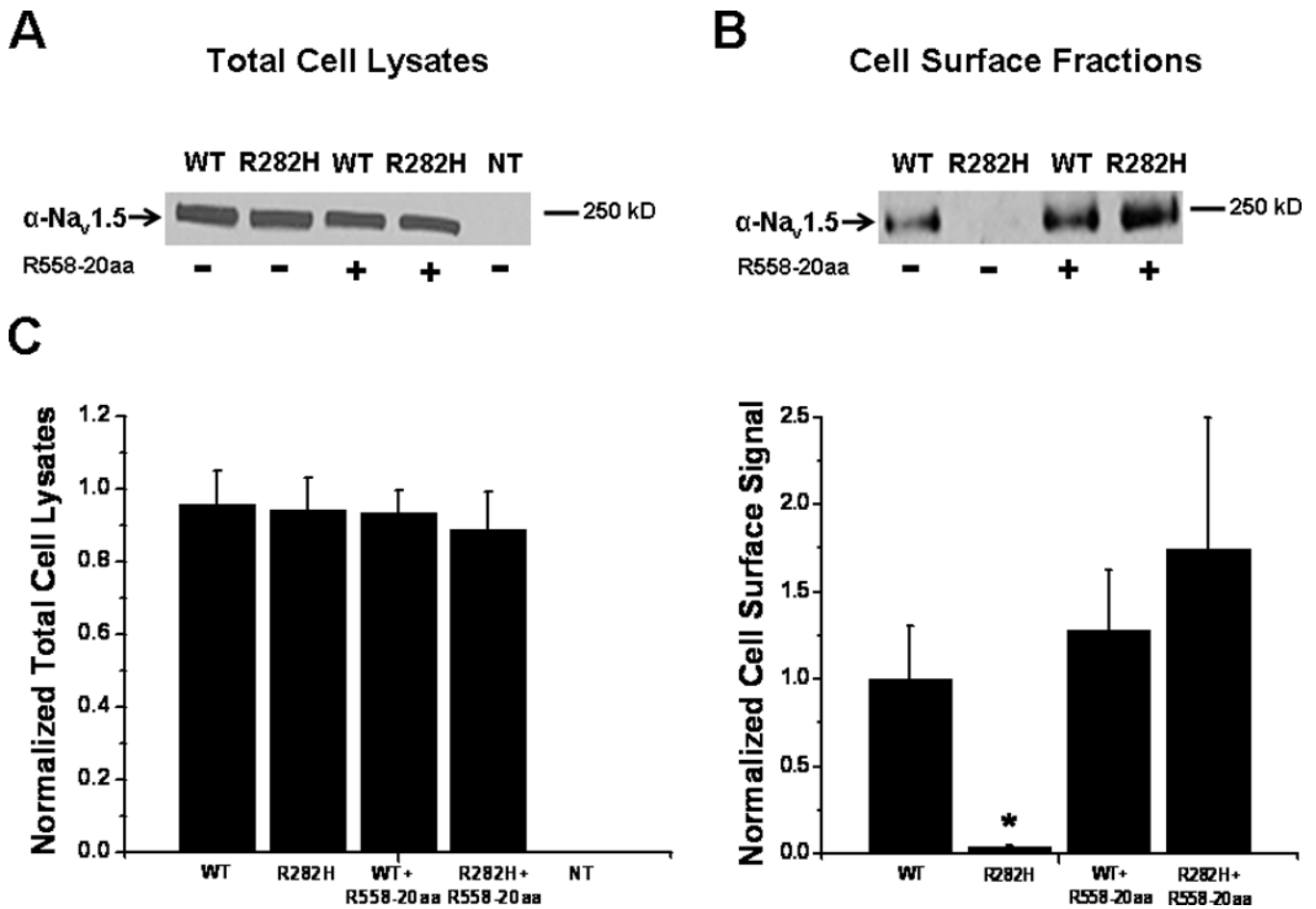


Figure 4. Biochemical Analysis A. Representative Western blot of three independent experiments showing WT and mutant proteins in total cell lysates in the presence or absence of the R558-containing peptide (R558-20aa). NT, non-transfected cells. B. Representative surface biotinylation experiments to investigate the absence or presence of the R282H mutant in the cell membrane with and without the peptide containing the polymorphism. Three independent biotinylation experiments were performed. C. Quantification normalized to WT. Data are presented as mean \pm SEM. * P <0.05 compared to WT.

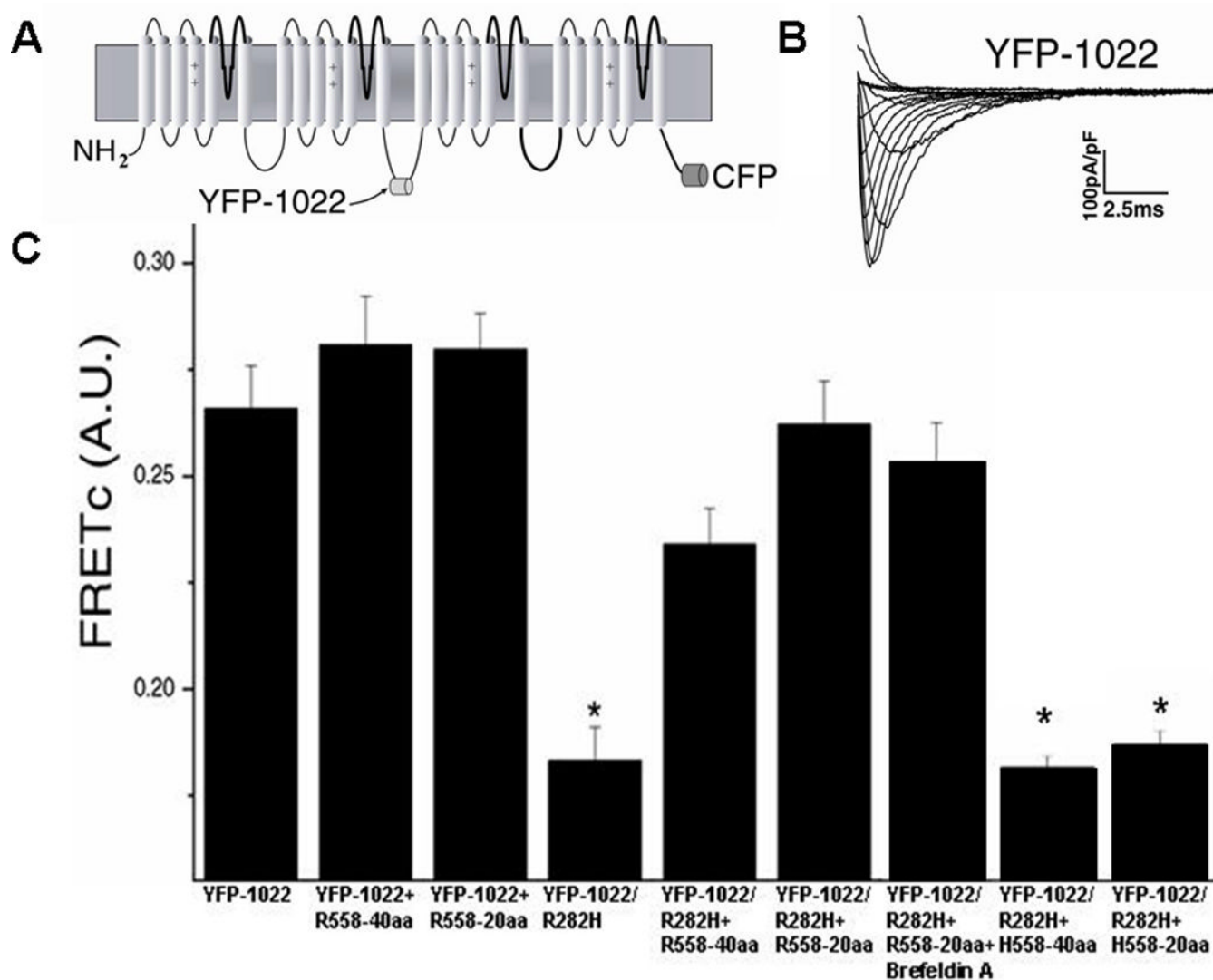


Figure 5. FRET in Normal and Mutant Channels **A.** Diagram of FRET construct containing CFP fused to the C-terminus of hNav1.5 and YFP inserted into the Domain II-III linker at amino acid position 1022. **B.** Currents elicited from the FRET construct are similar to WT currents. **C.** FRETc values for YFP-1022 (n=43) were similar to YFP-1022+R558-40aa (n=35), YFP-1022+R558-20aa (n=39), YFP-1022/R282H+R558-40aa (n=43), YFP-1022/R282H+R558-20aa (n=46), and YFP-1022/R282H+R558-20aa+Brefeldin A (n=34). FRETc of YFP-1022/R282H (n= 45) alone, YFP-1022/R282H+H558-40aa (n=36), and YFP-1022/R282H+H558-20aa (n=42) were significantly smaller than WT. * $P < 0.05$ compared to WT.

Table 1

Electrophysiological Properties for Wild-Type and BrS Mutant hNav1.5 Channels in the Presence or Absence of the Peptide Fragments.

Na Channels hNav1.5	Peak Current Density (pA/pF)	Steady-State Inactivation ($V_{1/2}$), mV	Recovery From Inactivation (τ_{rec}), ms
WT (n=7)	-337.7 ± 105.8	-91.9 ± 1.7	8.4 ± 1.0
WT+R558-40aa (n=9)	-474.1 ± 125.9	-90.0 ± 1.6	7.0 ± 0.7
R282H (n=10)	-9.7 ± 3.0^a	n.a.	n.a.
R282H+H558-20aa (n=12)	-9.9 ± 2.8^a	n.a.	n.a.
R282H+R558-40aa (n=9)	-101.9 ± 21.2^b	-80.6 ± 2.7^a	4.5 ± 0.4^a
R282H+R558-20aa (n=13)	-252.9 ± 71.8^b	-82.8 ± 1.5^a	5.0 ± 0.5^a

^a $P < 0.01$ compared with wild type.

^b $P < 0.01$ compared with mutant alone.

n.a. = not available due to the small currents.

Table 2

Electrophysiological Properties for BrS Mutant hNav1.5 Channels in the Presence or Absence of the R558-20aa Peptide.

Na Channels hNav1.5	Peak Current Density (pA/pF)	Steady-State Inactivation ($V_{1/2}$), mV	Recovery From Inactivation ^v (τ_{rec}), ms
G292S (n=10)	-129.2 ± 24.6^a	-84.6 ± 1.3^a	5.9 ± 0.6
G292S+R558-20aa (n=8)	-350.3 ± 25.5^b	-89.8 ± 1.6	6.2 ± 0.3
V294M (n=8)	-155.5 ± 24.8^a	-87.8 ± 1.7	6.3 ± 0.7
V294M+R558-20aa (n=10)	-339.7 ± 30.8^b	-89.0 ± 0.8	5.9 ± 0.8
K317N (n=7)	n.a.	n.a.	n.a.
K317N+R558-20aa (n=7)	-102.4 ± 14.8	-83.8 ± 0.2^a	5.2 ± 0.3
R376H (n=7)	n.a.	n.a.	n.a.
R376H+R558-20aa (n=7)	-110.3 ± 22.0	-93.1 ± 3.8	5.9 ± 0.5

^a $P < 0.01$ compared with wild type.

^b $P < 0.01$ compared with mutant alone.

n.a. = not available due to the absence of currents.

The following mutants did not produce currents when co-expressed with the R558-20aa peptide: L325R, G351V, D356N and R367H.