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γ_1 Subunit Interactions within the Skeletal Muscle L-type Voltage-gated Calcium Channels*

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Voltage-gated calcium channels mediate excitation-contraction coupling in the skeletal muscle. Their molecular composition, similar to neuronal channels, includes the pore-forming α_1 and auxiliary $\alpha_2\delta$, β, and γ subunits. The γ subunits are the least characterized, and their subunit interactions are unclear. The physiological importance of the neuronal γ is emphasized by epileptic stargazer mice that lack γ_2. In this study, we examined the molecular basis of interaction between skeletal γ_1 and the calcium channel. Our data show that the $\alpha_1$1.1, β_{1a}, and $\alpha_2\delta$ subunits are still associated in γ_1 null mice. Reexpression of γ_1 and γ_2 showed that γ_1, but not γ_2, incorporates into γ_1 null channels. By using chimeric constructs, we demonstrate that the first half of the γ_1 subunit, including the first two transmembrane domains, is important for subunit interaction. Interestingly, this chimera also restores calcium conductance in γ_1 null myotubes, indicating that the domain mediates both subunit interaction and current modulation. To determine the subunit of the channel that interacts with γ_1, we examined the channel in muscular dysgenesis mice. Cosedimentation experiments showed that γ_1 and $\alpha_2\delta$ are not associated. Moreover, $\alpha_1$1.1 and γ_1 subunits form a complex in transiently transfected cells, indicating direct interaction between the γ_1 and $\alpha_1$1.1 subunits. Our data demonstrate that the first half of γ_1 subunit is required for association with the channel through $\alpha_1$1.1. Because subunit interactions are conserved, these studies have broad implications for γ heterogeneity, function and subunit association with voltage-gated calcium channels.

The L-type voltage-gated calcium channels of the skeletal muscle serve both as a voltage-gated calcium channel and as a voltage sensor for excitation-contraction (EC)[1] coupling (1, 2). These channels serve to couple depolarization to intracellular calcium release via the ryanodine receptor. The sites of EC coupling in the skeletal muscle are the triads, which are highly organized junctions comprising the t-tubules and the underlying sarcoplasmic reticulum (3). The voltage-gated calcium channels are localized predominantly in the t-tubules in close association with the ryanodine receptor in the sarcoplasmic reticulum (4, 5).

At the molecular level, the calcium channel is composed of the pore-forming $\alpha_1$1.1 subunit and auxiliary $\alpha_2\delta$, β_1, and γ_1 subunits (6). This four-subunit composition of the channels is similar to that of the neuronal voltage-gated channels (7, 8). The auxiliary $\alpha_2\delta$ and β subunits enhance membrane trafficking of the $\alpha_1$1.1 subunit and modulate the voltage-dependent kinetics of the channel (9). In addition to the role of the β_1 subunit in the trafficking of the channel, it also has a crucial role in EC coupling as emphasized by the absence of EC coupling and early lethality in mice that lack the skeletal β_1 subunit (10). The subunit interactions of the $\alpha_2\delta$ and β subunits have been relatively well defined.

The γ_1 subunit is an auxiliary subunit with four predicted transmembrane domains and intracellular N and C termini. The role of the γ subunit in the membrane trafficking of the channels and subunit interaction remain unclear. The γ_1 subunit was the only γ subunit that was originally known and is associated with the skeletal muscle voltage-gated calcium channel (11, 12). However, the identification of a neuronal γ_2 subunit (13) renewed interest in the γ subunits and led to the identification of a number of γ subunits (14–16).

The β subunits of the voltage-gated calcium channels are known to be capable of forming heterogenous complexes both *in vivo* (17, 18) and *in vitro*. The β subunits and the α_1 subunit possess highly conserved interaction regions (19) allowing the formation of heterogeneous channel complexes. The $\alpha_2\delta$ subunits modulate different α_1 subunits *in vitro* (20), and presumably this heterogeneity extends to the *in vivo* situation. Such diversity of interaction would potentially multiply the number of possible combinations of channels with different biophysical and physiological properties, which could then translate into fine modulation of a variety of cellular responses.

γ subunits are relatively less well understood. Subunit interactions of γ subunits with the voltage-gated calcium channels are unknown, and their ability to form heterogeneous complexes is unclear. To address these questions, we took advantage of γ_1 null mice. Mice that lack γ_1 have been generated by use of conventional gene targeting strategy (21, 22). The mice have no detectable phenotype, and we have demonstrated previously (21) that the subunits of the skeletal muscle calcium channel are expressed at wild type levels in the γ_1 null mice. The lack of the γ_1 subunit also does not appear to diminish the

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[1] The abbreviations used are: EC, excitation-contraction; WGA, wheat germ agglutinin; GFP, green fluorescent protein; EGFP, enhanced fluorescent green protein; aa, amino acid.

ability of the other subunits to assemble together and conduct voltage-gated calcium currents. However, the absence of the γ_1 subunit slows the inactivation kinetics and increases the amplitude (21, 22) of L-type currents in the skeletal muscle. This led us to examine if the L-type calcium channel is maintained as a complex in the γ_1 null mice. We hypothesized that if the channel were still maintained as a complex in the γ_1 null mice, it would serve as a valuable tool offering the potential to explore subunit-subunit interactions in an *in vivo* environment.

Our data show that the skeletal L-type channel is indeed maintained as a complex in the γ_1 null mice. To examine the ability of the γ subunits to form heterogeneous complexes, the γ_1 and γ_2 subunits were introduced into the skeletal muscle of the γ_1 null mice via adenovirus-mediated expression, and their ability to incorporate into the channel was examined. We demonstrate that the γ_1 does incorporate, but the γ_2 does not. Consistent with these results, γ_1EGFP localizes to the t-tubules, whereas the γ_2EGFP does not demonstrate organized t-tubule localization. Furthermore, by using a γ_1/γ_2 chimeric strategy, we demonstrate that the first half of the γ_1 subunit mediates its interaction with the calcium channel. Examination of the electrophysiological characteristics of the chimeric subunits in γ_1 null myotubes showed that the first half of the protein is sufficient to restore calcium conductance of L-type currents in γ_1 null myotubes.

To examine the subunit of the channel with which the γ_1 subunit associates, we examined the channel in the muscular dysgenesis (*mdg*) mice. In the absence of $\alpha_1$1.1 in the *mdg* mice, γ_1 does not associate with $\alpha_2\delta$, indicating that $\alpha_1$1.1 is necessary for γ_1 to associate with the calcium channel. These predictions are confirmed by coimmunoprecipitation of $\alpha_1$1.1 and γ_1EGFP subunits from transiently transfected cells.

MATERIALS AND METHODS

Enrichment and Purification of the Skeletal Voltage-gated Calcium Channels—KCl microsomes were prepared from wild type and γ_1 null mice as described previously (23). The microsomes were solubilized twice in a buffer containing 50 mM Tris, 1% digitonin, 0.5 M NaCl, and seven protease inhibitors (benzamidine, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, calpain inhibitor, calpeptin, and pepstatin). The solubilized material was subjected to wheat germ agglutinin (WGA) chromatography. The material bound to the WGA column was eluted using *N*-acetylglucosamine and concentrated. In some cases, the material was used as the enriched voltage-gated calcium channel. For purification, the WGA-eluted material was diluted and applied to a diethylaminoethylcellulose column, and the bound material eluted with buffer containing 50 mM Tris, 0.1% digitonin, and 100 mM NaCl.

Constructs and Generation of Adenoviruses—All the adenoviruses and cell expression constructs used in this study expressed the protein under the control of the cytomegalovirus promoter. The cDNAs of interest were subcloned into the pAd5CMVK-NpA vector and used for generation of the adenovirus by standard homologous recombination techniques. The recombinant viruses were then purified using standard techniques. The Gene Transfer Vector Core at the University of Iowa, Iowa City, IA, generated all adenoviruses in this study. In some cases, the cDNAs in the adenovirus vectors were used for cell expression studies.

γ_1—The rabbit γ_1 subunit (GenBank™ accession number M32231) was subcloned into the *Bam*HI/*Bam*HI site of the adenovirus shuttle vector. The resulting construct was checked for appropriate orientation by restriction analysis.

γ_2—The mouse γ_2 subunit (GenBank™ accession number NM_007583) was excised from the pCDNA3 vector using *Hin*dIII and *Bam*HI and subcloned into the *Hin*dIII/*Bam*HI sites of the adenovirus vector.

γ_1EGFP and γ_2EGFP—The γ_1EGFP and γ_2EGFP constructs were generated by inserting the cDNA into the pEGFP-N1 vector (Clontech) in-frame with the enhanced fluorescent green protein (EGFP) using standard PCR techniques. The resulting construct encoded the γ subunit with the EGFP tag at the C terminus of the protein. The resulting constructs were excised and subcloned into the adenovirus vector.

γ_1/γ_2 Chimera—To generate this construct, a fragment of the rabbit γ_1 cDNA was amplified using a forward primer that includes a *Hin*dIII site and the start codon (γ_1 forward, 5′ CCC AAG CTT CCA CCA TGT CCC CGA CGG AAG CC 3′) and the reverse primer in the region after the second transmembrane domain (5′ GTT GTG GCG TGT CTT CCG CTT CTT CCT GAA GGC 3′). This reverse primer also includes a stretch of residues homologous to the γ_2 subunit. Another PCR fragment was generated using the γ_2 cDNA as a template, with the forward primer (5′ GCC TTC AGG AAG AAG CGG AAG ACA ACG CCA CAA C 3′) and a reverse primer that includes a stop codon and a *Bam*HI site (γ_2 reverse, 5′ CGG GAT CCC GTC ATA CGG GCG TGG TCC GGC G 3′). The products from the two PCRs were mixed and reamplified using the γ_1 forward and γ_2 reverse primers. The resulting product was subcloned into the adenovirus shuttle vector into the *Hin*dIII and *Bam*HI sites. The final construct was sequenced to verify integrity. This construct encodes a protein that includes the N terminus to aa 133 of γ_1 and aa 129 through the C terminus of the γ_2 subunit. This protein is detected by an antibody to the γ_2 subunit.

γ_2/γ_1 Chimera—A fragment of the γ_2 cDNA was amplified using the forward primer that includes a *Hin*dIII site and the start codon (γ_2 forward, 5′ CCC AAG CTT CCA CCA TGG GGC TGT TTG ATC GAG 3′) and the reverse primer (5′ CGG CCG CAG CAG GTA ATC GTA GAA CTC GCT CGC CGC 3′). A fragment of the γ_1 cDNA was amplified using the forward primer (5′ GCG AGC GAG TTC TAC GAT TAC CTG CTG CGG CCG 3′) and a reverse primer that includes a stop codon and a *Bam*HI site (γ_1 reverse, 5′ CGT GGA TCC CGT TAA TGC TCG GGT TCG GC 3′). The products from the two PCRs were mixed and reamplified with the γ_2 forward and γ_1 reverse primers. The resulting product was subcloned into the adenovirus shuttle vector into the *Hin*dIII and *Bam*HI sites. The final construct was sequenced to verify the integrity. This construct encodes a protein that includes the N terminus to aa 128 followed by aa 134 through the C terminus of the γ_1 subunit. This protein is detected by an antibody to the γ_1 subunit.

$\gamma_1$sspnEGFP Chimera—This construct was generated by standard PCR techniques to replace the first extracellular loop of the γ_1EGFP with the first extracellular loop of the unrelated four-transmembrane protein sarcospan (24). The first extracellular loop of sarcospan includes the six amino acids RTDPFW. The protein can be detected by an antibody to GFP.

$\alpha_1$1.1—This construct has been described previously (25).

Injection of Adenoviruses into Skeletal Muscle of the γ_1 Null Mice—10 μl of primary particles or diluted secondary particles ($\sim 10^{11}$ particles/ml) of the adenovirus were injected into the tibialis anterior and the quadriceps muscles of 2–5-day-old γ_1 null pups as described previously (26). 3 to 5 weeks later, the mice were sacrificed and the injected muscles collected. The tissue was processed for immunohistochemistry or biochemical analysis as described below.

Sucrose Gradient Fractionation—Wheat germ agglutinin-enriched material or KCl microsomes that were solubilized as described in the methods for the purification of the channel complex were concentrated and loaded on 5–30% linear sucrose gradients with 0.1% digitonin and 0.5 M NaCl. The gradients were centrifuged for 90 min at 50,000 rpm in a 65.2 Ti rotor (Beckman Instruments). 800-μl fractions were collected from the top from each tube.

SDS-PAGE and Immunoblotting—Samples were subjected to SDS-PAGE on gradient gels and transferred to polyvinylidene difluoride membranes for immunoblotting. The membranes were blocked and incubated with the primary antibody, followed by secondary antibody and detected by enhanced chemiluminescence.

Antibodies—The antibodies used in this study have been described previously, IID5E1, IIF7 ($\alpha_1$1.1 (26)), sheep 6 ($\alpha_1$1.1 and β_{1a} (27)), guinea pig 1 ($\alpha_2\delta$), guinea pig 11/15/16/77 (γ_1 (28)), rabbit 239 (γ_2/γ_3 (13)), and AP83 (β-dystroglycan (29)).

Immunofluorescence Analysis—Tissue samples (quadriceps) were frozen and processed for immunohistochemistry as described previously (26). The sections were labeled with a rabbit polyclonal antibody to β-dystroglycan (AP83). To visualize the $\alpha_1$1.1 subunit, the sections were labeled with the monoclonal antibody to the $\alpha_1$1.1 subunit (IIID5E1). To minimize the background, the sections were first treated with the Fab fragment using the conditions described by the manufacturer (The Jackson Laboratories). Cy3-conjugated secondary antibodies were used to detect the primary antibodies. EGFP fluorescence was used to detect the γ subunits. Immunolabeled sections were visualized with the ×60 objective using confocal microscopy (Bio-Rad). The *bar* represents 10 μm.

Primary Cultures and cDNA Transfection—Primary cultures were prepared from enzyme-digested hind limbs of late-gestation (E18) γ1 null embryos. Myoblasts were isolated by enzymatic digestion with 0.125% (w/v) trypsin and 0.05% (w/v) pancreatin. After centrifugation, mononucleated cells were resuspended in plating medium containing

78% Dulbecco's modified Eagle's medium with low glucose, 10% horse serum, 10% fetal bovine serum, and 2% chick embryo extract. Cells were then plated at a density of ~1 × 10^4 cells per dish on gelatin-coated plastic culture dishes (BD Biosciences). Cultures were grown at 37 °C in 8% CO_2, and after the fusion of myoblasts (5–7 days), the medium was replaced with fetal bovine serum-free medium, and CO_2 was decreased to 5%. In the case of cDNA expression, γ subunit cDNAs of interest and a separate plasmid encoding the T-cell membrane antigen CD8 were mixed at a 1:1 weight ratio and cotransfected with the polyamine LT-1 (Panvera, WI). CD8-transfected cells were identified by incubation with anti-CD8 antibody beads (Dynal, Norway).

Whole-cell Voltage Clamp and Solutions—Cells were voltage-clamped ~48 h after transfection. Transfected cells revealed by CD8 beads were voltage-clamped with an Axopatch 200B amplifier (Axon Instruments, CA) and a Digidata 1200 (Axon) pulse generator and digitizer. Linear capacitance, leak currents, and effective series resistance were compensated with the amplifier circuit. The voltage dependence of the Ca^{2+} conductance was fitted according to a Boltzmann distribution, $A = A_{max}/(1 + \exp(-(V - V_{½})/k))$. Where A_{max} is G_{max}; $V_{½}$ is the potential at which $A = A_{max}/2$; and k is the slope factor. The external solution in all cases was (in mM) 130 triethanolamine methanesulfonate, 10 $CaCl_2$, 1 $MgCl_2$, 0.001 tetrodotoxin (Sigma), and 10 HEPES titrated with triethanolamine(OH) to pH 7.4. The pipette solution was (in mM) 140 Cs^+-aspartate, 5 $MgCl_2$, 5 EGTA, 10 MOPS-CsOH, pH 7.2.

Analysis of mdg and Wild Type Muscle—Skeletal muscle from *mdg* pups or 1- to 2-day-old wild type mice were obtained, and KCl-washed microsomes were prepared as described above. The skeletal muscle calcium channel was enriched using a wheat germ agglutinin column after solubilization as described above. The enriched material was subjected to sucrose gradient fractionation as described, and the fractions were analyzed by SDS-PAGE and immunoblotting.

Transient Transfection in tsA201 Cells and Immunoprecipitation—tsA201 cells were transfected using the calcium phosphate method. Cells were lysed in buffer containing 50 mM Tris, 1% digitonin, 0.5 M NaCl, and protease inhibitors, and the solubilized material was isolated by centrifugation. The solubilized material was immunoprecipitated with beads to which the antibody to the $\alpha_1$1.1 subunit (IIF7) was coupled. The beads were then thoroughly washed in a buffer containing 0.1% digitonin, 0.5 M NaCl, 50 mM Tris, and two protease inhibitors (benzamidine and phenylmethylsulfonyl fluoride). The bound material was eluted using SDS loading buffer and immunoblotted. Aliquots of the cell lysate were also immunoblotted to examine the expression of the proteins.

Tunicamycin Treatment—TsA201 cells were transfected as described above. Tunicamycin (10 μg/ml) was then added to the medium, the cells were incubated for 2 days and immunoprecipitated as described above.

RESULTS

Skeletal Muscle L-type Calcium Channel Complex Is Maintained in γ_1 Null Mice—The generation of γ_1 null mice using conventional gene targeting strategy has been described previously (21). The subunits of the calcium channel are expressed at normal levels in these mice. This, in conjunction with the expression of robust voltage-gated calcium currents in the skeletal muscle (21, 22), led us to examine if the calcium channel subunits are maintained as a complex. The calcium channel was purified from the wild type and γ_1 null mice as described under "Materials and Methods." The presence of all the subunits in the complex was verified by Western blotting with antibodies specific to each subunit of the channel (Fig. 1). The γ_1 subunit can be detected in the complex from the wild type mice but not in the γ_1 null mice. The $\alpha_1$1.1, $\alpha_2\delta$, and β_{1a} subunits can be detected in the purified material from both the wild type and the γ_1 null mice, demonstrating that the residual calcium channel is maintained as a complex in the γ_1 null mice.

γ_1 Can Be Stably Incorporated into the L-type Calcium Channels of γ_1 Null Mice—The γ_1 subunit was reintroduced into the skeletal muscle of the γ_1 null mice, via recombinant adenoviruses, to determine whether it could be incorporated into the calcium channel of the γ_1 null mice. Expression of the protein was examined by immunoblot analysis (Fig. 2*A*). The muscle from the wild type mice showed expression of the γ_1 protein, whereas the γ_1 null mice did not express any γ_1 protein. Robust expression of γ_1 recombinant protein was detected in the muscle of the mice injected with the recombinant adenovirus, confirming that the adenovirus-mediated expression may be successfully used for the expression of this subunit in the skeletal muscle.

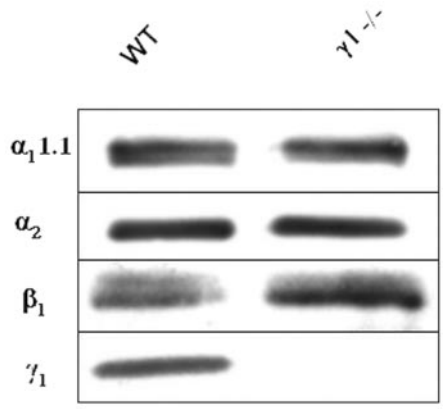

FIG. 1. **The skeletal muscle L-type calcium channel is maintained as a complex in the γ_1 null mice.** Purified calcium channels from the wild type (*WT*) and γ_1 null mice were immunoblotted to detect the presence of the subunits. The $\alpha_1$1.1, α_2, and β_{1a} subunits are detected in both the wild type and the γ_1 null mice, whereas γ_1 is only detected in the wild type mice, indicating that the channel is maintained as a complex in γ_1 null mice.

To examine if the virally expressed γ_1 is incorporated into the residual calcium channel complex of γ_1 null mice, a WGA-enriched preparation containing highly enriched material was examined for the presence of the γ_1. γ_1 is enriched by the WGA column, as is the rest of the channel as indicted by the presence of the $\alpha_2\delta$ subunit in the elution. This suggests that γ_1 may be incorporated into the channel complex (Fig. 2*B*). To confirm this further, the enriched material was subjected to sucrose gradient fractionation and immunoblot analysis (Fig. 2*C*). Our results clearly demonstrate that γ_1 cofractionates with the other subunits of the calcium channel, indicating that indeed it is stably incorporated into the calcium channel complex of γ_1 null mice. A fraction of γ_1 is not associated with the channel. It is possible that this pool represents free γ_1 that is not complexed with the channel due to overexpression of the protein, because it is not observed in similar preparations from older wild type skeletal muscle (data not shown).

To examine the localization of γ_1, a construct that encodes γ_1 with an EGFP at the C terminus was engineered. Expression of this protein in mammalian cells revealed that γ_1EGFP traffics to the plasma membrane, even in the absence of the other subunits of the calcium channel (data not shown). By using adenovirus-mediated expression, the EGFP tagged constructs were introduced in the muscle of the γ_1 null mice. Interestingly, the protein is also localized to the sarcolemma (Fig. 2*D*), as indicated by its colocalization with β-dystroglycan, a marker for the sarcolemma (30) (Fig. 2*D*). In addition, γ_1EGFP is localized to the t-tubules (Fig. 2, *D* and *E*), as confirmed by its colocalization with $\alpha_1$1.1 (Fig. 2*E*).

γ_2 Does Not Incorporate into the Skeletal L-type Calcium Channels of γ_1 Null Mice—The ability of γ_2 to incorporate into the calcium channel of γ_1 null mice was then examined using a similar approach. The adenovirus encoding γ_2 allows robust expression of the protein in the skeletal muscle of γ_1 null mice. There is no detectable endogenous protein in the skeletal muscle of either wild type or γ_1 null mice (Fig. 3*A*) as reported previously (13).

WGA enrichment of the channel complex indicates that, unlike γ_1, γ_2 is in the void, indicating that that γ_2 may not be

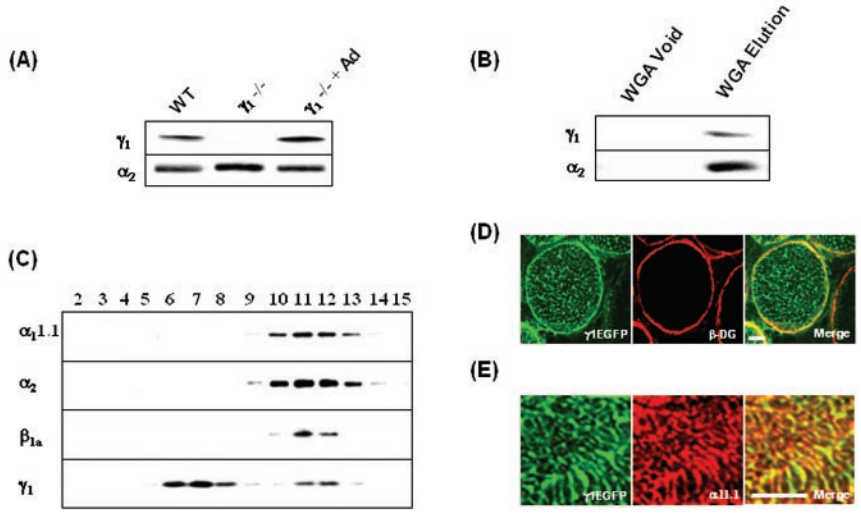

FIG. 2. **Incorporation of an adenovirally expressed γ_1 into skeletal L-type calcium channel of γ_1 null mice.** *A*, adenovirally expressed γ_1 in γ_1 null skeletal muscle was detected by immunoblot analysis. The blot was reprobed with an antibody to α_2. *WT*, wild type. *B*, calcium channels from solubilized microsomes were enriched using WGA chromatography and immunoblotted. The channel is enriched by WGA as indicated by the presence of α_2 in the WGA elution. γ_1 is also enriched by the WGA, suggesting association with the calcium channel. *C*, WGA-enriched material was subjected to linear sucrose gradient fractionation and immunoblot analysis. $\alpha_1$1.1, α_2, and β_{1a} subunits comigrate on the sucrose gradient. γ_1 migrates in the same fractions, confirming incorporation into the calcium channels of the γ_1 null mice. *D*, muscle sections from γ_1EGFP injected muscle were double labeled with an antibody to the sarcolemma protein, β-dystroglycan (β *DG*). *E,* muscle sections were double-labeled with an antibody to $\alpha_1$1.1. γ_1EGFP is localized to the t-tubules and colocalizes with $\alpha_1$1.1.

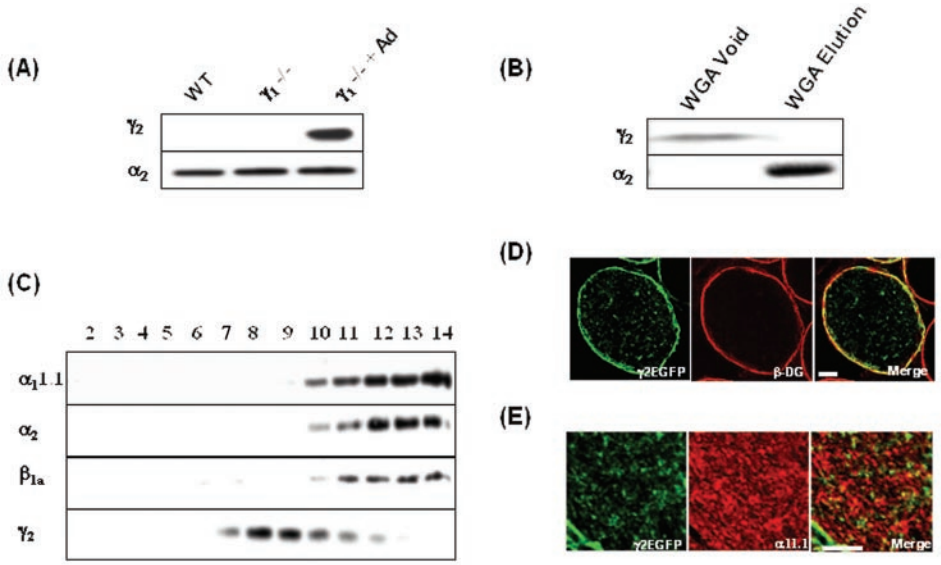

FIG. 3. **γ_2 subunit is not incorporated into the skeletal L-type calcium channel of γ_1 null mice.** *A*, adenovirally expressed γ_2 in γ_1 null skeletal muscle was detected by immunoblot analysis. The blot was reprobed with an antibody to α_2. *WT*, wild type. *B*, microsomes from the γ_1 null mice expressing γ_2 were solubilized, and the channel was enriched using WGA chromatography. The calcium channel is enriched by the WGA as indicated by the presence of the α_2 subunit in the WGA elution. The γ_2 subunit is not enriched by the WGA. *C*, solubilized microsomes were subjected to linear sucrose gradient fractionation and immunoblot analysis. $\alpha_1$1.1, α_2, and β_{1a} subunits comigrate on the sucrose gradient. γ_2 subunit does not comigrate with the calcium channel subunits, confirming that it is not incorporated into the channel of γ_1 null mice. *D*, muscle sections from γ_2EGFP-injected muscle were double-labeled with an antibody to the sarcolemma protein, β-dystroglycan (β *DG*). γ_2EGFP localizes to the sarcolemma. *E*, muscle sections were double-labeled with an antibody to $\alpha_1$1.1. γ_2EGFP is not localized to the t-tubules and shows a punctate pattern. γ_2EGFP does not colocalize with $\alpha_1$1.1.

incorporated into the complex (Fig. 3*B*). To ascertain if the γ_2 subunit is weakly associated with the complex and the steps leading to the WGA chromatography disrupt this association, sucrose gradient fractionation of solubilized microsomes from γ_2 injected mice was examined for the presence of γ_2 and the other subunits of the channel (Fig. 3*C*). These results demonstrate that γ_2 does not comigrate in the same fractions as the rest of the channel complex confirming that it is not incorporated into the channel complex in γ_1 null mice.

Similar to γ_1EGFP, γ_2EGFP traffics to the plasma membrane in transiently transfected mammalian cells (data not shown). Consistent with the biochemical data indicating the absence of an association of γ_2 with the calcium channel, the adenovirally expressed γ_2EGFP in the skeletal muscle of the γ_1 null mice does not demonstrate an organized t-tubule expression pattern (Fig. 3*D*). Interestingly, similar to that observed with the γ_1 subunit, the protein is also localized to the sarcolemma (Fig. 3*D*) and colocalizes with β-dystroglycan, a marker for the sarcolemma (Fig. 3*E*). In contrast to γ_1, γ_2 appears in punctate clusters within some muscle fibers (Fig. 3*E*). In other fibers, a more diffused pattern is observed (data not shown). However, the protein is not localized to the t-tubules as con-

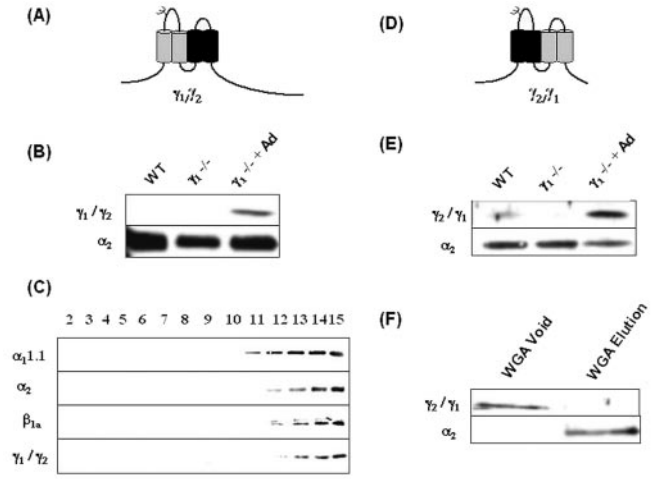

FIG. 4. **The first half of the γ_1 subunit interacts with the L-type calcium channels.** *A*, schematic of the γ_1/γ_2 chimera. The protein encodes the first half of the γ_1 subunit, including the N terminus, first extracellular loop, and first two transmembrane domains, fused to the second half of the γ_2 subunit. *B*, adenovirally expressed γ_1/γ_2 in γ_1 null skeletal muscle was detected by immunoblot analysis. The blot was reprobed with an antibody to the α_2 subunit. *WT*, wild type. *C*, solubilized microsomes were subjected to sucrose gradient fractionation and immunoblot analysis for subunits of the calcium channel. The γ_1/γ_2 comigrates with the subunits of the calcium channel, indicating that it is incorporated. *D*, schematic of the γ_2/γ_1 chimera. The protein encoded includes the first half of the γ_2 subunit followed by the second half of the γ_1 subunit. *E*, adenovirally expressed γ_2/γ_1 in γ_1 null skeletal muscle was detected by immunoblot analysis. The blot was reprobed with an antibody to the α_2 subunit. *F*, microsomes from γ_1 null mice expressing γ_2/γ_1 were solubilized, and the channels were enriched using WGA. The γ_2/γ_1 chimera is in the void of the WGA, whereas the channel is in the WGA elution as indicated by the presence of the α_2, indicating that the γ_2/γ_1 chimera is not incorporated.

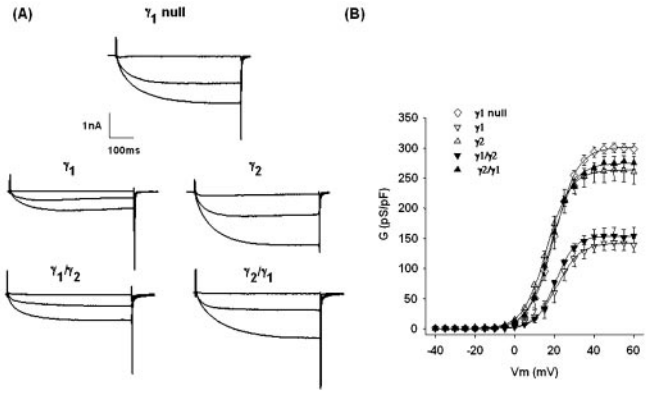

FIG. 5. **Electrophysiological characterization of the γ subunits in γ_1 null myotubes.** *A*, whole-cell calcium currents when γ_1 null or γ_1 null myotubes transfected with the indicated constructs are depolarized to 0, +20, or +40 mV from a holding potential of −40 mV. The pulse duration was 500 ms. *B*, voltage dependence of calcium conductance for γ_1 null myotubes or γ_1 null myotubes transfected with the indicated constructs. The curves correspond to a Boltzmann fit of the population mean with the parameters G_{max} (pS/pF), $V_{½}$ (mV), and k (mV), respectively, of 301.7, 19.8, and 6.1 for γ_1 null; 142.2, 21.4, and 5.8 for γ_1; 263.3, 16.1, and 5.9 for γ_2; 153.6, 20.5, and 4.8 for γ_1/γ_2 chimera; and 274.9, 18.2, and 5.9 for γ_2/γ_1.

firmed by the absence of colocalization with the $\alpha_1$1.1 subunit (Fig. 3*E*).

Chimeras of γ_1 and γ_2 Define Interaction Domain of γ_1 with the Calcium Channel—The γ subunits possess a similar four-transmembrane domain structure with intracellular N and C termini. The first extracellular loops of the γ subunits have charged regions that are conserved across the γ subunits (12). We therefore hypothesized that the first half of the γ_1 subunit, including the first two transmembrane domains, might mediate the interaction of the protein with the channel, placing the first loop in close proximity to the channel.

To test this hypothesis, we took advantage of the ability of the γ_1 subunit, but not the γ_2 subunit, to incorporate into the calcium channel. Chimeric proteins containing the first two transmembrane domains of the γ_1 subunit and the last two domains of the γ_2 subunit were generated (Fig. 4*A*). The ability of this protein to incorporate into the channel was examined by techniques similar to those described for the γ_1 and γ_2 subunits. The chimeric protein can be detected in microsomes from the injected muscle (Fig. 4*B*).

To examine incorporation of the chimeric γ_1/γ_2 protein into the calcium channel, the microsomes from such a preparation were solubilized and subjected to sucrose gradient fractionation and immunoblotting. Our data show that the γ_1/γ_2 chimera comigrates in the same fractions as the other subunits of the channel, confirming that it is indeed incorporated into the channel complex (Fig. 4*C*). Similar to that observed for the γ_1 studies described above, in some experiments a pool of the γ_1/γ_2 chimera that is not associated with the channel complex is observed (data not shown), presumably as a result of overexpression.

To assess if secondary interaction sites exist on the γ_1 subunit that allow interaction with the calcium channel, we generated a construct that encodes the first two transmembrane domains of the γ_2 subunit and the last two transmembrane domains of the γ_1 (Fig. 4*D*). Similar to the other adenovirally expressed proteins, the γ_2/γ_1 chimera showed robust expression in the skeletal muscle of the γ_1 null mice (Fig. 4*E*). The γ_2/γ_1 protein is not enriched with the calcium channel as indicated by its presence in the void of the WGA column, whereas the other subunits of the calcium channel are in the WGA elution, as indicated by the presence of the $\alpha_2\delta$ subunit (Fig. 4*F*). These results clearly demonstrate that the γ_2/γ_1 chimeric protein does not incorporate into the calcium channel of the γ_1 null mice.

γ_1 and γ_1/γ_2 Chimera Restore Conductance of L-type Calcium Channels in γ_1 Null Myotubes—We have demonstrated previously (21) that the L-type voltage-gated calcium current conductance is increased in γ_1 null myotubes. To examine the functional effects of the γ subunits and the chimeras, the different constructs were introduced into the myotubes of the γ_1 null mice (Fig. 5, *A* and *B*). In agreement with the biochemical studies, re-expression of γ_1 and γ_1/γ_2 chimera reduced the calcium conductance (142 ± 12.1 and 160.5 ± 16.7 pS/pF, respectively) significantly compared with that of γ_1 null myotubes (296.1 ± 10.8 pS/pF). Consistent with the lack of incorporation of γ_2 and γ_2/γ_1, these chimeras did not alter the conductance significantly (262.6 ± 20.4 and 276.4 ± 8.2 pS/pF, respectively). In all of the cases, no effect was observed on the voltage dependence of current conductance (*G-V* curve), the voltage of half-maximal conductance, $V_{½}$, and slope factor, k values (Table I). These results demonstrate that the first half of γ_1, which mediates subunit interaction, can modulate the functional properties of skeletal L-type voltage-gated calcium channels.

γ_1 Directly Interacts with $\alpha_1$1.1—To determine the subunit of the calcium channel that interacts with the γ_1 subunit, we took advantage of the muscular dysgenesis (*mdg*) mice. The *mdg* mouse arose as a spontaneous mutation in the gene that encodes the $\alpha_1$1.1 (31) that results in the loss of any detectable protein (32). In the absence of $\alpha_1$1.1, $\alpha_2\delta$ (32, 33) and β_{1a} are still expressed. We sought to examine γ_1 subunit in these mice and determine whether, in the absence of $\alpha_1$1.1, γ_1 is associated with $\alpha_2\delta$. The WGA-enriched material from *mdg* and 1–2-day-

old wild type muscle microsomes was subjected to sucrose gradient fractionation and immunoblot analysis. In the wild type mice, the $\alpha_1$1.1, $\alpha_2\delta$, and a fraction of the γ_1 subunits comigrate on the sucrose gradient (Fig. 6*A*), confirming that they are associated in a complex. Interestingly, a large fraction of the γ_1 subunit does not appear to be in the complex and migrates in the lower fractions, and presumably this is developmentally regulated. In contrast, in the sucrose gradient fractions from the *mdg* muscle, the $\alpha_1$1.1 is not detected. However, the $\alpha_2\delta$ and the γ_1 subunits do not comigrate (Fig. 6*A*), suggesting that the presence of the $\alpha_1$1.1 subunit may be necessary for the γ_1 subunit to associate with the calcium channel.

To examine if the γ_1 subunit directly interacts with the $\alpha_1$1.1 subunit, subunit associations were examined in transiently transfected mammalian cells. TsA201 cells were transiently transfected with cDNAs encoding the $\alpha_1$1.1 subunit, the $\alpha_1$1.1 subunit, and the γ_1EGFP or the γ_1EGFP subunit alone. Cell lysates were subjected to immunoprecipitation using an antibody to the $\alpha_1$1.1 subunit. Aliquots of the lysate and the immunoprecipitated material were examined for the presence of the $\alpha_1$1.1 and γ_1EGFP proteins (Fig. 6*B*). Our results show that in the cells transfected with the $\alpha_1$1.1 subunit, the $\alpha_1$1.1 subunit is immunoprecipitated, but the EGFP antibody does not detect any endogenous protein. Coexpression of $\alpha_1$1.1 and γ_1EGFP allows coimmunoprecipitation, indicating complex formation. The γ_1EGFP is not immunoprecipitated in the absence of the $\alpha_1$1.1 subunit, indicating specificity of interaction. These results confirm that the $\alpha_1$1.1 and the γ_1 directly associate in the absence of the other subunits of the voltage-gated calcium channel.

Role of the First Extracellular Loop of γ_1 in Subunit Interaction—Our studies demonstrate that the first half of the γ_1 subunit interacts with the calcium channel complex via the α_1 subunit. To examine the role of the first extracellular loop of the γ_1 subunit in subunit interaction, a chimeric subunit was generated. This chimeric subunit (Fig. 7*A*) has the first extracellular loop of the γ_1 subunit replaced by an extracellular loop of the unrelated four-transmembrane protein sarcospan (24) and EGFP at the C terminus. Transient transfection of tsA201 cells with the $\alpha_1$1.1 and $\gamma_1$sspn and immunoprecipitation demonstrated that the two proteins associate (Fig. 7*B*). These results further support a role for the first two transmembrane domains of the γ_1 subunit in subunit interaction.

The γ_1 subunit has an *N*-linked glycosylation site in the first extracellular loop and is glycosylated *in vivo*. To examine the role of this glycosylation in the interaction of the γ_1 subunit with the $\alpha_1$1.1 subunit, cells transiently transfected with the $\alpha_1$1.1 and γ_1EGFP constructs, as described above, were treated with the aminoglycoside antibiotic tunicamycin, an inhibitor of *N*-linked glycosylation. Examination of cell lysates from treated and untreated cells indicated that the γ_1EGFP existed in a smaller molecular weight form in tunicamycin-treated cells, indicating an absence of glycosylation (Fig. 7*C*). Furthermore, the γ_1EGFP could be immunoprecipitated by an antibody to the $\alpha_1$1.1 protein, indicating that the lack of glycosylation does not inhibit the association of the $\alpha_1$1.1 subunit with the γ_1 subunit (Fig. 7*D*).

TABLE I
Ca^{2+} conductance of the γ_1 null cells expressing γ_1, γ_2, γ_1/γ_2, and γ_2/γ_1 subunits

Entries correspond to mean ± S.E. of Boltzmann parameters fitted to each cell. The number of cells is in parentheses.

	G-V		
	G_{max} (pS/pF)	$V_{1/2}$ (mV)	*K* (mV)
γ_1 null	296.1 ± 10.8 (7)	23.9 ± 1.2	5.5 ± 0.7
γ_1	142 ± 12.1[a] (7)	22.2 ± 2.8	5.2 ± 0.5
γ_2	262.6 ± 20.4 (6)	20.7 ± 1.4	5.2 ± 0.5
γ_1/γ_2	160.5 ± 16.7[a] (6)	20.6 ± 1.8	4.3 ± 0.7
γ_2/γ_1	276.4 ± 8.2 (8)	22.1 ± 2.2	5.6 ± 0.3

[a] Data are compared with non-transfected cells with a *t* test significance of $p < 0.0001$.

DISCUSSION

The γ subunits of the voltage-gated calcium channels are the least characterized subunits of the calcium channels. In this study, we have dissected subunit interactions and domains mediating subunit interaction of the γ_1 subunit with the other components of the skeletal L-type voltage-gated calcium channel. Moreover, we provide evidence for restricted subunit heterogeneity of the γ subunits. The voltage-gated calcium channels have a similar structure and subunit interactions, hence these studies have broad implications for the interactions of the γ subunits with the voltage-gated calcium channels (Fig. 8).

The γ subunit was originally identified in skeletal muscle (12). In the recent past, a number of γ subunits that are expressed in a variety of tissues have been cloned (13–16), and their functional roles are only beginning to be revealed. In general, the γ subunits appear to be inhibitory (7, 22, 30, 34). The important physiological role for the γ subunit is emphasized by the epileptic phenotype in the stargazer mouse, a spontaneous mutant that lacks the γ_2 subunit (13).

It is well known that the γ_1 subunit modulates the inactivation kinetics and the current amplitude of the skeletal muscle

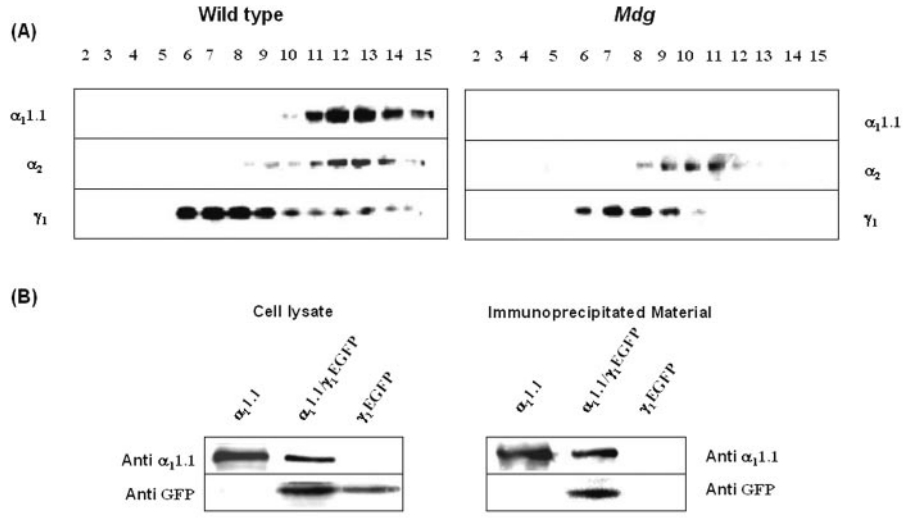

FIG. 6. **γ_1 interacts with the $\alpha_1$1.1.** *A*, enriched skeletal muscle calcium channels from muscular dysgenesis (*mdg*) and 1–2-day-old wild type mice were subjected to sucrose gradient fractionation and immunoblot analysis. In the wild type mice, α_2 and γ_1 comigrate, indicating the presence of an intact channel complex. In the *mdg* mice, γ_1 and α_2 do not comigrate, indicating that $\alpha_1$1.1 may be required for γ_1 to be associated with the channel. *B*, tsA201 cells were transiently transfected with the indicated constructs. Cell lysates were immunoprecipitated with an antibody to $\alpha_1$1.1. Aliquots of the lysate and the immunoprecipitated material were examined for the presence of the $\alpha_1$1.1 and the γ_1EGFP. The $\alpha_1$1.1 immunoprecipitates with the γ_1EGFP.

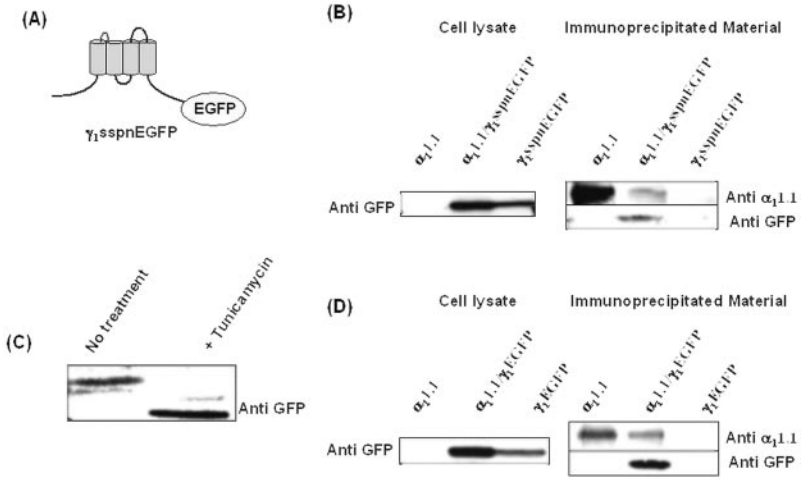

FIG. 7. **Role of the first extracellular loop in subunit interaction.** *A*, schematic of the $\gamma_1$sspnEGFP chimera. The first extracellular loop of γ_1 is replaced by an extracellular loop of the unrelated protein sarcospan. *B*, tsA201 cells were transiently transfected with the indicated constructs. Cell lysates were immunoprecipitated with an antibody to $\alpha_1$1.1. Aliquots of lysate and immunoprecipitated material were examined for the presence of the $\alpha_1$1.1 and the $\gamma_1$sspnEGFP. The $\alpha_1$1.1 immunoprecipitates with the $\gamma_1$sspnEGFP. *C*, cell lysates from cells transfected with the γ_1EGFP constructs and either treated with tunicamycin or untreated were immunoblotted with an antibody to GFP. Tunicamycin treatment inhibited *N*-linked glycosylation of γ_1EGFP. *D*, tsA201 cells were transiently transfected with the indicated constructs and treated with tunicamycin. Cell lysates were immunoprecipitated with an antibody to $\alpha_1$1.1. Aliquots of the lysate and the immunoprecipitated material were examined for the presence of the $\alpha_1$1.1 and the γ_1EGFP. The $\alpha_1$1.1 immunoprecipitates with the γ_1EGFP in the absence of *N*-linked glycosylation.

voltage-gated calcium channels (21, 22). The γ_1 subunit is not, however, required for maintaining the integrity of the channel complex or its targeting to the t-tubules. This is in sharp contrast to the distribution of the subunits in *mdg* mice and β_1 null mice. $\alpha_2\delta$ shows a punctate or diffused pattern in *mdg* mice (33), whereas $\alpha_1$1.1 is reduced or absent in β_1 null mice (10). Recent studies have indicated that, unlike β_1 (10), γ_1 does not have a major role in membrane trafficking of the $\alpha_1$1.1 subunit as assessed by gating current measurements (21) or in EC coupling (35). Our results clearly demonstrate that the first half of the γ_1 subunit that allows subunit interaction also allows the restoration of L-type currents in γ_1 null muscle. Interestingly, despite the differences in L-type currents in the wild type and γ_1 null mice, we have not observed any gross morphological changes in the muscle. Taken together, these data suggest that, at least in skeletal muscle, γ_1 is predominantly involved in modulating the biophysical properties of the channel.

Subunit interactions are conserved across the subunits of the voltage-gated calcium channels as indicated by the presence of highly conserved interaction sites on the β subunit (19) and *in vivo* and *in vitro* subunit heterogeneity (17, 18). We have identified the interaction site of the γ_1 with the calcium channel and predict that other γ subunits interact with α_1 subunits of the calcium channels similarly. Our studies indicate that the γ subunits may not be as capable of functional heterogeneity as β subunits, thereby restricting the number of possible subunit associations of the γ subunits with the voltage-gated calcium channels. However, the γ subunits have diverging homologies, with the γ_6 subunit being the phylogenetically most closely related subunit to the γ_1 subunit (15, 16). Hence, it is possible that the γ subunits might possess the ability to form heterogeneous complexes, albeit to a relatively limited extent as compared with the β subunits. This is also suggested by cell expression studies indicating the ability of the γ_1 subunit to associate with the $\alpha_1$1.2 subunit (36), although it is associated with the $\alpha_1$1.1 in native tissue. The γ_1 null mouse offers a good model system to test the ability of the other γ subunits to form heterogeneous complexes in an *in vivo* environment.

The t-tubule is a specialized organized structure continuous with the sarcolemma in the skeletal muscle. There is increasing evidence that the targeting of proteins to these structures involves determinants that are different from targeting to the sarcolemma (37). We have demonstrated the localization of the γ_1 subunit in the t-tubules by microscopy. To our knowledge, this is the first report of localization of the γ_1 subunit in the skeletal muscle. Interestingly, the γ_1 subunit is localized to both the sarcolemma and the t-tubules, unlike the other subunits of the calcium channel, which are predominantly localized in the t-tubules (4). Whether this is an effect of overexpression of the protein is unclear. However, the localization of the protein at the plasma membrane in transfected mammalian cells suggests that the γ_1 subunit contains the determinants for plasma membrane localization. In the future, it would be interesting to determine whether the ability of the γ_1 subunit to localize to the t-tubules of the skeletal muscle, in addition to the plasma membrane/sarcolemma, is a result of its

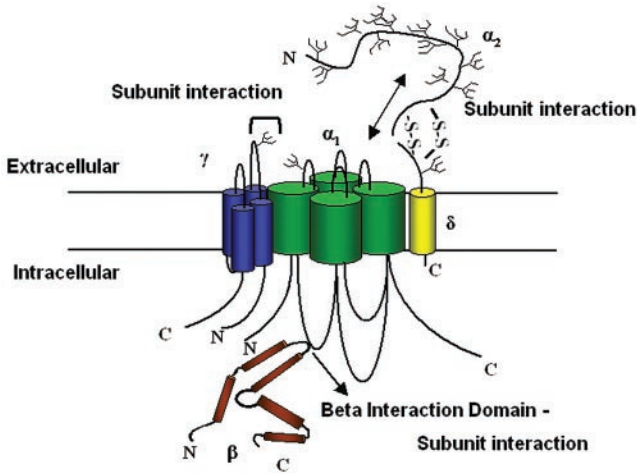

FIG. 8. **Model of subunit interactions of the high voltage-gated calcium channels.** The γ subunit is depicted as a four-transmembrane domain protein with predicted intracellular N and C termini. The first half of the γ subunit interacts with the α_1 subunit. The first extracellular loop contains charged residues and glycosylation sites. The interaction sites of the $\alpha_2\delta$ (Gurnett *et al.* (9)) and the β subunit (Beta Interaction Domain-Pragnell *et al.* (19)) with the α_1 subunit have been defined previously.

association with the subunits of the calcium channel or whether the protein has its own signals, like the $\alpha_1$1.1 subunit (38), to target it to the t-tubules, where it can then associate with the calcium channel complex.

We demonstrate that the first half of the γ_1 mediates the interaction of the subunit with the rest of the calcium channel. This subunit interaction also allows the restoration of L-type calcium conductance in γ_1 null myotubes. The absence of *N*-linked glycosylation of the γ_1 subunit does not prevent the protein from associating with the $\alpha_1$1.1 subunit, suggesting that the *N*-linked glycosylation is not the predominant mediator of the interaction between the $\alpha_1$1.1 and γ_1 subunits. Interestingly, the first extracellular loop of the γ_1 subunit is relatively negatively charged (12) and has been suggested to be important in mediating the biophysical properties of the γ_1 subunit (22). We demonstrate that the first extracellular loop of the γ_1 subunit is not required for subunit interaction. Because the first half of the γ_1 subunit interacts with the calcium channel, presumably via the transmembrane domains, the first extracellular loop would be predicted to be in close proximity to the $\alpha_1$1.1 subunit.

The subunit interactions of the γ subunits with the calcium channels have been contradictory, with suggestions of a requirement (7) or lack of requirement (36, 39) of the $\alpha_2\delta$ subunit in the association of the γ subunit with the voltage-gated calcium channel. The studies on the *mdg* mice indicate that the $\alpha_2\delta$ and γ_1 subunits are not associated, thereby precluding a direct interaction between the two subunits. Interestingly, in the 1–2-day-old mice, there is a pool of γ_1 subunit that is not associated with the calcium channel. The exact significance of this is unclear. It is possible that during development, there is an excess of the protein generated, and later γ_1 that is not incorporated into the calcium channel is degraded. Alternatively, it is possible that during the early stages of muscle development, γ_1 is associated with other proteins in addition to the calcium channel and involved in other unknown functions. Further studies are necessary to clarify the role of the γ_1 subunits during early development. Our cell expression studies indicate that the γ_1 subunit directly interacts with the α_1 subunit. This is consistent with studies that demonstrate a direct effect of the γ_6 subunit on the $\alpha_1$3.1 protein in the absence of the other known calcium channel subunits (40). It is therefore likely that the requirement of the $\alpha_2\delta$ subunit to mediate some of the electrophysiological properties contributed by the γ subunit reflect conformational changes rather than direct subunit interaction.

These studies provide valuable insights into the γ subunit interactions within the voltage-gated calcium channels. The past few years have seen the discovery of a number of different γ subunits and the confirmation of the four-subunit composition of the voltage-gated calcium channels. Understanding the subunit interactions of the γ subunits is an important step toward understanding the structural and functional subunit interactions within the voltage-gated calcium channels.

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