Voltage-gated calcium channels mediate excitation-contraction coupling in the skeletal muscle. Their molecular composition, similar to neuronal channels, includes the pore-forming $\alpha_1$ and auxiliary $\alpha_2\delta$, $\beta$, and $\gamma$ subunits. The $\gamma$ subunits are the least characterized, and their subunit interactions are unclear. The physiological importance of the neuronal $\gamma$ is emphasized by epileptic stargazer mice that lack $\gamma_2$. In this study, we examined the molecular basis of interaction between skeletal $\gamma_1$ and the calcium channel. Our data show that the $\alpha_1.1$, $\beta_1$, and $\alpha_2\delta$ subunits are still associated in $\gamma_1$ null mice. Reexpression of $\gamma_1$ and $\gamma_2$ showed that $\gamma_1$ but not $\gamma_2$ incorporates into $\gamma_1$ null channels. By using chimeric constructs, we demonstrate that the first half of the $\gamma_1$ subunit, including the first two transmembrane domains, is important for subunit interaction. Interestingly, this chimera also restores calcium conductance in $\gamma_1$ null myotubes, indicating that the domain mediates both subunit interaction and current modulation. To determine the subunit of the channel that interacts with $\gamma_1$, we examined the channel in muscular dysgenesis mice. Cosegregation experiments showed that $\gamma_1$ and $\alpha_2\delta$ are not associated. Moreover, $\alpha_1.1$ and $\gamma_1$ subunits form a complex in transiently transfected cells, indicating direct interaction between the $\gamma_1$ and $\alpha_1.1$ subunits. Our data demonstrate that the first half of $\gamma_1$ subunit is required for association with the channel through $\alpha_1.1$. Because subunit interactions are conserved, these studies have broad implications for $\gamma$ 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) 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 $\alpha_1.1$ subunit and auxiliary $\alpha_2\delta$, $\beta_1$, and $\gamma_1$ 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 $\beta$ subunits enhance membrane trafficking of the $\alpha_1.1$ subunit and modulate the voltage-dependent kinetics of the channel (9). In addition to the role of the $\beta_1$ 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 $\beta_1$ subunit (10). The subunit interactions of the $\alpha_2\delta$ and $\beta$ subunits have been relatively well defined.

The $\gamma_1$ subunit is an auxiliary subunit with four predicted transmembrane domains and intracellular N and C termini. The role of the $\gamma$ subunit in the membrane trafficking of the channels and subunit interaction remain unclear. The $\gamma_1$ subunit was the only $\gamma$ subunit that was originally known and is associated with the skeletal muscle voltage-gated calcium channel (11, 12). However, the identification of a neuronal $\gamma_2$ subunit (13) renewed interest in the $\gamma$ subunits and led to the identification of a number of $\gamma$ subunits (14–16).

The $\beta$ subunits of the voltage-gated calcium channels are known to be capable of forming heterogeneous complexes both in vivo (17, 18) and in vitro. The $\beta$ subunits and the $\alpha_1$ subunit possess highly conserved interaction regions (19) allowing the formation of heterogeneous channel complexes. The $\alpha_2\delta$ subunits modulate different $\alpha_1$ 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.

$\gamma$ subunits are relatively less well understood. Subunit interactions of $\gamma$ 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 the $\gamma_1$ null mice. Mice that lack $\gamma_1$ 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 $\gamma_1$ null mice. The lack of the $\gamma_1$ subunit also does not appear to diminish the
ability of the other subunits to assemble together and conduct voltage-gated calcium currents. However, the absence of the γ1 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 γ1 null mice. We hypothesized that if the channel were still maintained as a complex in the γ1 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 the L-type L-type subunits were still maintained as a complex in the γ1 null mice. To examine the ability of the γ subunits to form heterogeneous complexes, the γ1 and γ2 subunits were introduced into the skeletal muscle of the γ1 null mice via adeno-virus-mediated expression, and their ability to incorporate into the channel was examined. We demonstrate that the γ1 does incorporate, but the γ2 does not. Consistent with these results, γ1EGFP localizes to the t-tubules, whereas the γ2EGFP does not demonstrate organized t-tubule localization. Furthermore, by using a γ1/γ2 chimeric strategy, we demonstrate that the first half of the γ1 subunit mediates its interaction with the calcium channel. Examination of the electrophysiological characteristics of the chimeric subunits in γ1 null myotubes showed that the first half of the protein is sufficient to restore calcium conductance of L-type currents in γ1 null myotubes.

To examine the subunit of the channel with which the γ1 subunit associates, we examined the channel in the muscular dysgenesis (mdg) mice. In the absence of α1.1 in the mdg mice, γ1 does not associate with α2δ, indicating that α1.1 is necessary for γ1 to associate with the calcium channel. These predictions are confirmed by co-immunoprecipitation of α1.1 and γ1EGFP 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 γ1 null mice as described previously (23). The microsomes were solubilized in a buffer containing 50 mM Tris, 1% digitonin, and 0.5 mM 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 with a buffer containing 50 mM Tris, 0.1% digitonin, and 100 mM NaCl. Solubilized material was subjected to wheat germ agglutinin (WGA) chromatography. The material bound to the WGA column was eluted with a buffer containing 50 mM Tris, 0.1% digitonin, and 100 mM NaCl. Solubilized material was subjected to wheat germ agglutinin (WGA) chromatography. The material bound to the WGA column was eluted with a 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 pAd5CMV-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.

γ1—The rabbit γ1 subunit (GenBank™ accession number M32231) was subcloned into the BamHI/BamHI site of the adenovirus shuttle vector. The resulting construct was checked for appropriate orientation by restriction analysis.

γ2—The mouse γ2 subunit (GenBank™ accession number NM_007583) was excised from the pCDNA3 vector using HindIII and BamHI and subcloned into the HindIII/BamHI sites of the adenovirus vector.

γ1EGFP and γ2EGFP. The γ1EGFP and γ2EGFP 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.

γ1/γ2 Chimera—To generate this construct, a fragment of the rabbit γ1 cDNA was amplified using a forward primer that includes a HindIII site and the start codon (γ1 forward, 5′ CGC AAG CTT CCA CCA TGT CCC CGA CGG AAC CC 3′) and the reverse primer in the region after the second transmembrane domain (5′ GGT GTG CTT CTT CTC TCT GAA GAC CC G 3′). This reverse primer also includes a stretch of residues homologous to the γ2 subunit. Another PCR fragment was generated using the γ2 cDNA as a template, with the forward primer (5′ GCC TTC AGG AAG ACG AAG ACA ACG CCA CAA C 3′) and a reverse primer that includes a stop codon and a BamHI site (γ2 reverse, 5′ CGG GAT CCC GTA AGG GCG TGC TCC GCC G 3′). The products from the two PCRs were mixed and reamplified using the γ1 forward and γ2 reverse primers. The resulting product was subcloned into the adenovirus shuttle vector into the HindIII and BamHI sites. The final construct was sequenced to verify integrity. This construct encodes a protein that includes the N terminus to aa 133 of γ1 and aa 129 through the C terminus of the γ2 subunit. This protein is detected by an antibody to the γ1 subunit.

γ12EGFP—A fragment of the γ1 cDNA was amplified using the forward primer that includes a HindIII site and the start codon (γ1 forward, 5′ CGC AAG CTT CCA CCA TGT CCC CGA CGG AAC CC 3′) and the reverse primer (5′ CGG CCG CAG CAG GAT ATC GTA GAA CTC GCC GCG C 3′). A fragment of the γ1 cDNA was amplified using the forward primer (5′ CGC AGC GAG TTC TAC GAT TAC CTG CTG CCG C 3′) and a reverse primer that includes a stop codon and a BamHI site (γ1 reverse, 5′ GTG GAA TTC TGC TAA TGC TCG GGT TCG GCG C 3′). The products from the two PCRs were mixed and amplified with the γ1 forward and γ2 reverse primers. The resulting product was subcloned into the adenovirus shuttle vector into the HindIII and BamHI 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 γ1 subunit. This protein is detected by an antibody to the γ1 subunit.

| γ1α1EGFP Chimera—This construct was generated by standard PCR techniques to replace the first extracellular loop of the γ1EGFP with the first extracellular loop of the unrelated four-transmembrane protein sarcospan (24). The first extracellular loop of sarcospan includes the six amino acids RTDFFW. The protein can be detected by an antibody to GFP. | a1.1—This construct has been described previously (25). | Injection of Adenoviruses into Skeletal Muscle of the γ1 Null Mice—10 μl of primary particles or diluted secondary particles (−1011 particles/ml) of the adenovirus were injected into the tibialis anterior and the quadriceps muscles of 2–5-day-old γ1 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 5–50% linear sucrose gradients with 50 mM Tris, 0.5 mM 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, IF7 (α1.1 (26)), sheep 6 (α1.1 and β3.27), guinea pig 1 (α2δ), guinea pig 11/15/16/17 (γ1 (28)), rabbit 239 (γ1/γ2 (121)), AP63 (β-dystroglycan). | 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 (AP63). To visualize the α1.1 subunit, the sections were labeled with the monoclonal antibody to the α1.1 subunit (IID5E1). To minimize background, the sections were first treated with 5% nonfat dried milk and incubated with 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. | Protein Titrations and cDNA Transfections—Porcine 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... |
Whole-cell Voltage Clamp and Solutions—Cells were voltage-clamped -48 h after transfection. Transfected cells revealed by CD8 bead staining 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\textsuperscript{2+} conductance was fitted according to a Boltzmann distribution, \( \frac{A}{\max} = \frac{A}{\max} \cdot \exp\left(\frac{-\left(V - V_{1/2}\right)}{k}\right) \), where \( A_{\max} \) is \( G_{\max} \) at which \( A = A_{\max}/2; \) and \( k \) is the slope factor. The external solution in all cases was (in mM) 130 triethanolamine methanesulfonate, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 0.001 tetrodotoxin (Sigma), and 10 HEPES titrated with triethanolamine(OH) to pH 7.4. The pipette solution was (in mM) 140 Cs\textsuperscript{+}-aspartate, 5 MgCl\textsubscript{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, as is the rest of the channel as indicated by the presence of the subunits. The mdg protein was detected in the elution. This suggests that mdg may be incorporated into the channel complex (Fig. 2).

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, 0.5 mM NaCl, and protease inhibitors, and the solubilized material was isolated by centrifugation. The solubilized material was immunoprecipitated with the antibody to which the antibody to the \( \alpha_1 \) subunit (IIFP) was coupled. The beads were then thoroughly washed in a buffer containing 0.1% digitonin, 0.5 mM 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 \( \mu \)g/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 \( \gamma_1 \) Null Mice—The generation of \( \gamma_1 \) 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 channel calcium subunits are maintained as a complex. The calcium channel was purified from the wild type and \( \gamma_1 \) 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 \( \gamma_1 \) subunit can be detected in the complex from the wild type mice but not in the \( \gamma_1 \) null mice. The \( \alpha_1 \), \( \alpha_2 \), and \( \beta_1 \) subunits can be detected in the purified material from both the wild type and the \( \gamma_1 \) null mice, demonstrating that the residual calcium channel is maintained as a complex in the \( \gamma_1 \) null mice.

\( \gamma_1 \) Can Be Stably Incorporated into the L-type Calcium Channels of \( \gamma_1 \) Null Mice—The \( \gamma_1 \) subunit was reintroduced into the skeletal muscle of the \( \gamma_1 \) null mice, via recombinant adenoviruses, to determine whether it could be incorporated into the calcium channel of the \( \gamma_1 \) null mice. Expression of the protein was examined by immunoblot analysis (Fig. 2A). The muscle from the wild type mice showed expression of the \( \gamma_1 \) protein, whereas the \( \gamma_1 \) null mice did not express any \( \gamma_1 \) protein. Robust expression of \( \gamma_1 \) 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.

To examine if the virally expressed \( \gamma_1 \) is incorporated into the residual calcium channel complex of \( \gamma_1 \) null mice, a WGA-enriched preparation containing highly enriched material was examined for the presence of the \( \gamma_1 \). When examined by the WGA column, as is the rest of the channel as indicated by the presence of the \( \alpha_1 \) subunit in the elution. This suggests that \( \gamma_1 \) may be incorporated into the channel complex (Fig. 2B). To confirm this further, the enriched material was subjected to sucrose gradient fractionation and immunoblot analysis (Fig. 2C). Our results clearly demonstrate that \( \gamma_1 \) cofractionates with the other subunits of the calcium channel, indicating that indeed it is stably incorporated into the calcium channel complex of \( \gamma_1 \) null mice. A fraction of \( \gamma_1 \) is not associated with the channel. It is possible that this pool represents free \( \gamma_1 \) 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 \( \gamma_1 \), a construct that encodes \( \gamma_1 \) with an EGFP at the C terminus was engineered. Expression of this protein in mammalian cells revealed that \( \gamma_1 \)EGFP 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 \( \gamma_1 \) null mice. Interestingly, the protein is also localized to the sarcolemma (Fig. 2D), as indicated by its colocalization with \( \beta_1 \)-dystroglycan, a marker for the sarcolemma (30) (Fig. 2D). In addition, \( \gamma_1 \)EGFP is localized to the t-tubules (Fig. 2, D and E), as confirmed by its colocalization with \( \alpha_1 \) (Fig. 2E).

\( \gamma_2 \) Does Not Incorporate into the Skeletal L-type Calcium Channels of \( \gamma_1 \) Null Mice—The ability of \( \gamma_2 \) to incorporate into the calcium channel of \( \gamma_1 \) null mice was then examined using a similar approach. The adenovirus encoding \( \gamma_2 \) allows robust expression of the protein in the skeletal muscle of \( \gamma_1 \) null mice. There is no detectable endogenous protein in the skeletal muscle of either wild type or \( \gamma_1 \) null mice (Fig. 3A) as reported previously (13).

WGA enrichment of the channel complex indicates that, unlike \( \gamma_1 \), \( \gamma_2 \) is in the void, indicating that that \( \gamma_2 \) may not be

FIG. 1. The skeletal muscle L-type calcium channel is maintained as a complex in the \( \gamma_1 \) null mice. Purified calcium channels from the wild type (WT) and \( \gamma_1 \) null mice were immunoblotted to detect the presence of the subunits. The \( \alpha_1, \alpha_2, \beta_1 \) subunits are detected in both the wild type and the \( \gamma_1 \) null mice, whereas \( \gamma_1 \) is only detected in the wild type mice, indicating that the channel is maintained as a complex in the \( \gamma_1 \) null mice.
incorporated into the complex (Fig. 3B). To ascertain if the \( \gamma_2 \) 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 \( \gamma_2 \) injected mice was examined for the presence of \( \gamma_2 \) and the other subunits of the channel (Fig. 3C). These results demonstrate that \( \gamma_2 \) 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 \( \gamma_1 \) null mice.

Similar to \( \gamma_1 \)EGFP, \( \gamma_2 \)EGFP 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 \( \gamma_2 \) with the calcium channel, the adenovirally expressed \( \gamma_2 \)EGFP in the skeletal muscle of the \( \gamma_1 \) null mice does not demonstrate an organized t-tubule expression pattern (Fig. 3D). Interestingly, similar to that observed with the \( \gamma_1 \) subunit, the protein is also localized to the sarcolemma (Fig. 3D) and colocalizes with \( \beta \)-dystroglycan, a marker for the sarcolemma (Fig. 3E). In contrast to \( \gamma_1 \), \( \gamma_2 \) appears in punctate clusters within some muscle fibers (Fig. 3E). 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 γ₁ subunit interacts with the L-type calcium channels. A, schematic of the γ₁/γ₂ chimera. The protein encodes the first half of the γ₁ subunit, including the N terminus, first extracellular loop, and first two transmembrane domains, fused to the second half of the γ₂ subunit. B, adenovirally expressed γ₁/γ₂ in γ₁ null skeletal muscle was detected by immunoblot analysis. The blot was reprobed with an antibody to the α₂ subunit. WT, wild type. C, solubilized microsomes were subjected to sucrose gradient fractionation and immunoblot for subunits of the calcium channel. The γ₁/γ₂ comigrates in the same fractions as the other subunits of the calcium channel. The γ₁/γ₂ incorporates into the channel, confirming that it is indeed incorporated into the channel complex (Fig. 4C). Similar to that observed for the γ₁ studies described above, in some experiments a pool of the γ₁/γ₂ 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 γ₁ subunit that allow interaction with the calcium channel, we generated a construct that encodes the first two transmembrane domains of the γ₁ subunit and the last two transmembrane domains of the γ₁ (Fig. 4D). Similar to the other adenovirally expressed proteins, the γ₁/γ₂ chimera showed robust expression in the skeletal muscle of the γ₁ null mice (Fig. 4E). The γ₁/γ₂ 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 α₂, indicating that the γ₁/γ₂ chimera is not incorporated.

**Fig. 5.** Electrophysiological characterization of the γ subunits in γ₁ null myotubes. A, whole-cell calcium currents when γ₁ null or γ₁ 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 γ₁ null myotubes or γ₁ null chimeras with indicated constructs. The curves correspond to a Boltzmann fit of the population mean with the parameters G_{max} (pS/pF), V_{1/2} (mV), and k (mV), respectively, of 301.7, 19.8, and 6.1 for γ₁ null; 142.2, 21.4, and 5.8 for γ₁; 263.3, 16.1, and 5.9 for γ₁/γ₂; 153.6, 20.5, and 4.8 for γ₁/γ₂ chimera; and 274.9, 18.2, and 5.9 for γ₁/γ₂.

**11.1 Directly Interacts with γ₁ Subunit**—To determine the subunit of the calcium channel that interacts with the γ₁ subunit, we took advantage of the muscular dysgenesis (mdg) mice. The mdg mouse arose as a spontaneous mutation in the gene that encodes the α₁.1.1 (31) that results in the loss of any detectable protein (32). In the absence of α₁.1.1, α₂δ (32, 33) and β₃, are still expressed. We sought to examine γ₁ subunit in these mice and determine whether, in the absence of α₁.1.1, γ₁ is associated with α₂δ. The WGA-enriched material from mdg and 1–2-day-old α₁.1.1–/–; mdg mouse thoracic aortas was examined by immunoblot analysis. The blot was reprobed with an antibody to the γ₁ subunit. WT, wild type. D, schematic of the γ₁/α₂δ chimera. The α₂δ subunit follows by the first half of the γ₁ subunit, including the first two transmembrane domains. The chimera is in the void of the WGA elution, as indicated by the presence of the α₂δ, indicating that the γ₁/α₂δ chimera is not incorporated.

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*Subunit Interactions of the γ₁ Subunit*
old wild type muscle microsomes was subjected to sucrose gradient fractionation and immunoblot analysis. In the wild type mice, the α1.1, αδ, and a fraction of the γ1 subunits comigrate on the sucrose gradient (Fig. 6a), confirming that they are associated in a complex. Interestingly, a large fraction of the γ1 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 α1.1 is not detected. However, the αδ and the γ1 subunits do not comigrate (Fig. 6a), suggesting that the presence of the α1.1 subunit may be necessary for the γ1 subunit to associate with the calcium channel.

To examine if the γ1 subunit directly interacts with the α1.1 subunit, subunit associations were examined in transiently transfected mammalian cells. TA201 cells were transiently transfected with cDNAs encoding the α1.1 subunit, the α1.1 subunit, and the γ1EGFP or the γ1EGFP subunit alone. Cell lysates were subjected to immunoprecipitation using an antibody to the α1.1 subunit. Aliquots of the lysate and the immunoprecipitated material were examined for the presence of the γ1 subunit in subunit interaction. The α1.1EGFP existed in a smaller molecular weight form in tunicamycin-treated and untreated cells indicated that the γ1EGFP existed in a smaller molecular weight form in tunicamycin-treated cells, indicating an absence of glycosylation (Fig. 7A). Further, the γ1EGFP could be immunoprecipitated by an antibody to the α1.1 protein, indicating that the lack of glycosylation does not inhibit the association of the α1.1 subunit with the γ1 subunit (Fig. 7D).

**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 γ1 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 subunit (13). It is well known that the γ1 subunit modulates the inactivation kinetics and the current amplitude of the skeletal muscle

**TABLE I**

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<th>Subunit</th>
<th>G(V)</th>
<th>$G_{max}$ (nS/pF)</th>
<th>$V_{1/2}$ (mV)</th>
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<td>4.3 ± 0.7</td>
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<tr>
<td>γ1/γγ1</td>
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<td>22.1 ± 2.2</td>
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*Data are compared with non-transfected cells with a t test significance of p < 0.0001.*

**FIG. 6. γ subunit interacts with the α1.1.** 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, αδ and γ1 comigrate, indicating the presence of an intact channel complex. In the mdg mice, γ1 and αδ do not comigrate, indicating that α1.1 may be required for γ1 to be associated with the channel. B, TA201 cells were transiently transfected with the indicated constructs. Cell lysates were immunoprecipitated with an antibody to α1.1. Aliquots of the lysate and the immunoprecipitated material were examined for the presence of the α1.1 and the γ1EGFP. The α1.1 immunoprecipitates with the γ1EGFP.
Subunit Interactions of the $\gamma_1$ Subunit

Fig. 7. Role of the first extracellular loop in subunit interaction. A, schematic of the $\gamma_{1}$-spnEGFP chimera. The first extracellular loop of $\gamma_1$ 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 $\alpha_{1,1,1}$ and $\gamma_{1}$-spnEGFP. The $\alpha_{1,1,1}$ immunoprecipitates with the $\gamma_{1}$-spnEGFP. C, cell lysates from cells transfected with the $\gamma_{1}$-EGFP constructs and either treated with tunicamycin or untreated were immunoblotted with an antibody to GFP. Tunicamycin treatment inhibited N-linked glycosylation of $\gamma_{1}$-EGFP. 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 $\gamma_{1}$-EGFP. The $\alpha_{1,1,1}$ immunoprecipitates with the $\gamma_{1}$-EGFP in the absence of N-linked glycosylation.

The $\gamma_1$ 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 $\beta_1$ null mice. $\alpha_\beta\delta$ shows a punctate or diffused pattern in mdg mice (33), whereas $\alpha_{1,1,1}$ is reduced or absent in $\beta_1$ null mice (10). Recent studies have indicated that, unlike $\beta_1$ (10), $\gamma_1$ 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 $\gamma_1$ subunit that allows subunit interaction also allows the restoration of L-type currents in $\gamma_1$ null muscle. Interestingly, despite the differences in L-type currents in the wild type and $\gamma_1$ null mice, we have not observed any gross morphological changes in the muscle. Taken together, these data suggest that, at least in skeletal muscle, $\gamma_1$ 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 $\beta$ subunit (19) and in vivo and in vitro subunit heterogeneity (17, 18). We have identified the interaction site of the $\gamma_1$ with the calcium channel and predict that other $\gamma$ subunits interact with $\alpha_\beta$ subunits of the calcium channels similarly. Our studies indicate that the $\gamma$ subunits may not be as capable of functional heterogeneity as $\beta$ subunits, thereby restricting the number of possible subunit associations of the $\gamma$ subunits with the voltage-gated calcium channels. However, the $\gamma$ subunits have diverging homologies, with the $\gamma_6$ subunit being the phylogenetically most closely related subunit to the $\gamma_1$ subunit (15, 16). Hence, it is possible that the $\gamma$ subunits might possess the ability to form heterogeneous complexes, albeit to a relatively limited extent as compared with the $\beta$ subunits. This is also suggested by cell expression studies indicating the ability of the $\gamma_1$ 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 $\gamma_1$ null mouse offers a good model system to test the ability of the other $\gamma$ 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 $\gamma_1$ subunit in the t-tubules by microscopy. To our knowledge, this is the first report of localization of the $\gamma_1$ subunit in the skeletal muscle. Interestingly, the $\gamma_1$ 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 $\gamma_1$ subunit contains the determinants for plasma membrane localization. In the future, it would be interesting to determine whether the ability of the $\gamma_1$ subunit to localize to the t-tubules of the skeletal muscle, in addition to the plasma membrane/sarcolemma, is a result of its
association with the subunits of the calcium channel or whether the protein has its own signals, like the α1.1 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 γ1 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 γ1-null myotubes. The absence of N-linked glycosylation of the γ1 subunit does not prevent the protein from associating with the α1.1 subunit, suggesting that the N-linked glycosylation is not the predominant mediator of the interaction between the α1.1 and γ1 subunits. Interestingly, the first extracellular loop of the γ1 subunit is relatively negatively charged (12) and has been suggested to be important in mediating the biophysical properties of the γ1 subunit (22). We demonstrate that the first extracellular loop of the γ1 subunit is not required for subunit interaction. Because the first half of the γ1 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 α1.1 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 αδγ subunit in the association of the γ subunit with the voltage-gated calcium channel. The studies on the mdg mice indicate that the αδγ and γ1 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 γ1 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 γ1 that is not incorporated into the calcium channel is degraded. Alternatively, it is possible that during the early stages of muscle development, γ1 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 γ1 subunits during early development. Our cell expression studies indicate that the γ1 subunit directly interacts with the α1 subunit. This is consistent with studies that demonstrate a direct effect of the γ6 subunit on the α1.1 protein in the absence of the other known calcium channel subunits (40). It is therefore likely that the requirement of the αδγ 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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