A Carboxyl-terminal Region Important for the Expression and Targeting of the Skeletal Muscle Dihydropyridine Receptor*

We have used the yeast two-hybrid technique and expression of truncated/mutated dihydropyridine receptors (DHPRs) to investigate whether the carboxyl tail of the DHPR is involved in targeting to junctions between the sarcolemma and sarcoplasmic reticulum in skeletal muscle. The carboxyl tail was extremely reactive in yeast two-hybrid library screens, with the reactivity residing in amino acids 1621–1647 and abolished by a point mutation (V1642D). Dysgenic myotubes were injected with cDNA encoding green fluorescent protein fused to the amino terminus of DHPRs truncated after either residue 1620 (Δ1621–1873) or residue 1542 (Δ1543–1873) or of full-length DHPRs with the V1642D mutation (V1642D). For either Δ1621–1873 or V1642D, the restoration of excitation-contraction coupling was reduced ~40%, and the number of functional DHPRs in the sarcolemma was reduced ~30%, compared with the wild-type DHPR. The restoration of excitation-contraction coupling and surface expression was more drastically reduced (by ~90 and ~55%, respectively) for Δ1543–1873. Fluorescence microscopy revealed that Δ1621–1873 and V1642D were concentrated in a longitudinally restricted region near the injected nucleus, whereas wild-type DHPRs were present relatively uniformly along the length of a myotube. The intensity of fluorescence was greatly reduced for Δ1543–1873, indicating a low level of protein expression. Thus, residues 1543–1647 appear to play a role in the biosynthetic processing, transport, and/or anchoring of DHPRs, with residues 1543–1620 being particularly important for expression.

In skeletal muscle cells, electrical excitation of the sarcolemma causes Ca\(^{2+}\) to be released from intracellular stores within the sarcoplasmic reticulum, which in turn causes contraction. This process, termed excitation-contraction (EC)\(^1\) coupling, involves a functional interaction between dihydropyridine receptors (DHPRs), voltage-sensitive Ca\(^{2+}\) channels in the sarcolemma, and ryanodine receptors (RyRs), intracellular Ca\(^{2+}\) release channels in the sarcoplasmic reticulum membrane. The interaction between DHPRs and RyRs occurs at specialized junctions between the sarcolemmal and sarcoplasmic reticulum membranes and thus requires that both proteins be targeted to and anchored at the junctions. However, the mechanisms for targeting and anchoring are not understood. Although the two proteins may interact with one another directly, other molecular interactions must also be involved because RyRs target to junctions in dysgenic muscle cells (1), which lack DHPRs (2), and DHPRs target to junctions in dyspedic muscle cells, which lack RyRs (3, 4).

The targeting of the DHPR seems likely to depend on structures within the α\(_{1S}\) subunit, because GFP-tagged α\(_{1S}\) subunits show a very different subcellular distribution in dysgenic myotubes depending on whether they are the neuronal or muscle type (5). Furthermore, for several members of the voltage-gated ion channel superfamily (to which α\(_{1S}\) belongs), recent data suggest that the carboxyl tail is involved in subcellular localization, often via interactions with other cellular proteins. For instance, interactions of the carboxyl tail with cytoplasmic proteins have been implicated in the subcellular localization and clustering of Shaker potassium channels (6), Na\(^{+}\) channels (7), and Trp Ca\(^{2+}\) channels (8). Because the carboxyl tails are the largest and most highly divergent of the cytoplasmic domains of Ca\(^{2+}\) channel α\(_{1}\) subunits (9), it seems reasonable that they might play a similar role in isoform-dependent channel targeting. As encoded by the mRNA found in skeletal muscle, the carboxyl tail of α\(_{1S}\) comprises residues 1393–1873. However, residues beyond 1662 are unlikely to play an important role in the anchoring of the mature protein because the predominant form of α\(_{1S}\) in mature skeletal muscle is truncated at a site between amino acids 1685 and 1699 (10) and because an α\(_{1S}\) truncated just after residue 1662 and expressed in dysgenic myotubes functions normally as both calcium channel and voltage sensor for EC coupling (11). The ~160 residues just beyond IVS6 (up to and including residue 1542 in α\(_{1S}\)) are also unlikely to be involved in isoform specific targeting, since they are well conserved between the high voltage-gated α\(_{1}\) isoforms (9). Thus, residues 1543–1662 appear to be a good candidate to contain domains critical for the targeting of α\(_{1S}\).

In order to identify domains of the carboxyl tail that might be important for the expression and targeting of DHPRs and to determine whether these domains interact with other muscle proteins, we have used the yeast two-hybrid method and electrophysiological assays of truncated and mutated DHPRs. The two-hybrid approach revealed that the carboxyl tail of α\(_{1S}\) is highly reactive in screens of a skeletal muscle cDNA library and that this reactivity resides in a small region (residues 1621–1647) near the end of the truncated form of the mature channel. The reactivity of the carboxyl tail was abolished by a valine to aspartate point mutation in a consensus PDZ-binding motif that is located within the reactive region. Expression of

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1 The abbreviations used are: EC, excitation-contraction; DHPR, dihydropyridine receptor; RyR, ryanodine receptor; GFP, green fluorescent protein.

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DHPRs in dysgenic myotubes showed that either truncation or mutation of the carboxyl tail resulted in a decreased efficiency for restoration of EC coupling, a reduction of functional DHPRs expressed in the sarcolemma, and a reduced expression of DHPRs at sites longitudinally distant from the nucleus injected with cDNA. Taken together, these results suggest that residues 1543–1662 are important in the expression of functional DHPRs in the sarcolemma, perhaps as a consequence of interaction with other proteins.

**EXPERIMENTAL PROCEDURES**

Molecular Biology—For yeast two-hybrid library screens, portions of the rabbit skeletal muscle α1S subunit of the DHPR (12) were inserted in-frame into the GAL4 binding domain plasmid, pGBT9 (CLONTECH, Palo Alto, CA) by means of restriction sites added to the ends of fragments generated by polymerase chain reaction. All inserts were confirmed by sequencing (Macromolecular Resources, Fort Collins, CO). The mammalian expression plasmid GFP-α1S, which encodes green fluorescent protein (GFP) fused in-frame to the amino terminus of α1S, was described previously (5). The plasmid Δ1621–1873, in which the carboxyl tail of GFP-α1S is terminated after residue 1620, was constructed by ligating a PCR product encoding α1S residues 1428–1620, followed by a stop codon, into GFP-α1S, by means of a naturally occurring BamHI site (at codon 1428) and an EcoRI partial digest of GFP-α1S, which generated a cut in the polynucleotide at position 5676 in GFP-α1S. The plasmid V1642D encoding GFP-α1S with the point mutation was generated with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) using GFP-α1S as a template. Δ1543–1873 was generated using the QuikChange mutagenesis kit to introduce a stop codon into GFP-α1S just downstream of codon 1542.

**RESULTS**

**The Carboxyl Tail of α1S Is Highly Reactive in Yeast Two-hybrid Library Screens**—In an attempt to identify unknown proteins that localize DHPRs to junctions between the sarcolemma and the sarcoplasmic reticulum, we used the yeast two-hybrid system to screen a skeletal muscle cDNA library for proteins that interact with each of the putative cytoplasmic regions of α1S. In library screens using the amino-terminal domain (residues 1–51), I-I loop (residues 331–432), II-III loop (residues 654–797), or III-IV loop (residues 1059–1126) as “bait,” fewer than 12 interacting colonies appeared on nutritional selection media (data not shown). By contrast, the carboxyl-terminal domain of the DHPR (residues 1381–1873) was extremely reactive in two-hybrid library screens, giving an average of about 19,000 positive interactors in each of five independent screens. This profuse reactivity is illustrated in Fig. 1 by the density of colonies (>4.9 colonies/cm²) appearing on a representative section of a plate containing SD−Trp−Leu−His nutritional selection medium. Transformation of yeast with only the carboxyl tail or co-transformation with the carboxyl tail plus the activation domain vector lacking library cDNA did not permit growth on the nutritional selection medium (Fig. 1), indicating that the high reactivity did not result from spurious activation of the reporter gene.

To define the domain of the α1S carboxyl tail responsible for its high reactivity, further library screens were carried out with a series of carboxyl tail fragments. All segments that contained residues 1621–1647 had reactivity equivalent to that of the full-length carboxyl tail (Fig. 2). Carboxyl tail segments lacking residues 1621–1647 did not have high reactivity (<12 colonies/cm²). In an attempt to identify “fish” proteins (encoded by library inserts in the activation domain vector) that interacted with carboxyl tail “baits” containing the reactive domain, we sequenced library inserts from the colonies. These were sequenced at random from colonies that were positive in library screens for both the HIS3 and lacZ reporter gene activation as assayed by survival on SD−Trp−Leu−His medium and by β-galactosidase activity. Of the sequenced interactors, 10 contained sequences of genes encoding mitochondrial or developmental proteins, which are unlikely physiological interaction partners of...
DHPRs at triad junctions in skeletal muscle. Nine of the sequenced interactors encoded 8 residues or fewer in-frame with the coding sequence for the GAL4 activation domain, indicating that the reporter gene activation was probably spurious. Nine of the interactors contained nucleotide sequences from known genes inserted backward with respect to the GAL4 activation domain, again indicating unlikely physiological interaction partners. All of the open reading frames of the sequenced interactors were aligned, but no common sequence or motif was identified.

The Reactivity of the αS Carboxyl Tail Depends on Val1642—Residues 1621–1647 contain a consensus PDZ binding motif (Fig. 3A), which is known to be a protein interaction module (for reviews, see Refs. 16 and 17). To test the importance of this PDZ binding motif for the reactivity of residues 1621–1647, we introduced a point mutation (V1642D) which corresponds to one that had been introduced into the Trp Ca2+ channel and shown to disrupt its interaction with the PDZ domain scaffolding protein, INAD (8). The V1642D mutation abolished the reactivity of residues 1621–1647 as indicated by both nutritional selection (Fig. 3B, inset) and β-galactosidase activity (Fig. 3B).

Δ1621–1873, V1642D, and Δ1543–1873 Restore EC Coupling Less Effectively than αS—To examine the functional significance of the carboxyl tail of the DHPR, we constructed mammalian expression vectors encoding GFP fused in-frame to the amino terminus of several variants of αS: “Δ1540–1873” and “Δ1621–1873,” in which αS terminates, respectively, at residue 1542 (just beyond the region of the carboxyl tail conserved between α isoforms) or residue 1620 (just before the yeast two-hybrid reactive domain), and “V1642D,” in which αS is full-length but contains the point mutation V1642D (Fig. 4A). These constructs, as well as GFP-αS (5), were expressed in dysgenic myotubes, which lack the αS subunit endogenous to normal myotubes (18). As the simplest measure of the function of the expressed constructs, we measured the percentage of green fluorescing cells that contracted in response to electrical stimulation. Compared with GFP-αS, this percentage was substantially reduced for Δ1621–1873 and for V1642D and was reduced even further for Δ1543–1873 (Fig. 4B and Table I). Thus, truncation prior to the nonconserved domain (Δ1543–1873) has a more pronounced effect than truncation just prior to, or mutation of, the yeast two-hybrid reactive domain (Δ1621–1873 and V1642D, respectively).

Membrane Currents and Surface Expression Are Altered for Δ1621–1873, Δ1543–1873, and V1642D—To characterize fur-
other the function of the mutant DHPRs, whole-cell L-type Ca\(^{2+}\) currents and immobilization-resistant charge movements were recorded from myotubes expressing GFP-\(\alpha_{1S}\), \(\Delta 1621\)–1873, V1642D, or \(\Delta 1543\)–1873 (Fig. 5A). To facilitate comparisons, the normalized maximal Ca\(^{2+}\) conductance (\(G_{\text{max}}\)) and maximal charge movement (\(Q_{\text{max}}\)) were determined for each cell. Compared with GFP-\(\alpha_{1S}\), \(G_{\text{max}}\) was not significantly reduced for \(\Delta 1621\)–1873 but was reduced ~28% for V1642D and ~39% for \(\Delta 1543\)–1873; \(Q_{\text{max}}\) was reduced by 22% for \(\Delta 1621\)–1873, 25% for V1642D, and 54% for \(\Delta 1543\)–1873 (Fig. 5, B and C, and Table I). Since charge movement is an indirect measure of the number of DHPRs in the membrane, this reduction of \(Q_{\text{max}}\) is suggestive of a reduced surface expression for \(\Delta 1621\)–1873, V1642D, and \(\Delta 1543\)–1873, which probably contributes to the reduced restoration of EC coupling by these constructs. The reduction in surface expression may actually be larger than it would appear from the comparison of total charge (\(Q_{\text{max}}\)), because a component of charge in dysgenic myotubes (\(Q_{\text{dys}}\sim 2.5\) nanocoulombs/microfarad) appears to be independent of DHPRs (13). If \(Q_{\text{dys}}\) is subtracted from the \(Q_{\text{max}}\) values in Table I (to yield \(Q_{\text{max}}\)), the estimated reduction of DHPRs in the sarcolemma is about 30% for both \(\Delta 1621\)–1873 and V1642D and about 70% for \(\Delta 1543\)–1873.

An alteration in membrane expression was also apparent when cells expressing \(\Delta 1621\)–1873, \(\Delta 1543\)–1873, and V1642D were viewed with fluorescence optics. In total, we collected fluorescent images from 31 cells expressing GFP-\(\alpha_{1S}\), 30 cells expressing \(\Delta 1621\)–1873, 34 cells expressing V1642D, and 14 cells expressing \(\Delta 1543\)–1873. Although there was some variability in the patterns observed, representative images are shown in Fig. 6. For GFP-\(\alpha_{1S}\), the fluorescence was present at a relatively uniform level along the entire length of the injected myotube. In contrast, the fluorescence for \(\Delta 1621\)–1873 and V1642D was more restricted to the area immediately surrounding the injected nucleus. Consistent with the large reduction in \(Q_{\text{max}}\), the intensity of green fluorescence was much lower in myotubes expressing \(\Delta 1543\)–1873 than for any of the other constructs. This dim fluorescence made it problematic to determine the pattern of subcellular distribution for \(\Delta 1543\)–1873.

### DISCUSSION

In this study, we have shown that the carboxyl tail of \(\alpha_{1S}\) is highly reactive in yeast two-hybrid screens of a skeletal muscle cDNA library, that this reactivity resides in residues 1621–1647, and that the reactivity is abolished by the V1642D point mutation. In functional assays, we have shown that introduction of the V1642D mutation into the full-length DHPR or truncation after residue 1620 (just before the reactive region) reduces the fraction of dysgenic myotubes with restored EC coupling and lowers \(Q_{\text{max}}\), suggesting a decreased surface expression of the altered DHPRs. Truncation of an additional 79 residues (after residue 1542) results in a much larger reduction in the restoration of EC coupling and in \(Q_{\text{max}}\). Compared with myotubes expressing GFP-\(\alpha_{1S}\), the green fluorescence in cells expressing \(\Delta 1621\)–1873 or V1642D is more longitudinally restricted (suggestive of altered targeting). Consistent with the large reduction in \(Q_{\text{max}}\), cells expressing \(\Delta 1543\)–1873 have substantially reduced fluorescence intensity (indicative of greatly diminished expression).

The ratio of \(G_{\text{max}}\) (maximal Ca\(^{2+}\) conductance) to \(Q_{\text{max}}\) (maximal charge after correction for dysgenic charge) provides an indirect estimate of the product of \(P_{o}\) (open probability) and \(\gamma\) (single channel conductance) of the DHPR Ca\(^{2+}\) channel (15). Interestingly, the \(G_{\text{max}}/Q_{\text{max}}\) ratio is lower for GFP-\(\alpha_{1S}\) than previously reported (20). A possible explanation is that the previous measurements were made 48–72 h after cDNA injection, whereas the measurements reported here were obtained 24 h after injection. Analysis at 24 h was chosen because it is relatively early with respect to the appearance of the expressed proteins in the sarcolemma and would thus be expected to emphasize differences in targeting. However, it is possible that at 24 h fewer DHPRs were associated with RyRs than at 48–72 h. Such a difference in association would be significant because previous work (19, 20) has shown that the \(G_{\text{max}}/Q_{\text{max}}\) ratio is higher for DHPRs that are functionally associated with RyRs than for DHPRs that are not functionally associated. Truncations of the carboxyl tail could affect the \(G_{\text{max}}/Q_{\text{max}}\) ratio independently of effects on targeting, because it has been shown that removal of the final 439 residues from the carboxyl tail of \(\alpha_{1C}\) (the pore-forming subunit of the cardiac DHPR) causes an increase in the \(G_{\text{max}}/Q_{\text{max}}\) measured after expression.

### Table I: Targeting of Dihydropyridine Receptors

| Clone | Restoration of ECC | \(G_{\text{max}}\) | \(Q_{\text{max}}\) |
|-------|-------------------|------------------|------------------|
| GFP-\(\alpha_{1S}\) | 70% (23) | 127.7 ± 12.2 (24) | 10.1 ± 0.7 (12) |
| \(\Delta 1621\)–1873 | 27% (79) | 115.2 ± 7.5 (32) | 7.9 ± 0.3 (15) |
| V1642D | 23% (39) | 92.1 ± 7.0 (21) | 7.6 ± 0.8 (17) |
| \(\Delta 1543\)–1873 | 9% (54) | 78.2 ± 14.2 (14) | 4.6 ± 0.7 (15) |

### Figure 5: Macroscopic Ca\(^{2+}\) currents and charge movements of GFP-\(\alpha_{1S}\), \(\Delta 1621\)–1873, \(\Delta 1543\)–1873, and V1642D.

- **A**: Representative traces of macroscopic Ca\(^{2+}\) currents (left) and charge movements (right) in dysgenic myotubes expressing the indicated constructs. The test depolarization was +40 mV for all traces. Data are indicated as current density after normalization by linear cell capacitance.
- **B**: Comparison of \(G_{\text{max}}\) and \(Q_{\text{max}}\) for GFP-\(\alpha_{1S}\), \(\Delta 1621\)–1873, \(\Delta 1543\)–1873, and V1642D. A double asterisk indicates a statistically significant difference from both \(\Delta 1621\)–1873 and V1642D (p < 0.05).
Why Does the Carboxyl Tail Have Such High Yeast Reactivity?—Because both yeast two-hybrid reactivity and DHPR surface expression are influenced by a domain that contains a consensus PDZ binding motif ((S/T)XV), it is tempting to speculate that interaction of the DHPR carboxyl tail with a PDZ protein is important for expression of functional DHPRs. Interactions with PDZ proteins have been implicated in the localization of other ion channels, including NMDA receptors (22) and Shaker K⁺ channels (6). Although both of these channels have a PDZ binding motif at the very end of the carboxyl tail, the Trp Ca²⁺ channel in Drosophila photoreceptors interacts with the PDZ scaffolding protein INAD via a PDZ binding domain 19 residues from the carboxyl terminus (8). There is an even greater distance between the PDZ binding motif (1640–1642) and the terminal residue (1873) of the DHPR that is encoded by the mRNA present in skeletal muscle. However, the predominant form of the DHPR in adult skeletal muscle, which is thought to arise by post-translational proteolytic cleavage, terminates somewhere between residues 1685 and 1699 (10). It is possible that one function of the post-translational truncation of the DHPR is to expose a protein interaction domain.

Although both Δ1621–1873 and V1642D have a diminished ability to restore EC coupling, as well as reduced surface expression and altered subcellular distribution, the yeast two-hybrid reactive region (residues 1621–1647) cannot be solely responsible for controlling surface expression/targeting of the DHPR, because both constructs retain appreciable levels of function. Thus, one or more additional regions of the DHPR (which are upstream from residue 1621) must also be involved. The domain encompassing residues 1543–1620 is a candidate both because it is just downstream from the region of the carboxyl tail that is highly conserved among calcium channel isoforms and because removal of this region caused a large reduction in restoration of EC coupling and surface expression compared with Δ1621–1873. It should be noted that cells expressing Δ1543–1873 did not display any obvious retention of fluorescence in the Golgi apparatus. Thus, if the primary effect of removing residues 1543–1620 was to prevent targeting, then this mistargeting must have resulted in rapid breakdown of the protein. Alternatively, the removal of these residues might have caused rapid breakdown as a consequence of misfolding. To resolve these alternatives, it may be useful in the future to complement the loss-of-function approach employed here with gain-of-function approaches. For example, previous work showed that neuronal α₁ subunits do not target to sarcosomal-sarcoendoplasmic reticulum junctions (5). Thus, it would be valuable to determine if targeting to junctions would occur for a neuronal α₁ subunit carrying residues from the α₁S carboxyl tail.
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