Metabolic Biotinylation as a Probe of Supramolecular Structure of the Triad Junction in Skeletal Muscle*

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Excitation-contraction coupling in skeletal muscle involves conformational coupling between dihydropyridine receptors (DHPRs) in the plasma membrane and ryanodine receptors (RyRs) in the sarcoplasmic reticulum. However, it remains uncertain what regions, if any, of the two proteins interact with one another. Toward this end, it would be valuable to know the spatial interrelationships of DHPRs and RyRs within plasma membrane/sarcoplasmic reticulum junctions. Here we describe a new approach based on metabolic incorporation of biotin into targeted sites of the DHPR. To accomplish this, cDNAs were constructed with a biotin acceptor domain (BAD) fused to selected sites of the DHPR, with fluorescent protein (XFP) attached at a second site. All of the BAD-tagged constructs properly targeted to junctions (as indicated by small puncta of XFP) and were functional for excitation-contraction coupling. To determine whether the introduced BAD was biotinylated and accessible to avidin, even though the DHPRs were present within fully assembled (RyR1-containing) junctions, but not to the α1S C-terminal, which appears to be occluded by the presence of RyR1.

In skeletal muscle, two major proteins involved in excitation-contraction (EC) coupling are the dihydropyridine receptor (DHPR), a voltage-gated calcium channel in the plasma membrane, and the ryanodine receptor (RyR), a calcium release channel in the sarcoplasmic reticulum (SR). Physically, the skeletal DHPRs are grouped in “tetrads,” and each DHPR within a tetrad is located in exact correspondence to one of the four subunits of RyR1 (1, 2). Functionally, a bidirectional interaction occurs in skeletal muscle between the DHPR and RyR1. Depolarization of the plasma membrane causes transmission of an orthograde signal from the DHPR (3, 4) to the RyR; this in turn causes Ca2+ release via RyR1 that does not require the entry of extracellular Ca2+ (5, 6). In addition to this orthograde signal, there is a retrograde signal whereby RyR1 increases the magnitude of the voltage-gated calcium current carried through the DHPR (L-type current, Ref. 7).

Despite a large number of studies involving functional analyses of intact cells and biochemical analyses of cell fractions and isolated proteins, the identity of the protein-protein links necessary for the functional and morphological coupling between DHPRs and RyRs in skeletal muscle remains unresolved. The preceding paper (8) described a new approach in which the fluorescent resonance energy transfer efficiency of a cyan fluorescent protein-yellow fluorescent protein (CFP-YFP) tandem was used as an indirect indicator of sites of possible proximity between the DHPR and RyR. Here we report on another novel approach in which the topology of the plasma membrane/SR junctions is probed by determining whether avidin can access biotin introduced at different sites of the DHPR. The site-specific introduction of biotin was based on the metabolic incorporation that normally occurs only for the small number of cellular enzymes that contain biotin as an essential co-factor. The biotin is incorporated into these enzymes by the catalytic action of biotin protein ligase, with the result that biotin is attached to a lysine contained within a biotin acceptor domain (BAD). In mammalian cells, metabolic biotinylation is effective for fusion proteins containing a minimal BAD (9). We constructed cDNAs that encoded a 70- or 97-residue BAD fused to sites of the DHPR, as well as a variant of green fluorescent protein (XFP) fused at a second site as an independent reporter of localization. It was then possible to determine whether the BAD had been biotinylated and was accessible for binding of fluorescently labeled avidin.

The XFP/BAD/DHPR fusions were found to be functional in EC coupling and to target to discrete foci indicative of membrane junctions. Moreover, effective, metabolic biotinylation occurred for BAD at the N- and C-terminals of the β1a and α1S subunits of the DHPR, as well as at an internal site within the α1S II–III loop. Nearly all of these sites were accessible to avidin, even though the DHPRs were present within fully assembled junctions. In contrast, the C-terminal of α1S was inaccessible to avidin in junctions that contained RyR1, but was accessible in junctions lacking RyR1. Thus, RyR1 appears to occlude access to the C-terminal of α1S but not to the other sites.
of the DHPR (β1a, N- and C-terminals, α1S N-terminal and II–III loop). However, a surprising number of sites within DHPRs localized to fully assembled junctions are accessible to avidin, a molecule of substantial size.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs**

**Biotin Acceptor Domain**—The BAD was extracted from the PinPoint Xa-1 expression vector (Promega, Madison, WI) containing the sequence encoding the PSTCD (Propionibacterium shermanii transcarboxylase domain). This PinPoint vector encodes a BAD of 123 amino acids in length with an approximate molecular mass of 13 kDa. The crystal structure of this biotin acceptor domain (Protein Data Bank code 1DCZ, Ref. 10) is shown in Fig. 1. The biotinylated lysine is located at position 89 of this sequence (highlighted in yellow). Fig. 2 summarizes the positions at which the BAD was incorporated into the α1S, β1a, and subunits of the skeletal muscle DHPR. The biotin acceptor domain in these constructs was either 70 or 97 amino acids in length. The cDNAs for the fusion proteins were constructed as follows, with restriction digests and sequencing used for verification.

**GFP-α1S-BAD**—For this and all other C-terminal fusions (except for GFP-BAD, as described later), PCR mutagenesis (QuickChange Kit, Stratagene, La Jolla, CA) was used to introduce two KpnI restriction sites into the PSTCD sequence of the PinPoint Xa-1 plasmid. One KpnI site was inserted directly before amino acid Gln212 (by inserting GGAGTACC-5’ to nucleotide G213, where 1 indicates the first nucleotide of the PSTCD coding sequence). The second KpnI site, preceded by a stop codon, was introduced after amino acid Gly122 by inserting TAGGTAC-3’ to nucleotide G686. The 219-bp KpnI BAD fragment was ligated to KpnI-digested XFP-α1a-BAD (constructed as described in Ref. 8). The 70-residue BAD was fused to the C-terminal of β1a via a one-residue linker.

**BAD-α1a/YFP**—To fuse the BAD to the N-terminal of α1a, the PinPoint Xa-1 plasmid was digested with HindIII and SalI. This BAD sequence codes for residues 26–133 of the original PSTCD sequence. The α1a-short-YFP plasmid (8) was digested with XhoI and HindIII removing a small plasmid fragment before the α1a N-terminal. The resulting α1a plasmid and the BAD fragment were co-ligated producing BAD-α1a-short-YFP with an 18-residue linker connecting the 97-residue BAD to the N-terminal of α1a-short-YFP.

**BAD-β1a-YFP**—The β1a-YFP plasmid (8) was opened using restriction cuts with EcoRI and Nhel. A 389-bp BAD fragment (encoding residues 23–133) was isolated from BAD-α1a-short-YFP with EcoRI and Nhel. The opened β1a plasmid and the BAD fragment were co-ligated producing BAD-β1a-YFP. A 13-residue linker connected the 97-residue BAD to the N-terminal of β1a-YFP.

**BAD-α1a(III–IV)-YFP**—A 347-bp BAD fragment (encoding residues 26–128) was isolated from BAD-α1a-short-YFP by restriction cuts with SacI and Nhel. The plasmid α1a(III–IV)-YFP (8) was digested with SacI and Nhel, opening the plasmid before the N-terminal of α1a, repeat III, and the BAD cDNA was inserted into this site. A 6-residue linker connected the 97-residue BAD to the N-terminal of α1a(III–IV)-YFP. This construct was co-expressed with the plasmid α1a(I–II) (8). The α1a(I–II)-BAD-α1a(III–IV)-YFP plasmid (see above) with Nhel and BamHI at the multiple cloning region preceding the α1a coding sequence. The α1a(I–II) coding sequence was isolated from a α1a(III–IV)-YFP plasmid by restriction cuts with Nhel and BamHI. These two fragments were co-ligated to obtain α1a(I–II)-BAD-III–IV–YFP. A 14-residue linker connected α1a(I–II) to the 79-residue BAD, which was connected via a 6-residue linker to α1a(III–IV)-YFP.

**GFP-BAD**—Using PCR mutagenesis, a KpnI restriction site was introduced into the PSTCD sequence of the PinPoint Xa-1 plasmid directly before amino acid Gln212 (by inserting GGAGTACC-5’ to nucleotide G213). A stop codon followed by a KpnI site was introduced after amino acid Gly686 by inserting TAGGTACCG-3’ to nucleotide G686. The modified plasmid was digested with KpnI, and the 218-bp fragment was inserted into the KpnI-cut multiple cloning site of pEGFP-C1 (BD Biosciences). A 17-residue linker connected the 70-residue BAD to the C-terminal of GFP.

**Expression of cDNA**

Primary cultures of myotubes isolated from newborn dysgenic, dyspedic, or β1a-null mice were prepared as described previously (11). Myoblasts were plated on ECL-coated (Upstate Biotechnology, Lake Placid, NY) 35-mm plastic culture dishes or dishes with glass coverslip bottoms (MatTek, Ashland, MA) and grown for 6–7 days in a humidified 37 °C
incubator with 5% CO₂. Approximately 1 week after plating, myotubes were microinjected (12) in a single nucleus with one of the above cDNA constructs (5–100 ng/μl). After injection, the cells were changed into a culture medium containing normal levels of biotin (1 μM). For Western blot analyses, immortal dysgenic myotubes were transfected with cDNA constructs using LT-1 transfection reagent (Mirus, Madison, WI).

Electrocally evoked contractions

Contractions were elicited by 10-ms, 55–100 V stimuli applied via an extracellular pipette placed near intact myotubes expressing constructs of interest. Images of these myotubes were acquired at a rate of 40–50 Hz. The contractions were quantified by measuring the movement of an identifiable portion of a myotube across the visual field.

NeutrAvidin Staining

Two days after injection, myotubes were washed in PBS (calcium- and magnesium-free), and then fixed with 4% paraformaldehyde in PBS for 20 min. The cells were then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 30 min and incubated in PBS blocking solution for 1 h. The cells were then exposed to NeutrAvidin-tetramethylrhodamine (hereafter referred to as “avidin”); Molecular Probes, Eugene, OR) or streptavidin-Cy3 (Jackson ImmunoResearch, West Grove, PA) (1:2000–5000) in 0.1% Triton X-100/PBS blocking solution for 1 h in the dark. The cells were washed with 0.1% Triton X-100 in PBS followed by PBS.

Western Blot Analysis

Samples were run on SDS-PAGE gels (4–20% precast Tris-HCl; Bio-Rad) in a Mini-PROTEAN II electrophoresis cell (Bio-Rad) according to the instructions. Protein was transferred to nitrocellulose using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) at 100 V for 1 h. Blots for GFP detection were blocked using Startingblock (1:1,000 in 3% milk/PBS) (Chemicon, Temecula, CA) overnight at room temperature. The blot was washed with 0.1% Tween 20, PBS and then incubated with goat anti-mouse secondary antibody (1:10,000 in 3% milk/PBS) (Pierce, Rockford, IL) for 30 min. Blots for biotin detection were blocked using Streptalking (Pierce), and then incubated with streptavidin-poly-HRP (Pierce), diluted (1:10,000 in Poly-HRP Dilution buffer, Pierce) for 30 min. Both GFP and biotin detection blots were then washed with 0.1% Tween 20, PBS. Blots were developed with Super Signal West Femto detection kit (Pierce).

Confocal Microscopy

Fluorescence was analyzed using an Axiovert/LSM 510 META laser-scanning confocal microscope (Zeiss, Thornwood, NY). Excitation and emission parameters for each fluorophore were set as follows: CFP, excitation at 488 nm (488/543 nm dual dichroic, and emission using a 505 nm band pass filter); YFP, excitation at 488 nm (488/543 nm dual dichroic, and emission with a 530-nm band pass filter); GFP, excitation at 488 nm (488/543 nm dual dichroic, and emission with a 530 nm band pass filter; YFP, excitation at 488 nm (488/543 nm dual dichroic, and emission using a 505 nm band pass filter). Confocal images were acquired at 1.3 NA oil immersion objective.

RESULTS

As a tool for probing the supramolecular architecture of DHPRs and RyRs assembled into plasma membrane/SR junctions, we introduced a BAD at different sites of the DHPR (Fig. 2) to determine whether a 60-kDa avidin molecule could access those sites. BAD fusion proteins were expressed in myotubes from dysgenic mice (lacking functional α₁S channels), dyspedic mice (lacking RyR1), or β₁-null mice (lacking β₁ subunits), which were cultured in the presence of normal biotin levels (1 μM). Previous studies have shown that a shortened 70-amino acid PSTCD is sufficient for endogenous enzymatic biotinylation in mammalian cells (9), and our fusion constructs included BADs of either 70 or 97 residues in length. As a test for endogenous biotinylation activity, we overexpressed a GFP-BAD fusion protein in dysgenic myotubes. The presence of biotinylated proteins was then assayed by fixing and permeabilizing the cells, exposing them to streptavidin-Cy3, and imaging with confocal microscopy. Cells displaying strong green fluorescence also displayed bright red fluorescence (Fig. 3), whereas cells without detectable green fluorescence showed only a low level of background, red fluorescence (not shown). Additionally, within cells expressing GFP-BAD, there was a strong correlation between the regions displaying higher intensities of green and those displaying higher intensities of red fluorescence (Fig. 3), with one important exception. Specifically, the nuclei displayed stronger GFP fluorescence than Cy3 fluorescence, indicating that streptavidin-Cy3 has restricted access to the inside of nuclei. As a second confirmation of endogenous biotinylation, Western blot analysis of GFP-BAD expression in transiently transfected immortal dysgenic myotubes showed a ~37-kDa biotinylated protein when probed with anti-GFP antibody or streptavidin-Poly-HRP (Fig. 3). Thus, histochemistry and Western blot analysis demonstrated that the metabolic biotinylation pathway in myotubes is able to effectively biotinylate the BAD fusion proteins.

Previous studies have shown that α₁S and β₁A subunits contained in junctionally targeted DHPRs are clustered in punctate foci, typically near the cell surface (13). Such punctate foci of XFP fluorescence were observed for all of the constructs examined here in which XFP and BAD were fused to sites of α₁S or β₁A (Figs. 5–10). Moreover, all of these constructs were able to support EC coupling, as indicated by electrically evoked contractions. In particular, evoked contractions were observed (Fig. 4) in both β₁-null myotubes expressing XFP-β₁A-BAD (14/21 cells) and BAD-β₁A-YFP (16/21) and in dysgenic myotubes expressing BAD-α₁S-YFP (20/28), XFP-α₁S(II–III)-BAD + α₁S(III–IV) (36/45), α₁S(II–III) + BAD-α₁S(III–IV)-YFP (14/27), α₁S(II–III)-BAD(III–IV)-YFP (42/52), GFP-α₁Slong-BAD (37/39), and GFP-α₁Sshort-BAD (20/27). Moreover, these same constructs mediated Ca²⁺ currents that did not differ dramatically from those of the wild-type constructs expressed in myotubes (14). Thus, the incorporation of a BAD did not appear to interfere with the targeting or essential function of the α₁S or β₁A fusion proteins.

Fig. 5 illustrates confocal fluorescent images from a dysgenic myotube in which YFP-β₁A-BAD was co-expressed with unlabeled α₁S. Because β₁A is freely diffusible in the absence of α₁S, the presence of yellow fluorescent foci is consistent with junctional targeting of DHPRs containing both the unlabeled α₁S and β₁A fusion proteins.
and YFP-β14-BAD. To determine whether BAD on the C-terminal had been biotinylated and was accessible to avidin, myotubes were fixed, permeabilized, and then exposed to avidin-rhodamine. Fig. 5 shows that red fluorescent foci were both present and co-localized with the yellow foci, as was observed in 28/35 dysgenic myotubes examined. A similar pattern of co-localizing red and yellow foci was also present when XFP-β14-BAD was expressed in β₁-null myotubes (9/9 cells). Thus, a large avidin molecule has access to sites located very near to the C-terminal of β₁α.

In endogenous biotin-containing proteins, the BAD is usually located near the C-terminal. Nonetheless, effective biotinylation was achieved when a BAD was fused to the N-terminal of the β₁α subunit, as is indicated by the occurrence of co-localizing red and yellow fluorescent puncta in β₁-null myotubes expressing BAD-β₁α-YFP (Fig. 6; 18/18 cells) and in dysgenic myotubes co-expressing both BAD-β₁α-YFP and unlabeled α₁S (12/12 cells). Moreover, coincident red and yellow foci were also observed in dysgenic myotubes expressing BAD-α₁S-YFP (Fig. 7; 19/19 cells). Thus, avidin has access to sites not only near both the N-terminal of BAD but also near the C-terminal of β₁α.
and C-terminals of \( \beta_{1a} \) and the N-terminal of \( \alpha_{1S} \) within fully assembled junctions.

An important goal of the present work was to probe the environment of the \( \alpha_{1S}(II-III) \) cytoplasmic loop. Because the results described above showed that either N- or C-terminal BAD could be effectively biotinylated, the initial approach was to separate \( \alpha_{1S} \) into two fragments (one containing repeats I–II with the proximal portion of the loop and the other containing the distal portion of the loop together with repeats III–IV). These constructs allowed attachment of a BAD to either the C-terminal (residue 671) of the proximal II–III loop, “\( XFP-\alpha_{1S}(I-II)-\text{BAD} \)” or the N-terminal (residue 686) of the distal II–III loop, “\( \text{BAD-}\alpha_{1S}(III-IV)-\text{YFP} \)” (residues 672–685) were omitted). These BAD containing constructs were then expressed with the appropriate complementary fragment: \( \alpha_{1S}(III-IV) \) (in some instances N-terminal tagged with YFP) or \( \alpha_{1S}(I-II) \), respectively. Previous studies have shown that two fragment constructs of \( \alpha_{1S} \) (without a BAD) are able to restore EC coupling in dysgenic myotubes (15, 16). Indeed, co-expression of either YFP-\( \alpha_{1S}(I-II) \)-BAD + \( \alpha_{1S}(III-IV) \), or \( \alpha_{1S}(I-II) + \text{BAD-}\alpha_{1S}(III-IV)-\text{YFP} \), in dysgenic myotubes resulted in restoration of EC coupling (see above) and the appearance of yellow punctate foci (Fig. 8, A and B), indicating that the BAD-tagged fragments were correctly targeted to junctions. After avidin-rhodamine staining, red fluorescent puncta co-localized with the foci of the XFP fluorescence (XFP-\( \alpha_{1S}(I-II) \)-BAD + \( \alpha_{1S}(III-IV) \), 22/22 cells; \( \alpha_{1S}(I-II) + \text{BAD-}\alpha_{1S}(III-IV)-\text{YFP} \), 9/9 cells; Fig. 8). Thus, avidin has access to sites near residues 671 and 686 of the II–III loop in two-fragment \( \alpha_{1S} \) constructs.

As a test of the ability of a BAD to become biotinylated when placed in the center of an intact protein, and of the environment of the II–III loop in a one-piece \( \alpha_{1S} \) construct, we constructed \( \alpha_{1S}(I-II)-\text{BAD-(III-IV)-YFP} \), where the BAD replaced residues 672–685 of \( \alpha_{1S} \). The BAD introduced into the II–III loop was biotinylated and accessible to avidin-rhodamine (11/11 cells), as illustrated by the tightly co-localized puncta of YFP and rhodamine fluorescence in Fig. 9. Thus, the proximal portion of the \( \alpha_{1S}(II-III) \) cytoplasmic loop is accessible to 60-kDa avidin molecules both when \( \alpha_{1S} \) is expressed as a single protein or as two-protein fragments divided at the proximal II–III loop.

The results above indicate that avidin has access to several sites of \( \alpha_{1S} \) and \( \beta_{1a} \). In contrast, the C-terminal of \( \alpha_{1S} \) appears to be inaccessible. Fig. 10 illustrates dysgenic myotubes expressing GFP-\( \alpha_{1S}\text{long-BAD} \) and GFP-\( \alpha_{1S}\text{short-BAD} \) (BAD following residues 1800 and 1667, respectively). Both constructs were functional in EC coupling (see above) and produced distinct green fluorescent puncta. However, there were no puncta of avidin staining that co-localized with the green puncta, a result that was consistently observed. Specifically, there was a clear absence of any co-localized puncta in 29/29 cells expressing GFP-\( \alpha_{1S}\text{long-BAD} \) and in 30/32 cells expressing GFP-\( \alpha_{1S}\text{short-BAD} \), with the other two cells showing some regions of aggregated red and green fluorescence that were difficult to interpret unambiguously as either being puncta or not. In principle, the lack of co-localized red and green puncta for GFP-\( \alpha_{1S}\text{long-BAD} \) could have been a consequence of proteolytic cleavage of the C-terminal, which has been reported to cause truncation (between residues 1685 and 1699) of the vast majority of \( \alpha_{1S} \) in adult skeletal muscle (17). However, green
fluorescence appears in junctional puncta after expression in myotubes of full-length α1S tagged on the C-terminal with GFP (18), which indicates either that relatively little truncation occurs in myotubes or that the distal segment remains anchored to the DHPR (19). In any case, red puncta coincident with green puncta were also not observed when the BAD was fused to the C-terminal (18), which indicates either that relatively little truncation occurred or that the distal segment remains anchored to the DHPR (19). In any case, red puncta coincident with green puncta were also not observed when the BAD was fused to the C-terminal (18), which indicates either that relatively little truncation occurred or that the distal segment remains anchored to the DHPR (19).

A second possibility, a lack of biotinylation, could explain the absence of co-localizing red and green puncta for BAD fused to the C-terminal. However, near the site of cDNA injection, there was both strong (but non-punctate) green fluorescence and red fluorescence with similar intensity and subcellular distribution. Farther from the injected nucleus, the rhodamine-avidin staining had a granular appearance, which as already mentioned did not overlap with the green puncta. Similar granular binding of avidin was also observed in non-injected myotubes. The differential pattern of avidin staining at sites near and far from the injected nucleus suggests that early in the biosynthetic pathway, BAD on the C-terminal of α1S is biotinylated but becomes inaccessible once α1S is inserted into fully assembled junctions. A third possibility, which could explain the lack of avidin binding to the C-terminal of junctionally targeted α1S constructs, is that RyR1 occludes access to the α1S C-terminal. This possibility was tested by expression of GFP-α1S-BAD in RyR1-lacking (dysgenic) myotubes. Indeed, as shown in Fig. 11, punctate avidin staining that co-localized with green fluorescent puncta was observed both when GFP-α1S-long-BAD (3/3 cells) and GFP-α1S-short-BAD (13/19 cells) were expressed in dysgenic myotubes. Thus, the presence of the Ryanodine receptor appears to prevent avidin from accessing the α1S C-terminal.

**DISCUSSION**

To probe the topology of the proteins at the triad junction, we have used endogenous biotinylation to investigate the ability of avidin, a 60-kDa molecule, to access specific sites of the DHPR within junctions. Effective biotinylation in living myotubes occurred as a consequence of insertion of a modestly sized BAD sequence (70 or 97 residues) at the N- and C-terminals of both α1S and β1a, as well as within the cytoplasmic II–III loop of α1S. The DHPR/BAD fusion proteins were correctly targeted as determined by punctate XFP fluorescence near the cell surface and by restoration of excitation-contraction coupling. To test whether avidin could access sites of the DHPR within fully assembled (RyR1-containing) junctions, permeabilized myotubes were exposed to rhodamine-avidin. As judged by the occurrence of red fluorescent foci that co-localized with the XFP foci, avidin had access to all but one of the sites tested for both β1a and α1S. The only exception was the C-terminal of α1S, which was inaccessible in junctions containing RyR1, but accessible in junctions lacking RyR1. These results are summarized in Fig. 12.

In thin section electron micrographs, much of the junctional gap between the SR and plasma membrane is filled by electron dense material contributed by the foot region of RyR1. Despite the presence of RyR1, however, it appears that these junctions can accommodate substantial additional mass (~60 kDa), inasmuch as avidin had access to the N- and C-terminals of β1a, and to the N-terminal and II–III loop of α1S. It is useful to compare this accessibility with results of other studies aimed at identifying potential sites of interaction between the DHPR and RyR1. The β1a subunit is required for trafficking of α1S to the plasma membrane (20) and also has modulatory effects on kinetics and voltage dependence of the L-type calcium current (reviewed in Ref. 21). Moreover, functional analyses of β1 cDNA constructs expressed in β1-null myotubes have revealed an important role for the β1a C-terminal in EC coupling (22). Indeed, deletion of the final 29 residues of β1a largely eliminates skeletal-type EC coupling (23). In addition, preliminary studies have shown that the β1a subunit binds to RyR1 (24). These results raise the possibility that the distal portion of the β1a C-terminal interacts with RyR1. However, if this interaction does occur, it is not sufficient to occlude access of avidin to a site nearby since BAD fused to the C-terminal of the β1a subunit did not grossly affect EC coupling and was accessible for avidin binding.

A number of studies have suggested that the II–III loop of α1S plays an important role in the orthograde and retrograde signaling between the DHPR and RyR1. For example, application of small peptides corresponding to the proximal portion of the II–III loop (α1S residues 671–690; “peptide A”) activates RyR1, as measured by rymyodine binding, single channel activity, and calcium release (25–28). However, when the DHPR is directed in dysgenic myotubes, scrambling the peptide A sequence, replacing it with non-related sequence, or deleting it entirely, does not appear to impair function of α1S as voltage sensor or Ca^2+ channel (15, 16, 29, 30). Consistent with these results, insertion of BAD in place of α1S residues 671–686 (α1S–II-BAD–III–IV)-YFP does not interfere with the ability of α1S to mediate EC coupling. An additional argument that this general region of the II–III loop does not interact with RyR1 is that BAD introduced into this site is accessible to avidin binding. Moreover, the accessibility in three different constructs (YFP-α1S-II-BAD, BAD-α1S-III–IV-YFP, α1S-II–III-BAD–III–IV-YFP) suggests that this accessibility is not an artifact of a particular construct. It is also important to consider the site of the BAD placement with respect to downstream residues that are important in EC coupling. In particular, α1S residues 720–765 (31, 32), or more minimally 734–748 (33) in α1C chimeras, are able to restore full orthograde and retrograde DHPR/RyR1 coupling. Moreover, yeast two-hybrid analyses indicate a weak interaction between α1S loop residues 720–765 and RyR1 residues 1837–2168 (34). In the constructs BAD-α1S-III–IV-YFP and α1S-II–III-BAD–III–IV-YFP, the BAD was attached at α1S residue 686 that was 49 residues upstream of the minimal sequence identified by Kugler et al. (33). Of course, the three-dimensional structural relationship between BAD and this minimal sequence remains uncertain. However, the accessibility of avidin makes it unlikely that this minimal sequence binds to a deep pocket within RyR1, particularly because biotin is almost completely embedded within the binding pocket of avidin (35).

In regard to the α1S C-terminal, the distal portion (residues...
expressing GFP-α1S, long-BAD. Right, dysgenic myotube expressing GFP-α1S, short-BAD. Although puncta of green fluorescence were present for both constructs, there were no co-localized foci of avidin-rhodamine fluorescence. A complete lack of biotinylation did not appear to be responsible for the absence of punctuate avidin-rhodamine staining. In particular, there was a similar pattern of diffuse fluorescence for GFP and avidin-rhodamine in regions of highly expressed protein not yet in junctions (arrows). Scale bars = 5 μm.

![Image](image_url)

**Fig. 10.** The C-terminal of α1S is biotinylated but inaccessible to avidin within fully assembled junctions. Left, dysgenic myotubes expressing GFP-α1S, long-BAD. Right, dysgenic myotube expressing GFP-α1S, short-BAD. Although puncta of green fluorescence were present for both constructs, there were no co-localized foci of avidin-rhodamine fluorescence. A complete lack of biotinylation did not appear to be responsible for the absence of punctuate avidin-rhodamine staining. In particular, there was a similar pattern of diffuse fluorescence for GFP and avidin-rhodamine in regions of highly expressed protein not yet in junctions (arrows). Scale bars = 5 μm.

An important caveat for the present studies is that most of the experiments were done on fixed and permeabilized myotubes. Thus, it is uncertain which functional states of the DHPR and RyR1 are responsible for the observed avidin binding. Because of these limitations, questions still remain: does avidin binding depend on the functional state, and does avidin binding alter function? However, the results from the BAD experiments involving fixed and permeabilized myotubes are supported by complementary studies of living myotubes (8) expressing DHPR constructs in which a CFP-YFP tandem was inserted at the same sites described in this paper for BAD insertion. In all cases, function of the DHPR as channel and voltage sensor for EC coupling did not appear to be disrupted. Moreover, for all but two of these sites, the fluorescent resonance energy transfer efficiency of the inserted CFP-YFP tandem was unaffected by whether or not RyR1 was present, indicating that it is unlikely that RyR1 is in close proximity to any of these sites. This conclusion is strengthened by the present results showing that avidin has access to the BAD at all of these sites, taking into account that avidin (8 × 8 × 8.5 nm; Protein Data Bank code 1AVD, Ref. 35) is larger than either CFP or YFP (5 × 6 × 7 nm; Protein Data Bank code 1EMA, Ref. 39). Thus, an important conclusion from both the CFP-YFP experiments and the BAD experiments is that functional junctions can accommodate substantial additional mass at a number of sites.

For two sites (N-terminal of β1a, shortened C-terminal of α1S), the fluorescent resonance energy transfer efficiency of the inserted CFP-YFP did depend strongly on the presence of RyR1 as evidenced by a substantial increase in efficiency in dyspedic myotubes compared with dysgenic myotubes (8). Thus, both might represent sites at which the DHPR approaches closely to RyR1. It is interesting, however, that avidin was able to access biotinylated BAD at the β1a N-terminal, which raises the possibility that this site opens freely into the myoplasm. However, it is possible to reconcile both the CFP-YFP and BAD results if one were to assume that BAD on the β1a N-terminal lies in a fairly compact space but is oriented such that biotin extends sufficiently out of this space to allow avidin binding. In the case of the C-terminal of α1S, the CFP-YFP and BAD results are both consistent with localization within a fairly compact space, particularly because avidin lacked access to either the α1S short or long C-terminal. This postulated occlusion could occur either because the α1S C-terminal lies within a pocket of RyR1 or because RyR1 causes the DHPR to assume a configuration such that another protein (perhaps α1S itself) occludes the C-termin-

1543–1647) has been shown to be important for targeting to junctions (29, 36), which may indicate a binding interaction with other junctional proteins. However, this putative targeting interaction seems unlikely to account for the differential ability of avidin to bind to BAD at the α1S C-terminal in dyspedic but not dysgenic myotubes because α1S targets to junctions in both cell types. Besides being important for targeting, several studies suggest that the α1S C-terminal may interact with RyR1. For example, a peptide corresponding to α1S residues 1487–1506 inhibits RyR1 in vitro (37). Moreover, α1S residues 1393–1527 bind calmodulin and, in the absence of calmodulin, bind directly to RyR1 residues 3609–3643 (38). Thus, the inability of avidin to access the C-terminal of α1S within RyR1-containing junctions may be a reflection of this binding interaction.

![Image](image_url)

**Fig. 11.** The presence of the ryanodine receptor is responsible for the inaccessibility of the α1S C-terminal to avidin. In contrast to expression in dysgenic myotubes, expression of GFP-α1S, short-BAD in a dyspedic (RyR1-null) myotube resulted in co-localized fluorescent puncta of both GFP and avidin-rhodamine. Scale bar = 5 μm.

![Image](image_url)

**Fig. 12.** The C-terminal of α1S is occluded from avidin binding, most likely because of the presence of the ryanodine receptor. Schematic of avidin molecules (orange circles) binding to biotin (brown line) on the BADs (red circles) of BAD-DHPR fusion proteins in a dysgenic myotube. Note that avidin did not bind to the BAD attached at the C-terminal of α1S.

Note that avidin did not bind to the C-terminal of α1S within RyR1-containing junctions may be a reflection of this binding interaction.
nal. It is important to state the obvious point that both the CFP-YFP tandem and BAD can at best be inserted near sites of interaction between junctional proteins because interrupting these sites would abolish function.

One can expect that future studies will provide improved information about the three-dimensional structure of both the DHPR and RyR1 and about the localization within these structures of specific sites (e.g., αT(II–III) loop). Obviously this information will be of considerable value for the interpretation of both the CFP-YFP and BAD studies. Conversely, because the structural studies will likely be limited to the individual proteins (either DHPR or RyR), the CFP-YFP and BAD methods will be important for understanding the disposition of the DHPR and RyR with respect to one another within functioning cells. It will also be important to determine whether the access of avidin to biotinylated BAD depends on the functional status of the EC coupling apparatus (resting, activated, and inactivated) and whether the fluorescent resonance energy transfer efficiency of the CFP-YFP tandem displays changes that are correlated with such functional changes. However, even without awaiting these further refinements, the ability to introduce specific biotinylation at both terminal and internal sites of cellular proteins should provide a useful tool for studying the architecture of macromolecular assemblies in diverse cell types.

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