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 correctly 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 junctions, but not to the other sites of localization. It was then possible to determine whether the introduced BAD was biotinylated and was accessible for binding of fluorescently labeled avidin.

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 Ca\(^{2+}\) release via RyR1 that does not require the entry of extracellular Ca\(^{2+}\) (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 energy transfer efficiency of a 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 avidin can access biotin introduced at different sites of the DHPR.

The XFP/BAD/DHPR fusions were found to be functional in EC coupling and to target to discrete focal indicators of membrane junctions. Moreover, effective, metabolic biotinylation occurred for BAD at the N- and C-terminals of the β\(_{1α}\) and α\(_{1S}\) subunits of the DHPR, as well as at an internal site within the α\(_{1S}\) 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 α\(_{1S}\) was inaccessible to avidin in dysgenic myotubes (containing RyR1). In contrast, this site was accessible to avidin when the identical construct was expressed in dysgenic myotubes lacking RyR1. These results indicate that avidin has access to a number of sites of the DHPR within fully assembled (RyR1-containing) junctions, but not to the α\(_{1S}\) C-terminal, which appears to be occluded by the presence of RyR1.

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of the DHPR (β1a N- and C-terminals, α1α 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 1DC2, 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 α1α and β1a 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-α1α-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 G157 (by inserting GGGG-TACC-CCG-5′ to nucleotide G227, 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 TGGGATCCCGG-3′ by inserting TAGGTACCGG-3′ to nucleotide G276. The 219-base pair (bp) fragment yielded by the PSTCD coding sequence. The 219-bp KpnI BAD fragment was ligated to the N-terminal of GFP-α1α to which it is covalently attached, but the flexibility of the linkage suggests substantial mobility. The modified plasmid was digested with KpnI, and the 218-bp fragment was ligated to the N-terminal of the 219-bp BAD fragment to produce BAD-α1α-BAD with a 16-residue linker connecting the 97-residue BAD to the N-terminal of α1α-BAD.

**BAD-β1a-YFP**—The β1a-YFP plasmid (8) was opened using restriction cuts with EcoRI and Nhel. A 369-bp BAD fragment (encoding residues 112–153) was isolated from BAD-β1a-BAD-YFP with EcoRI and Nhel. The opened β1a plasmid and the BAD fragment were co-ligated producing BAD-β1a-BAD-YFP. A 13-residue linker connected the 97-residue BAD to the N-terminal of β1a-YFP.

**BAD-α1α(III–IV)-YFP**—A 347-bp BAD fragment (encoding residues 126–128) was isolated from BAD-α1α-BAD-YFP by restriction cuts with SacI and Nhel. The plasmid α1α(III–IV)-YFP (8) was digested with SacI and Nhel, opening the plasmid before the N-terminal of α1α 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-BAD-β1a-YFP. This construct was co-expressed with the plasmid α1α(II–III)-YFP (8). The α1α(II–III)-YFP plasmid was digested with restriction cuts with Nhel and BamHI. These two fragments were co-ligated to obtain α1α(II–III)-BAD-III–IV–YFP. A 14-residue linker connected α1a-BAD-β1a(II–III)-BAD-III–IV–YFP plasmid (see above) with Nhel and BgIII at the multiple cloning regions preceding the α1α coding sequence. The α1α(II–III)-BAD-β1a(II–III)-BAD-III–IV–YFP plasmid was digested with restriction cuts with Nhel and BamHI. Two fragments were co-ligated to obtain α1α(II–III)-BAD-III–IV–YFP. A 14-residue linker connected α1α(II–III)-BAD-β1a(II–III)-BAD-III–IV–YFP plasmid with a 6-residue linker to α1α(II–III)-BAD-β1a-BAD-YFP. The modified plasmid was digested with KpnI, and the 218-bp fragment was inserted into the KpnI-cleavage site of pEFP-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/ml). 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).

**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 (here after referred to as “avidin”); Molecular Probes, Eugene, OR) or streptavidin-Cy3 (Jackson ImmunoResearch, West Grove, PA). After 5400–15000 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 in 3% milk/PBS, and exposed to monoclonal GFP antibody (1:1,000 in 3% milk/PBS) (Chemicon, Temecula, CA) overnight at room temperature. The blot was washed with 0.1% Tween 20, PBS, 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 Streptavidin-Biotin (Pierce), and then incubated with streptavidin-poly-HRP (Pierce), diluted (1:10,000 in Poly-HPHR 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: GFP: excitation at 458 and 458 nm dichroic, and emission using a 465–485 nm band pass filter (Chroma Technology Corp., Rockingham, VT); GPP, excitation at 488 and 488/543 nm dual dichroic, and emission with a 505–530 nm band pass filter; YFP, excitation 488 and 488/543 nm dual dichroic, and emission with a 505–530-nm band pass filter (Chroma Technology Corp., Rockingham, VT); and EGFP, excitation at 488 and 488/543 nm dual dichroic, and emission with a 495-nm band pass filter (Chroma Technology Corp., Rockingham, VT). For detection of Alexa Fluor 594, a 560-nm long pass filter was needed for detection. Cells were viewed with a ×40 (1.3 NA) or ×63 (1.4 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 DHPR) to determine whether a single fragment α₁S construct (top, “α₁S–II–BAD–(III–IV)”), or double fragment constructs (“α₁S–II–BAD plus α₁S–IV”), respectively) were used. In all of the II–III loop constructs, α₁S residues 672–687 were absent. To probe the α₁S C-terminal, BAD was attached to either after residue 1860 (“α₁S–long-BAD”) or after residue 1667 (“α₁S–Short-BAD”). In addition to containing a BAD, each construct also contained a fluorescent protein attached to either the N- or C-terminal as an independent report of localization.

**Electrically evoked contractions**

Contractions were elicited by 10-ms, 55–100 V stimulus 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.

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FIG. 2. Schematic illustration of the BAD-DHPR fusion constructs. All eight sites of BAD (red circles) attachment are shown although each individual construct contained only one BAD. In the text, the constructs are designated by the site of BAD attachment (e.g., BAD–β₁a represents BAD attached to the N-terminal of β₁a). To probe the α₁S–II–BAD–(III–IV) loop, either a single fragment α₁S construct (top, “α₁S–II–BAD–(III–IV)”), or double fragment constructs (“α₁S–II–BAD plus α₁S–IV”), or “α₁S–II–BAD plus α₁S–IV”, “middle and bottom”, respectively) were used. In all of the II–III loop constructs, α₁S residues 672–687 were absent. To probe the α₁S C-terminal, BAD was attached either after residue 1860 (“α₁S–long-BAD”) or after residue 1667 (“α₁S–Short-BAD”). In addition to containing a BAD, each construct also contained a fluorescent protein attached to either the N- or C-terminal as an independent reporter of localization.
and YFP-β1a-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-β1a-BAD was expressed in β1-null myotubes (9/9 cells). Thus, a large avidin molecule has access to sites near to the C-terminal of β1a.

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 β1a subunit, as is indicated by the occurrence of co-localizing red and yellow fluorescent puncta in β1-null myotubes expressing BAD-β1a-YFP (Fig. 6; 18/18 cells) and in dysgenic myotubes co-expressing both BAD-β1a-YFP and unlabeled α1S (12/12 cells). Moreover, coincident red and yellow foci were also observed in dysgenic myotubes expressing BAD-α1S(III–IV)-YFP (Fig. 7; 19/19 cells). Thus, avidin has access to sites near both the N-terminal and the C-terminal of β1a.
and C-terminals of $\beta_{1a}$ and the N-terminal of $\alpha_{18}$ within fully assembled junctions.

An important goal of the present work was to probe the environment of the $\alpha_{18}$(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 $\alpha_{18}$ 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-$\alpha_{18}$(I–II)-BAD,” or the N-terminal (residue 686) of the distal II–III loop, “BAD-$\alpha_{18}$(III–IV)-YFP” (residues 672–685 were omitted). These BAD containing constructs were then expressed with the appropriate complementary fragment: $\alpha_{18}$(III–IV) (in some instances N-terminal tagged with YFP) or $\alpha_{18}$(I–II), respectively. Previous studies have shown that two fragment constructs of $\alpha_{18}$ (without a BAD) are able to restore EC coupling in dysgenic myotubes (15, 16). Indeed, co-expression of either YFP-$\alpha_{18}$(I–II)-BAD + $\alpha_{18}$(III–IV), or $\alpha_{18}$(I–II) + BAD-$\alpha_{18}$(III–IV)-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-$\alpha_{18}$(I–II)-BAD + $\alpha_{18}$(III–IV), 22/22 cells; $\alpha_{18}$(I–II) + BAD-$\alpha_{18}$(III–IV)-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 $\alpha_{18}$ 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 $\alpha_{18}$ construct, we constructed $\alpha_{18}$(I–II)-BAD-(III–IV)-YFP, where the BAD replaced residues 672–685 of $\alpha_{18}$. 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 $\alpha_{18}$(II–III) cytoplasmic loop is accessible to 60-kDa avidin molecules both when $\alpha_{18}$ 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 $\alpha_{18}$ and $\beta_{1a}$. In contrast, the C-terminal of $\alpha_{18}$ appears to be inaccessible. Fig. 10 illustrates dysgenic myotubes expressing GFP-$\alpha_{18}$long-BAD and GFP-$\alpha_{18}$short-BAD (BAD following residues 1860 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-$\alpha_{18}$long-BAD and in 30/32 cells expressing GFP-$\alpha_{18}$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-$\alpha_{18}$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 $\alpha_{18}$ 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 at a position upstream of the potential proteolytic site (i.e., GFP-α1Sshort-BAD in Fig. 10).

A second possibility, a lack of biotinylation, could explain the absence of co-localizing red and green puncta for BAD fused to the α1S 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 foci was observed both when GFP-α1Slong-BAD (3/3 cells) and GFP-α1Sshort-BAD (13/19 cells) were expressed in dysgenic myotubes. Thus, the presence of the ryonodine 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 β1c 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 ryanodine binding, single channel activity, and calcium release (25–28). However, when the DHPR is expressed 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 Ca²⁺ channel (15, 16, 29, 30). Consistent with these results, insertion of BAD in place of α1S residues 671–686 (α1S(II–III)-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–III)-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
Thus, the inability of avidin to access the C-terminal of calmodulin, bind directly to RyR1 residues 3643 (37). 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-termi-
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., αII(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.

Acknowledgment—We thank Kathy Parsons for technical assistance with the myotubes cultures.

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