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DISSERTATION

YEAST TWO-HYBRID ANALYSIS OF SKELETAL MUSCLE TRIAD JUNCTION PROTEINS

Submitted by

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Department of Physiology

In Partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall, 1999

COLORADO STATE UNIVERSITY

NOVEMBER 8, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CATHERINE PROENZA ENTITLED **YEAST TWO-HYBRID ANALYSIS OF SKELETAL MUSCLE TRIAD JUNCTION PROTEINS** BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

YEAST TWO-HYBRID ANALYSIS OF SKELETAL MUSCLE TRIAD JUNCTION PROTEINS

Excitation-contraction (EC) coupling in muscle is the process by which electrical stimulation releases calcium from the sarcoplasmic reticulum (SR), thereby allowing cross-bridge cycling between actin and myosin to occur. EC coupling occurs at specialized membrane regions called triad junctions. In this dissertation, the yeast two-hybrid system was used to search for interactions among known and novel proteins of triad junctions. In direct interaction tests, no strong interaction was detected between the dihydropyridine receptor (DHPR) II-III loop and the skeletal muscle ryanodine receptor (RyR); however, a possible weak interaction between physiologically important parts of the two receptors was investigated. In screens of a skeletal muscle cDNA library, no interactors were detected for the N-terminus, the I-II, II-III, or III-IV loops of the skeletal muscle DHPR. Some potential interactors of s53, of RyR1 and of junctin were identified.

The carboxyl terminals of both skeletal and cardiac DHPRs were extremely reactive in two-hybrid library screens. The reactive region of the skeletal DHPR was

identified as residues 1621-1647. Five candidate interaction partners of the reactive region, including the glycolytic enzymes aldolase and enolase, were identified. A hypothesis for the physiological basis of the reactivity of segment E is discussed. A GFP-labeled α_{1s} subunit truncated at residue 1620, just before the reactive region, was found to have qualitatively normal expression, current, and EC coupling when expressed in dysgenic myotubes.

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OVERVIEW

Excitation-contraction (EC) coupling is the transduction mechanism that converts electrical excitation of a muscle to the release of intracellular calcium that triggers cross bridge cycling. Dihydropyridine receptors (DHPRs) are L-type Ca^{2+} channels in the plasmalemma that act as voltage sensors for EC coupling (Rios and Brum, 1987). Ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR) membrane are the intracellular calcium release channels for EC coupling (Nabauer et al., 1989). In mature skeletal muscle, EC coupling occurs at specialized membrane regions called triad junctions. A triad junction is composed of two SR terminal cisternae which flank an invagination of the plasmalemma called a transverse tubule (T-tubule; Fig. 1). In cardiac muscle, similar junctions exist between the surface membrane and the SR (peripheral couplings), and between T-tubules and single SR (dyads). DHPRs and RyRs are localized to these junctional membranes in both skeletal and cardiac muscle. However, different DHPR and RyR isoforms exist in heart and skeletal muscle, and the mechanism of EC coupling differs in the two tissues. In cardiac muscle, voltage-gated Ca^{2+} entry through the DHPR triggers the opening of ryanodine receptors; whereas, in skeletal

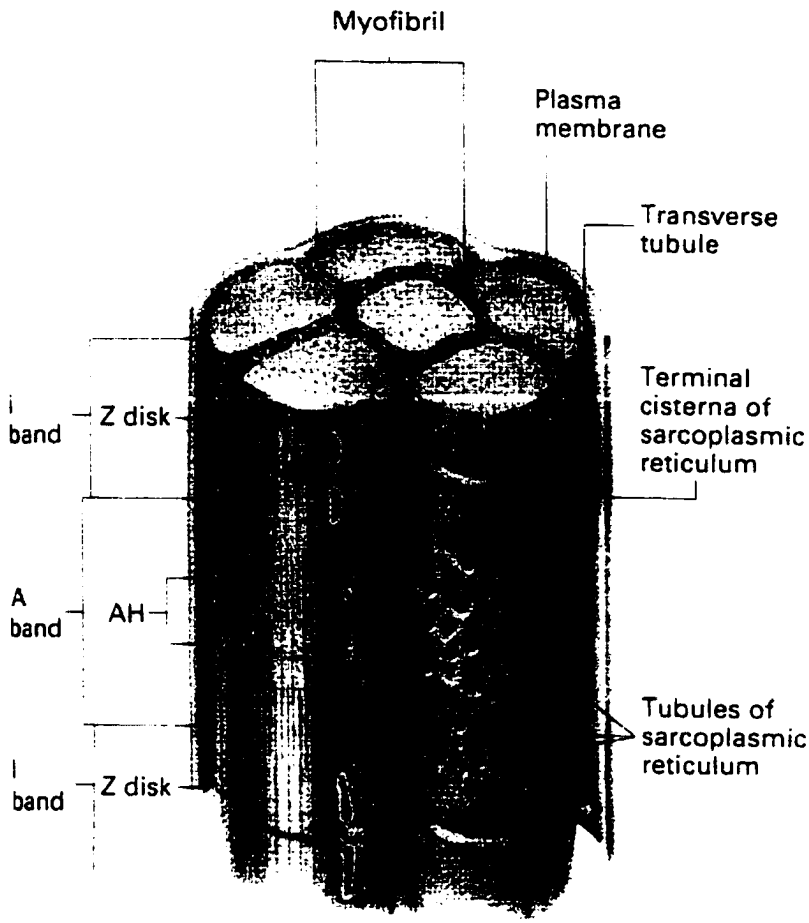


Figure 1. Arrangement of transverse tubules and sarcoplasmic reticulum to form the triad junction. Transverse tubules poke into the myocyte and are surrounded by the terminal cisternae of the sarcoplasmic reticulum. From Darnell et al., 1990.

muscle, depolarization alone is sufficient to trigger SR Ca^{2+} release even in the absence of Ca^{2+} entry (Armstrong et al., 1972; Rios and Brum, 1987; Tanabe et al., 1988). The mechanism of this communication between DHPRs and RyRs in skeletal muscle remains unknown. The "induced ligand hypothesis" postulates that a voltage-gated

conformational change in the DHPR allosterically controls ryanodine receptor gating (Schneider and Chandler, 1973).

This dissertation is not only about excitation-contraction coupling. It is also about using and interpreting the yeast two-hybrid system (Fields and Song, 1989). As is the case for any complex technique, a thorough understanding of the two-hybrid system is essential for interpretation of data. Consequently, chapter 1 of this dissertation is a description and discussion of the methods employed, with special emphasis on the design of the two-hybrid system and on interpretation of its results.

In chapter 2, experimental data are presented to test the "induced ligand hypothesis," which proposes a direct interaction between DHPRs and RyRs. Chapter 3 addresses the structure of the triad junction itself — what are the basic molecules required for the close apposition of the plasmalemma and the SR membrane, and what interactions underlie the formation of DHPR tetrads? Finally, in chapter 4, a reactive region of the DHPR C-terminus is identified and its effects on the function and distribution of the channel are assessed.

CHAPTER 1
EXPERIMENTAL PROCEDURES

INTRODUCTION

The yeast two-hybrid system exploits the modular nature of transcriptional activators as a measure of protein-protein interactions *in vivo* (Fields and Song, 1989). As illustrated in figure 1.1, two sets of fusion proteins are constructed: cDNA for one target protein is inserted into a plasmid encoding the binding domain of a transcriptional activator protein and cDNA encoding a test second protein — or a fragment from a cDNA library — is fused in-frame with the activation domain coding region in a second plasmid. If the target proteins interact with one another, they tether the two domains of the transcription factor together and reconstitute its ability to activate transcription of reporter genes.

The two-hybrid system used in this dissertation is based on the GAL4 promoter. The native GAL4 protein in yeast is a 881 residue, galactose-responsive regulatory protein that includes an N-terminal 147 amino acid DNA binding domain and a C-terminal 113 amino acid activation domain. When galactose is present, the GAL4

protein binds to a consensus 17-bp DNA sequence upstream of several genes involved in galactose metabolism and activates transcription. In the absence of galactose, a second regulatory protein, GAL80, binds to GAL4 and blocks transcriptional activation. The yeast strains used in this system carry deletions of the *gal4* and *gal80* genes to avoid interference by endogenous GAL4 and GAL80 proteins. Thus, no induction of the engineered downstream genes should be observed unless a two-hybrid interaction occurs.

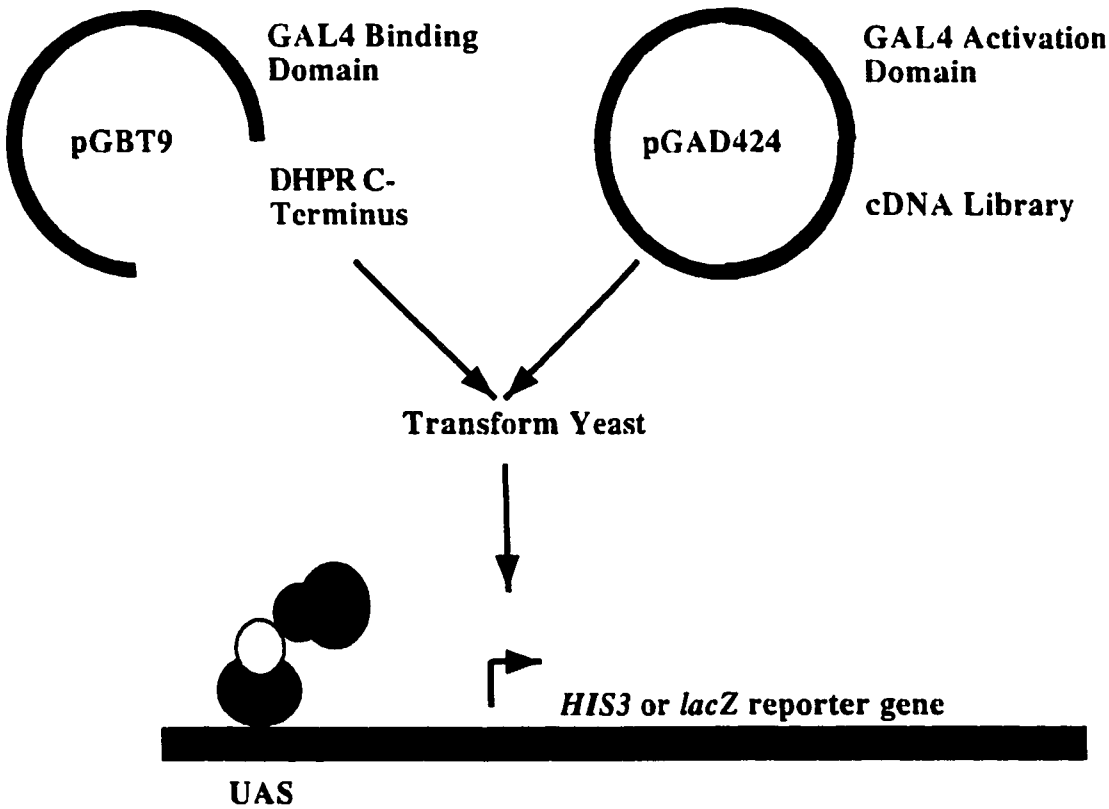


Figure 1.1 Yeast two-hybrid method. Yeast is cotransformed with two plasmids encoding fusions of proteins of interest and either the activation domain or DNA binding domain of the GAL4 transcription factor. Upon translation, interaction of the test proteins tethers the two parts of the transcription factor together, enabling them to activate transcription of reporter genes. In strain HF7c, *HIS3* and *lacZ* are the two reporter genes. *HIS3* encodes the His3p protein which allows the cells to grow on plates lacking histidine; *lacZ* encodes the b-galactosidase protein.

The yeast shuttle vectors used were pGBT9 and pGAD424/pGAD10. The GAL4 binding domain plasmid, pGBT9, contains sequence encoding the GAL4 DNA-binding domain (GAL4-BD, residues 1-147 of GAL4), and pGBT9 carries the *TRP1* nutritional gene which allows yeast auxotrophs transformed with the plasmid to grow on synthetic defined medium lacking tryptophan. A nuclear localization sequence is an intrinsic part of the GAL4-BD. pGAD10 and pGAD424 are identical except for their multiple cloning sites. They encode fusion proteins in-frame with the GAL4 activation domain (GAL4-AD, residues 768-881 of GAL4). pGAD10 and pGAD424 include the nuclear localization sequence from the SV40 T-antigen upstream of the AD sequence, and they carry the *LEU2* nutritional gene that allows transformed yeast auxotrophs to grow on synthetic defined medium lacking leucine.

MATERIALS AND METHODS

Cloning

Domains of interest from skeletal and cardiac DHPRs and RyRs, from junctin, from FKBP, and from sLIM were amplified from the templates indicated in Table 1.1 by PCR and inserted into pGBT9 and pGAD424 by standard means using restriction sites added to the ends of the PCR products as indicated in Table 1.1. pCAC6 is the skeletal DHPR receptor inserted into the expression plasmid pCRH2 (Tanabe et al., 1990).

Table 1.1 PCR Primers

Clone	Foreward Primer	Reverse Primer	Template (amplified region, bp)	Sites	Vector(s)
αID	GGAATTCGAGAAGGCCAAGT CCAGGGGAACC	CGGGATCCTCTCAGGTCCTC CACGTCCATGAC	pCAC6 (1024-1146)	EcoRI-BamHI	pGBT9
βID	GGAATTCAAGGAGGGCTGC GAGGTT	CGGGATCCTGCTGGGGTTGT TGAGGA	pCAS14 (933-1055)	EcoRI-BamHI	pGAD424, pGBT9
N-term	GGGAATTCATGGAGCCATCC TCACCCCAG	CCGGATCCTCATTTCCATTCC ACGAGCTGAT	pCAC6 (1-153)	EcoRI-BamHI	pGBT9
I-II loop	CGGGATCCTCCTGAGTGGGG AATTC	AACTGCAGCTCTCGACTTCA CCAGGTCATG	pCAC6 (995-1296)	EcoRI-SalI	pGBT9
II-III loop	GGGAATTCGTCTTCCTGGCC ATCGCCGTG	CCGAATTCTCAGTTGACGAT GCGGTGACACAG	pCAC6 (1963-2391)	EcoRI-BamHI	pGBT9
III-IV loop	GGAATTCCTTGTGGGCTTTG TCATCGTC	CGGGATCCCGTGAGGGCGA ACATCAGGTATT	pCAC6 (3179-3379)	EcoRI-BamHI	pGBT9
cardiac II-III	CCGAATTCGTGTTCTTGGCC ATTGCTGTG	CCGAATTCTCAGTTGACGAT ACGGTGACACTG	pCARD1 (2352-2790)	EcoRI-BamHI	pGBT9
s53	GGAATTCAAGCTCAAGGTCG ATGAG	CGGGATCCTTGAGCTGCAGC TCGGC	pCAC6 (2147-2312)	EcoRI-BamHI	pGAD424, pGBT9
s31	GGAATTCGAGGCGGAGAGC CTGACTTCC	CGGGATCCGGGCTTCTGCTC CAGCTTCTT	pCAC6 (1996-2127)	EcoRI-BamHI	pGAD424, pGBT9
s58	GGAATTCGAATCTAACGTCA ACGAGGTG	CGGGATCCCGATCATCCCCT GGGAAGTCAGC	pCAC6 (2178-2235)	EcoRI-BamHI	pGBT9
cardiac 53	GGAATTCAAGATCAACATGG ATGAC	CGGGATCCTTAAGGTGCAGC TCGGAG	pCARD1 (2547-2692)	EcoRI-BamHI	pGBT9
sR16	GCGAATTCCAGTTCGTGCCG GTGCTCAAG	GCGGATCCGAGCGGCATCTT GGCGAACTCGTT	pCIneo-RyR1 (5509-6504)	EcoRI-BamHI	pGAD424, pGBT9
sR16 A	GCGAATTCCAGTTCGTGCCG GTGCTCAAG	CGGGATCCGCACATCTGTAG CTTCACGGA	pCIneo-RyR1 (55109-5820)	EcoRI-BamHI	pGAD424
sR16 B	GGAATTCTCCGTGAAGCTAC AGATGTGC	CGGGATCCTCCACAGTGCGC CAG	pCIneo-RyR1 (5800-6129)	EcoRI-BamHI	pGAD424
sR16 C	GGAATTCCTGGCGCACTGTG GAAT	GCGGATCCGAGCGGCATCTT GGCGAACTCGTT	pCIneo-RyR1 (6115-6504)	EcoRI-BamHI	pGAD424

Clone	Foreward Primer	Reverse Primer	Template (amplified region, bp)	Sites	Vector(s)
D2	GGAATTCTGGGGCGAGGCT GAAGGGGGC	CGGGATCCTGGCTGAGTCAT CATGGCGGC	pCIneo-RyR1 (4024-4206)	EcoRI-BamHI	pGAD424
D3	GGAATTCACCGAGGAAGAG GAGGAGGAA	CGGGATCCCTCCAAATCTTCT TTTTCCCC	pCIneo-RyR1 (5614-5769)	EcoRI-BamHI	pGAD424
cR16	GCGAATTCCTCTTTGTGCCTC TCATC	CGGGATCCCATAAGCTTCTC TTCCTCTTT	pCIneo-R14 (5458-6425)	EcoRI-BamHI	pGAD424, pGBT9
R10A	GGAATTCCCCGTGACCATGA TGGCG	CGGGATCCATCCTCATCGCC AAAGATGC	pCIneo-RyR1 (4899-5574)	EcoRI-BamHI	pGAD424
R10B	GGAATTCGTGCTCATCGTGC AGATGG	CGGGATCCTTCTTCAGGTGG CTCCTCCCC	pCIneo-RyR1 (6495-7242)	EcoRI-BamHI	pGAD424, pGBT9
R10C	GGAATTCGAGGAGCCCCCTG AAGAAAAC	CGGGATCCGAACTCGTTCAG GATGGGC	pCIneo-RyR1 (7224-7908)	EcoRI-BamHI	pGAD424, pGBT9
R9A	GGAATTCTTCGCCAAGATGC CGCTCA	CGGGATCCGGCGAACCGCTT CTCAATGGA	pCIneo-RyR1 (7906-8868)	EcoRI-BamHI	pGAD424, pGBT9
R9B	GGAATTCTCCATTGAGAAGC GGTTCGCC	CGGGATCCGTCTGCCATGAG CCGATCCAG	pCIneo-RyR1 (8848-9566)	EcoRI-BamHI	pGAD424, pGBT9
R9C	GGAATTCCTGGATCGGCTCA TGGCAGAC	CGGGATCCAAATTCCCGGAC CTCCTCGTC	pCIneo-RyR1 (9736-10685)	EcoRI-BamHI	pGAD424, pGBT9
human enolase-β A	GGAATTCATGGCCATGCAGA AAATCTTTGCC	CGGGATCCGCCGATCACCAC CTTGTCTGG	muscle cDNA library(1-729)	EcoRI-BamHI	pGAD424, pGBT9
human enolase-β B	GGAATTCCCAGACAAGGTGG TGATCGGC	CGGGATCCCTTGGCCTTCGG GTTACG	muscle cDNA library(709-1302)	EcoRI-BamHI	pGAD424, pGBT9
FKBP	GGAATTCCATGGGAGTGCAG GTGGA	CGGGATCCCGGAGGAGGCC ATTCCTGTCA	muscle cDNA library(1-520)	EcoRI-BamHI	pGBT9
Junctin	GGAATTCATGGCTGAAGAGA CAAAGCAT	CGGGATCCTCAGTTCTTCCTC TTCTGGTT	dog junctin clone (1-633)	EcoRI-BamHI	pGBT9
Cytojunctin	GGAATTCATGGCTGAAGAGA CAAAGCAT	CGGGATCCTGAACTTCCAGA AAGTCCTCC	muscle cDNA library(1-66)	EcoRI-BamHI	pGBT9

Clone	Foreward Primer	Reverse Primer	Template (amplified region, bp)	Sites	Vector(s)
sLIM 154-280	GGAATTCCATGAGACCAAGT TGGCC	CGGGATCCCAGCTGTTTGGC AGAGTCGGG	muscle cDNA lib. (448-840)	EcoRI-BamHI	pGBT9
sk C term	GGAATTCGACCAACTTTGAC TACCTGACA	AACTGCAGCGGCCTGGGAG GAATAAGG	pCAC6 (4143-5619)	EcoRI-PstI	pGBT9
C6Δ1	GGAATTCGACCAACTTTGAC TACCTGACA	AACTGCAGGTTGGTATTGGC ACGAGCGAG	pCAC6 (4143-4986)	EcoRI-PstI	pGBT9
sk c16	GGAATTCCCTCTCGCTCGTG CCAATACC	AACTGCAGCGGCCTGGGAG GAATAAGG	pCAC6 (4965-5619)	EcoRI-PstI	pGBT9
sk c13	GGAATTCGACCAACTTTGAC TACCTGACA	AACTGCAGGAAGTTACCTTC TGTCTTGAT	pCAC6 (4143-4449)	EcoRI-PstI	pGBT9
sk c14	GGAATTCACAGAAGGTAACT TCGAGCAGGCC		pCAC6 (4437-4986)		pGBT9
Hamilton Inhibitory region	GGAATTCACAGAAGGTAACT TCGAGCAGGCC	AACTGCAGTCACCTCGTCAT CTCCTATGG	pCAC6 (4437-4557)	EcoRI-PstI	pGBT9
sk c15	GGAATTCATAGGAGATGACG AGGTGACC	AACTGCAGGTTGGTATTGGC ACGAGCGAG	pCAC6 (4539-4986)	EcoRI-PstI	pGBT9
sk C-term A	GGAATTCATAGGAGATGACG AGGTGACC	AACTGCAGCTCCTCCTCTATG GTCCGCAGC	pCAC6 (4539-4695)	EcoRI-PstI	pGBT9
sk C-term B	GGAATTCCAGATCCAGGCTG GGCTCCGG	AACTGCAGCACCTGGCCAAA CAGGCCTCC	pCAC6 (4662-4824)	EcoRI-PstI	pGBT9
sk C-term C	GGAATTCAGGACCGGAGGC CTGTTTGGC	AACTGCAGGTTGGTATTGGC ACGAGCGAG	pCAC6 (4800-4986)	EcoRI-PstI	pGBT9
sk C-term D	GGAATTCAGGACCGGAGGC CTGTTTGGC	AACTGCAGTTGGTTGGCCAT CACCGG	pCAC6 (4875-4860)	EcoRI-PstI	pGBT9
sk C-term E	GGAATTCGTGATGGCCAACC AAAGACCG	AACTGCAGGAAGTCCTCCAA GAAGACAGG	pCAC6 (4861-4941)	EcoRI-PstI	pGBT9
sk C-term F	GGAATTCCCTGTCTTCTTGGA GGACTTC	AACTGCAGGTTGGTATTGGC ACGAGCGAG	pCAC6 (4921-4986)	EcoRI-PstI	pGBT9

Clone	Foreward Primer	Reverse Primer	Template (amplified region, bp)	Sites	Vector(s)
sk C-term E→Q mutant	1.GGAATTCGTGATGGCCAAC CAAAGACCG 2.AGACCGCTCCAGTTTGCTC AGATACAAATGCAAATGCAA CAGCTG 3.GGAATTCGTGATGGCCAAC CAAAGACCG	1.CTCCAAGAAGACAGGCGAC TGCAGCTGTTGCATTTGTAT 2.AACTGCAGGAAGTCCTCCA AGAAGACAGG 3.AACTGCAGGAAGTCCTCCA AGAAGACAGG	pCAC6 (4861-4941) for PCR#1, products from 1 and 2 for PCR #3	EcoRI-PstI	pGBT9
cardiac C-terminus A	GGAATTCGACAACTTTGACT ACCTG	CGGGATCCCTGAGCTTCATG CTGC	pCARD1 (4518-5526)	EcoRI-BamHI	pGBT9
cardiac C-terminus B	GGAATTCGCACAGGAGGCA GCATGGAAGCTCAGC	CGGGATCCCAGGCTGCTGAC GCCGGCCCTGCG	pCARD1 (5499-6513)	EcoRI-BamHI	pGBT9

pCARD1 is the cardiac DHPR coding sequence in pCRH2 (Mikami et al., 1989). "cDNA library" refers to an oligo(dT) and random-primed human skeletal muscle cDNA library expressed in pGAD10 (catalog # HL4010AB, Clontech, Palo Alto, Ca).

All pGBT9 constructs were tested for independent activation of the *HIS3* reporter gene and for interaction with the pGAD424; none of the transformants behaved anomalously in these assays. Similarly, all pGAD424 constructs were tested for independent activation of *HIS3* and for interaction with pGBT9.

Yeast two-hybrid assays

Yeast two-hybrid library screens and most direct interaction tests were performed in *S. cerevisiae* strain, HF7c (Clontech, Palo Alto, CA); some direct interaction tests for chemiluminescent β-galactosidase assays were conducted in strain Y190. Yeast were transformed by the lithium acetate method according to manufacturer's directions (Clontech). For direct interaction tests, small-scale, simultaneous co-transformations were performed using 0.1-0.2 μg of each plasmid DNA. For RyR epitope library screening, large-scale simultaneous transformations were performed using 50 μg epitope library DNA, and 100 μg bait DNA. For library screens, large-scale sequential transformations were performed. First yeast were transformed with the bait plasmid in a small scale transformation (0.1-0.2 μg cDNA) and the cells grown on SD-Trp plates for 3-7 days. Cells were then inoculated into liquid SD/-Trp, grown overnight, and then

transformed with 100 μg library cDNA. Transformants were plated on SD/-Trp/-Leu/-His and incubated at 30°C for 7-14 days.

β-Galactosidase Assays

β-galactosidase filter lift assays were performed according to the manufacturer's protocol (Clontech). A modified filter assay was also employed, using a toothpick to transfer colonies from plates to filter paper rather than transferring by adherence. This allowed multiple interactions to be evaluated side-by-side under identical assay conditions.

Semi-quantitative β-galactosidase assays were performed using the Galacto-Star chemiluminescent substrate (Tropix, Bedford, MA) according to the manufacturer's directions. Luminescence was measured on a Turner TD 20/20 luminometer 1 hour after addition of yeast extracts to the substrate. Cell number was approximated by measuring OD_{600}.

Isolation, Amplification And Sequencing Of Yeast Plasmids

Plasmids were isolated from colonies that activated both the *HIS3* and *lacZ* reporter genes by a modified PerfectPrep Plasmid DNA kit (5'3', Boulder, CO). Briefly, the protocol provided with the kit was followed with the addition of a 2' vortex with glass beads in order to disrupt the yeast cell wall.

cDNA library inserts were amplified by PCR using the primer pairs pGAD.F/ pGAD.R and pGBT9.F/pGBT9.R (pGBT9.F CTGATATGCCTCTAACATTGAGA; pGBT9.R TTCGTTTTAAAACCTAAGAGTCAC). Single band PCR products were purified using the QIAQuick PCR purification kit (Qiagen); multiple bands were separated by electrophoresis on low melting agarose and purified with the Wizard purification kit (Promega, Madison, WI). PCR products were sequenced by automated sequencing (Macromolecular Resources, Fort Collins, CO). Sequences and their deduced amino acids were compared with the GenBank nucleotide and protein databases using the BLAST family of programs on the National Center for Biotechnology world wide web site (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/) using the BLOSUM62 matrix with an expect value of 10 (occasionally e=1 or e=100), default word size, and no filtering. Sequence alignments were performed using Geneworks software (Oxford Molecular Group, Oxford, England).

RyR Epitope Library Construction

A RyR epitope library was prepared following the protocol of Seeburg (Kornau et al.. 1995). Briefly, pCIneo-RyR1 was fragmented by brief sonication and size-selected to approximately 300-1200 Kb by purifying fragments of that size from a low-melting agarose gel. The size-selected DNAs were ligated into SmaI-digested pGAD424 and used to transform competent Max Efficiency *E. coli* strain DH5α (Life-Technologies).

Aliquots of mini-prep DNA were analyzed for inserts by restriction digest using EcoRI and SalI (NEB) to free the inserted fragments. 11 of the 16 colonies (69%) contained inserts with an average size of approximately 700 bp (Fig. 1.2A).

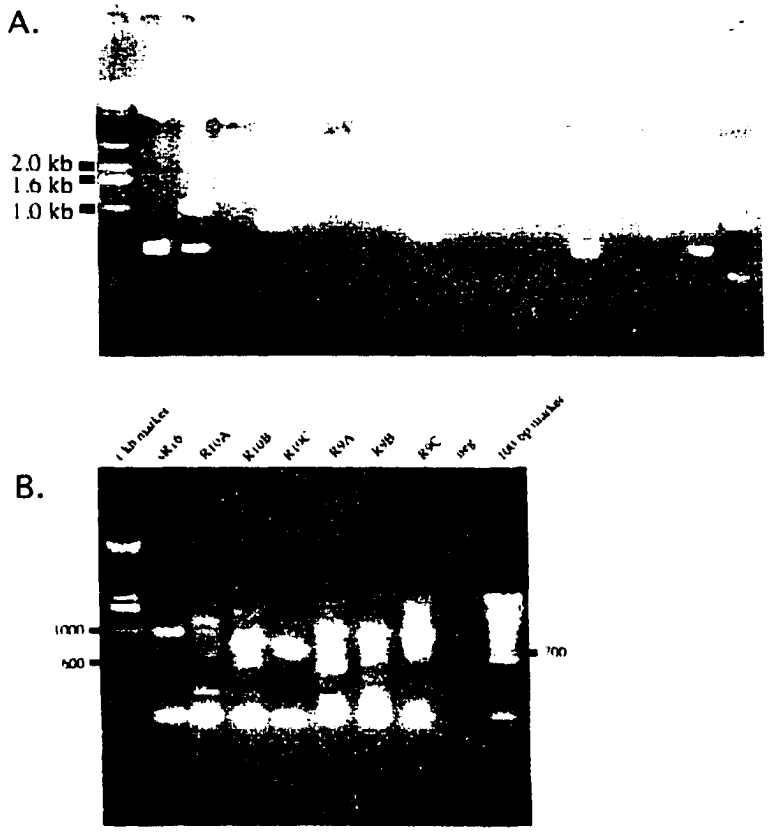


Figure 1.2 Analysis ofRyR epitope library.
A. Percentage of inserts. Sixteen transformed colonies were digested with EcoRI and SalI to release the size-selected library inserts. Eleven of the sixteen colonies (69%) contained inserts.
B. PCR analysis of RyR epitope library. Physiologically important regions of RyR1 were amplified from the RyR epitope library by PCR. Expected sizes for fragments (indicated by *): sR16, 997 bp; R10A, 693 bp; R10B, 762 bp; R10C, 702 bp; R9A, 978 bp; R9B 924 bp; R9C 936 bp.

A calculation for determining whether a sequence of interest is represented in a genomic DNA library (Ausubel, 1998) was adapted to the case of a particular piece of the

ryanodine receptor being present in the correct orientation and in-frame with the GAL4 activation domain. Under the assumption of a Poisson distribution of the size of the cloned fragments, the number of independent clones, N, that must be screened to isolate a particular sequence with probability, P, is given by

$$N = (6)(\text{fraction of inserts})^{-1}(\ln(1-P)/\ln(1-I/G))$$

where I is the size of the average cloned fragment (700 bp) and G is the size of the target sequence (15,000 base pairs). The factor 6 accounts for the one in six chance that the insert will be in the correct orientation and reading frame. Thus, to ensure with 99.9% probability that the epitope library with 69% of the clones containing inserts would express a given fragment, a total of 1301 colonies were needed for each library screen. To control for non-random fractionation of the RyR DNA, the calculated number of colonies was increased 50-fold for RyR epitope library screens. The library was subsequently checked by PCR to ensure that it contained full-length clones encoding physiologically important fragments of RyR1 (Fig. 1.2B).

Preparation Of DIG-RyR Probe And Dot-Blot Protocol

In order to quickly determine if interactors isolated from cDNA library screens contained fragments of RyR1, a random-primed digoxigenin- (DIG-) labeled ryanodine receptor probe was generated following the protocol in the Boehringer-Mannheim "Genius"™ system protocol. RyR1 cDNA was digested with NcoI to generate fragments

<5 kb in size. Following purification and concentration, the DNA was heat denatured in a boiling waterbath for 10 minutes, chilled in a dry ice/ethanol bath, and then incubated with dNTPs including DIG-11-dUTP (Genius kit) and Klenow DNA polymerase fragment. The probe yield was estimated to be 12 ng by comparison to dilutions of DIG standards on a dot blot (data not shown). The specificity of the DIG-RyR was determined by comparing its binding to DHPR and RyR cDNAs fixed to nylon membrane by UV irradiation and probed with anti-DIG, affinity-purified antibody (Boehringer). Figure 1.3 shows that the DIG probe specifically labeled RyR cDNA and did not label DHPR cDNA.

Functional analysis of GFP-DHPRs

Primary cultures of dysgenic myotubes were prepared and injected with plasmid DNA encoding either GFP-α_{1s} (Grabner et al., 1998) or GFP-δ1620 (Table 1.1) fusion proteins as previously described (Tanabe et al., 1988). Cells expressing the injected plasmids were identified by green fluorescence 24-48 hours post-injection.

The ability of GFP-δ1620 to mediate excitation-contraction coupling and to produce Ca^{2+} currents was compared to that of GFP-α_{1s} by examining electrically induced contraction in the presence and absence of extracellular Ca^{2+}, and by characterization of whole cell currents as previously described (Nakai et al., 1998). The

subcellular distribution of GFP-δ1620 was compared to that of GFP-α_{1s} using confocal microscopy as previously described (Grabner et al., 1998).

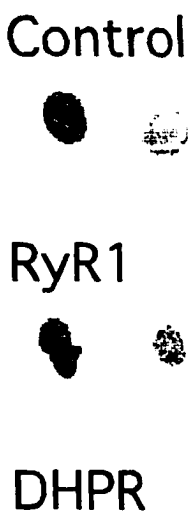


Figure 1.3 Digoxigenin-labeled RyR probe specifically binds to ryanodine receptor DNA.
Upper row: DIG-labeled control DNA. *Middle row:* dilution series of pCI-neo-RyR1 (90 ng, 9 ng, 900 pg, 90 pg). *Bottom row:* dilution series of DHPR clone CAC6 (70 ng, 7 ng, 700 pg, 7 pg).

DISCUSSION

Why use two-hybrid?

The yeast two hybrid system is a good choice for an exploration of interactions at the triad junction for several reasons. First, the two-hybrid system may detect weak or transient interactions such as those that might constitute a signaling interaction between

DHPRs and RyRs in EC coupling — interactions that may have been missed by the less sensitive affinity chromatography and protein overlay techniques (e.g.: Brandt et al., 1990) which are about an order of magnitude less sensitive than two-hybrid assays (Phizicky and Fields, 1995). As an example of this, two groups (Ohtsuka et al., 1997; Ohtsuka et al., 1997; Sorimachi et al., 1997) have recently reported an interaction between titin and α-actinin using two-hybrid assays despite the fact that previous attempts to demonstrate such an interaction using peptide fragments in overlay, dot blot, and immunoprecipitation experiments failed to detect the same interaction. A contributing factor to the increased sensitivity of two-hybrid assays for some interaction pairs may reside with the potential for the test segments to assume a more native-like conformation within a living cell that they can *in vitro*. Second, the likelihood that unidentified proteins interact with DHPRs and RyRs at the triad junction makes the two-hybrid system attractive because genes encoding novel proteins are immediately available.

Limitations of the yeast two-hybrid system

False-Positives

There are downsides to any technique. One of the best known drawbacks to the yeast two-hybrid system is that it is prone to false positives. In the design of the experiments reported in this dissertation, pains were taken to avoid false positives. The

single most important factor is the yeast strain chosen. Depending on the promoter structure, the reporter genes in some strains, such as Y190, are expressed at high levels. This is advantageous in detection of weak interactions, but can lead to an overwhelming number of false positives, making determination of physiological interactors problematic. On the other hand, tightly regulated expression from promoters strains in such as HF7c prevents spurious expression of the reporter genes.

Yeast two-hybrid library screens and most direct interaction tests were performed in strain HF7c which has *HIS3* and *lacZ* (β-galactosidase) reporter genes under the control different GAL4 promoters. Expression of the *lacZ* reporter gene in HF7c is regulated by binding of the GAL4-BD protein to three 17 bp GAL4 consensus binding sites upstream of the extremely weak CYC1 minimal promoter; thus, constitutive *lacZ* gene expression in HF7c is very low. The *HIS3* gene in HF7c is controlled by GAL4-BD binding to the native GAL1 upstream activating site which contains four GAL4 binding sites. Since the binding of GAL4 to the consensus promoter site is additive, and the promoter's activity is roughly proportional to the number of GAL4s bound (Giniger and Ptashne, 1988), the *HIS3* reporter gene in HF7c is theoretically more activatable than is the *lacZ* reporter gene. Indeed, a ten-fold difference in sensitivity is reported (Clontech, 1996). However, as discussed in chapter 4, I have observed that the theoretical sensitivity of the two reporter genes in strain HF7c is not a good predictor of their actual behavior.

A few direct interaction tests were conducted in *S. cerevisiae* strain Y190 in order to provide an independent and quantitative test of reporter activation. In contrast to HF7c, Y190 has leaky *HIS3* gene expression due to a high level of constitutive expression from the native *HIS3* minimal promoter which contains two TATA boxes in addition to the GAL1 upstream activation site. One of the TATA boxes (TR) is regulated while the other TATA box (TC) drives low-level constitutive expression of *HIS3* . Thus, *HIS3* gene expression in Y190 is not a good assay of interaction. This problem was addressed by inclusion of 10-25 mM 3-Amino-1,2,4-triazole (3-AT) in the plating media. 3-AT is a competitive inhibitor of the yeast *HIS3* protein (His3p). The concentration of 3-AT in the media was experimentally titrated to a level that allowed detection of the αID x βID control interaction. The *lacZ* gene in Y190 is controlled by a promoter identical to the one that regulates *HIS3* gene expression in strain HF7c. Thus, β-galactosidase activity in test constructs expressed in Y190 is theoretically comparable to His3p activity of the same test pairs expressed in HF7c, depending, of course, on the detection threshold of the assays.

False-Negatives

Interactions can be missed by two-hybrid assay for many reasons, including the following. (i) Binding sites on proteins are often composed of residues that are not contiguous in the primary amino acid sequence. Interaction of such interactions would

not be detected in two-hybrid assays if the test protein does not fold correctly when expressed as a fusion protein, or if, as is the case for the DHPR and RyR in this dissertation, test proteins must be subdivided since the largest piece that can be expressed as a two-hybrid fusion protein is about 400 residues. (ii) Detection of interaction in the two-hybrid system is dependent on translocation of fusion protein pairs to the nucleus. It is possible that some fusion proteins could be unstable, that the test segment of the fusion protein could introduce a tertiary structure that bound to or hindered normal functioning of the nuclear translocation signal, or that a fusion protein could be trapped in the yeast cytoplasm by binding to the cytoskeleton or to endogenous proteins.

False False-Positives

In the course of examining many "false positives" in the library screens outlined in subsequent chapters of this dissertation, one observation was made: many doubly-selected, putative interactors contained coding sequence for a gene inserted backwards with respect to the GAL4-AD. These inserts usually only encoded tiny open reading frames (ORFs) adjacent to and in frame with the GAL4-AD coding sequence. Now, most of these are *bona fide* false positives. But several such interactors were fished from the library multiple times and could be authentic interactors via mechanisms of reporter gene activation apart from the normal case of functional reconstitution of the GAL4 transcription factor protein. (i) An ORF in the antisense strand can be transcribed in the

reverse orientation from a cryptic promoter within the ADH1 terminator (Chien et al., 1991). The protein encoded by such a transcript could result in a positive signal if it functions as a transcriptional activator in addition to binding to the bait protein. Highly acidic proteins are a common class of transcriptional activators that could display this behavior (Ma and Ptashne, 1987;Ruden, 1992). It should be noted, however, that not all acidic proteins can function as transcriptional activators. (ii) An endogenous yeast protein, which itself can function as, or bind to, a transcriptional activator, could bind to the GAL4-BD-fusion protein. In this case, GAL4-AD-fusion proteins that bound the endogenous yeast protein would be the positive interactors in two-hybrid assays. Significantly, there could be a large complex formed that would result in a diversity of "interactors" being isolated. (iii) An endogenous yeast protein could specifically bind the mRNA transcript of certain GAL4-AD fusions. This could result in a very high level of expression of particular library proteins which would increase their probability of being detected frequently. (iv) Some transcripts could undergo post-transcriptional processing in the yeast, so that inserts that do not encode a theoretical open reading frame could, in fact, produce an in-frame ORF.

CHAPTER 2
TESTING THE INDUCED LIGAND HYPOTHESIS: DO PHYSIOLOGICALLY IMPORTANT DOMAINS OF SKELETAL MUSCLE DHPRs AND RYRs INTERACT?

INTRODUCTION

Determination of DHPR domains important for skeletal-type EC coupling

Like pore-forming subunits of other voltage-gated channels, the pore-forming α_1 subunit of the DHPR has four repeats each composed of six putative transmembrane domains (Fig. 2.1A). The putative intracellular loop between repeats I and II (I-II loop) contains the binding site for the regulatory β subunit (Pragnell et al., 1994). The interaction between the alpha interaction domain (αID) and the beta interaction domain (βID) was used as a control in two hybrid experiments reported in this dissertation. The putative cytoplasmic loop between repeats II and III (II-III loop) is an important determinant of the type of EC coupling (Tanabe et al., 1990).

The hypothesis that cytoplasmic portions of the DHPR might interact with the RyR to effect EC coupling has been studied using several approaches. Chimeric skeletal-

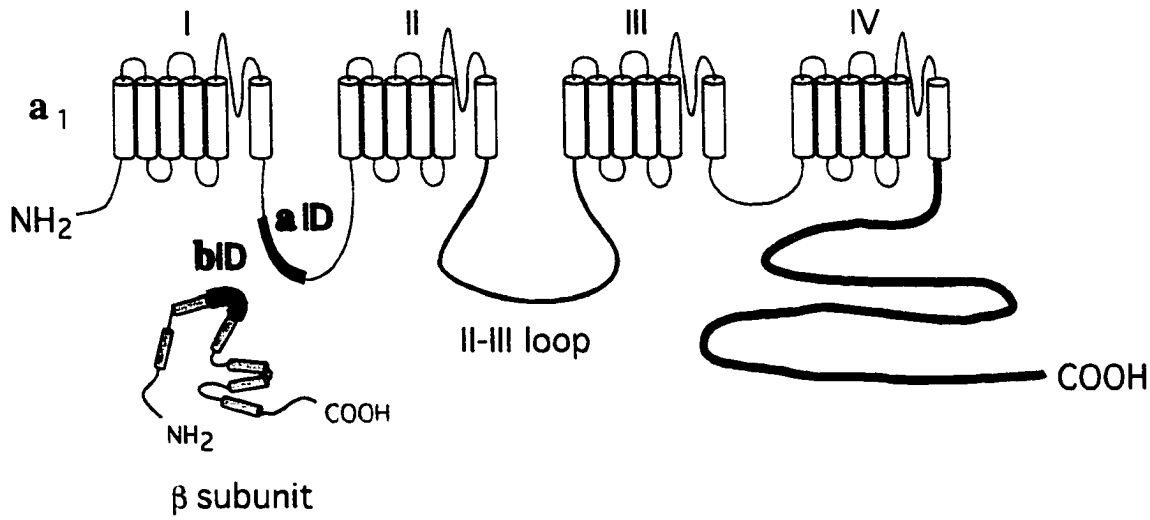
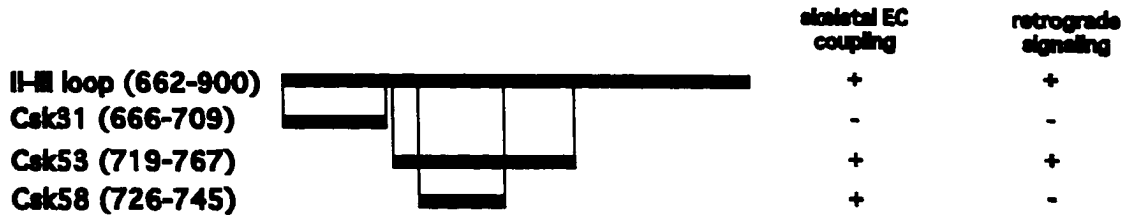


Figure 2.1 Schematic representation of the dihydropyridine receptor (DHPR) α1s subunit. The β subunit interacts with α1s as indicated. The interactive domains of the alpha and beta subunits are termed αID and βID, respectively (Pragnell et al., 1994). The II-III loop is important for skeletal-type EC coupling (Tanabe et al., 1990). The C-terminus is drawn with a bold line.

cardiac DHPRs have been tested for their ability to restore skeletal-type EC coupling to dysgenic myocytes that lack endogenous DHPRs (1990; Nakai). This approach has identified physiologically important segments of the II-III loop, but it can not establish whether they interact directly with the RyR. On the other hand, biochemical experiments have established regions of the II-III loop which can to bind to and activate RyRs *in vitro*, but these experiments cannot address whether the identified regions are important *in vivo*. Figure 2.2 is a schematic representation illustrating the relative positions of some important fragments of the DHPR II-III loop. s53 (residues 720-765 of the skeletal II-III loop) is necessary and sufficient to confer skeletal-type EC coupling to

A.



B.

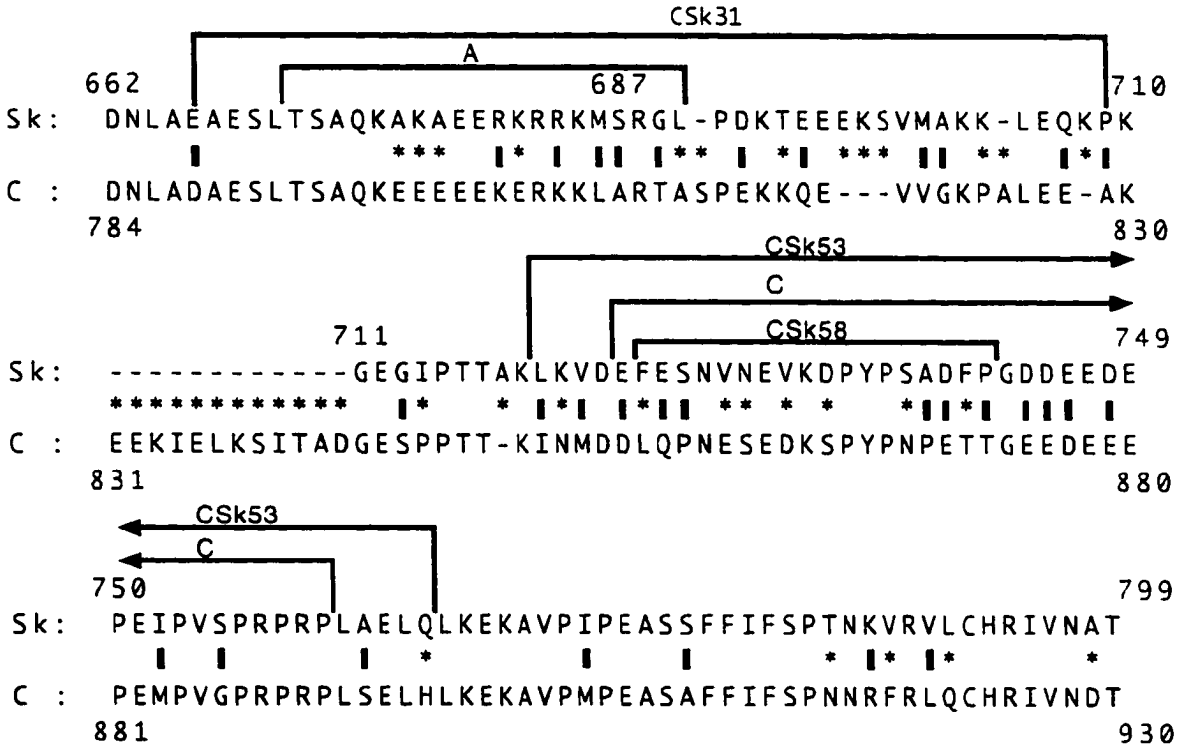


Figure 2.2 Important regions of the DHPR II-III loop.

A. Schematic illustration of regions of the DHPR II-III loop tested in chimeric receptors (Tanabe et al., 1990; Nakai et al., 1998).

B. Amino acid sequence of the DHPR II-III loop showing active chimeras and

an otherwise cardiac DHPR Nakai et al., 1998. Moreover, s53 is responsible for receiving the retrograde signal from the RyR (DHPRs in dyspedic myotubes have very

small calcium currents which are enhanced by coexpression of RyR1 but not RyR2; Nakai et al., 1996). c53 is the corresponding segment of the cardiac DHPR which is unable to support either skeletal-type EC coupling or retrograde signaling when substituted into the skeletal DHPR (Grabner et al., 1999). s58 is a smaller region within s53 (residues 725-742) that confers weak skeletal EC coupling to chimeric DHPRs; and, s31 (residues 666-709) is a segment of the II-III loop that not important for skeletal-type EC coupling in chimeric DHPRs (Nakai et al., 1998).

Determination of the region of the DHPR II-III loop that is important for skeletal-type EC coupling using II-III loop peptides is in apparent contradiction to the results observed in chimeric DHPRs. Peptide A (residues 671-690) has been identified as an activator of ryanodine binding to, and Ca^{2+} release from, SR vesicles (El-Hayek et al., 1995). Peptide C (residues 724-760) was found to antagonize the actions of peptide A, suggesting that either or both of these could bind to RyR1, or that they may bind to each other. These results are supported by the independent observation that Lys677 and Lys682, which are contained within peptide A, are important determinants of direct binding of DHPR peptides to RyR peptides in protein affinity chromatography experiments (Leong and MacLennan, 1998; Leong and MacLennan, 1998). However, recent data show that a smaller fragment of peptide A, "peptide As-10," is an even better activator of ryanodine binding and calcium release than is peptide A itself (El-Hayek and Ikemoto, 1998). However, peptide As-10 does not include the first of the two lysine

residues shown to be important for DHPR-RyR peptide binding in the affinity columns (Leong and MacLennan, 1998).

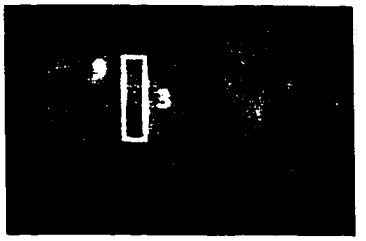
Determination of RyR domains important for skeletal-type EC coupling.

RyRs are humongous homotetramers. A single channel is composed of 20,148 amino acids and has a mass of about 2 million daltons. While the molecular architecture of the RyR at the amino acid level is not nearly so well established as that of the DHPR, single RyRs can be resolved by electron microscopy. Three dimensional EM reconstructions show that the RyRs are composed of a relatively small transmembrane region plus a huge cytoplasmic bolus and appear as four large subunits surrounding a central pore (Fig. 2.3A). The C-terminal portion of RyR1 alone forms a functional channel (Bhat et al., 1997) and is predicted to contain four (Takeshima et al., 1989) to ten (Zorzato et al., 1990) membrane spanning domains. The remaining amino terminal 90% of the RyR probably forms the large cytoplasmic "foot" structure observed in electron micrographs.

Three RyR isoforms have been identified: RyR1 is primarily expressed in skeletal muscle, RyR2 is the predominant form in cardiac muscle and brain, and RyR3 is the primary isoform in tonic muscle and is a minor component of smooth and skeletal muscle and of brain. The three isoforms share approximately 70% overall homology; however, there are three regions of significant sequence divergence (D1, D2, and D3; Sorrentino and Volpe, 1993) which have been investigated as potentially important for

skeletal-type EC coupling. Deletion of the D2 region (amino acids 1303-1406 in RyR1) resulted in functional channels that are unable to support EC coupling in myocytes cultured from RyR1/RyR3 double knockout mice, suggesting that D2 is essential for skeletal EC coupling (Yamazawa et al., 1997). Affinity chromatography demonstrated binding of DHPR II-III and III-IV loop peptides to adjacent sequences in RyR1 (residues 954 - 1112; Leong and MacLennan. 1998), suggesting that this region of RyR1 may be important for skeletal-type EC coupling. Yet, a different important region was determined by using chimeric RyR1/RyR2 channels expressed in dyspedic myocytes (RyR1 knockouts). In contrast to the deletion mutant, chimeras in which the D2 region had cardiac sequence were still able to support skeletal-type EC coupling. On the other hand, the R10 region (amino acids 1635-2636) which includes D3, was found to be essential for skeletal-type EC coupling (Nakai et al., 1998). Preliminary results suggest that R16 (residues 1837-1940) within R10 is the part responsible (Proenza and Nakai, unpublished observations). So, as for the case of the DHPR, different methods point to different regions of the RyR that may be important for EC coupling. The results of these studies are summarized in figure 2.3B.

A.



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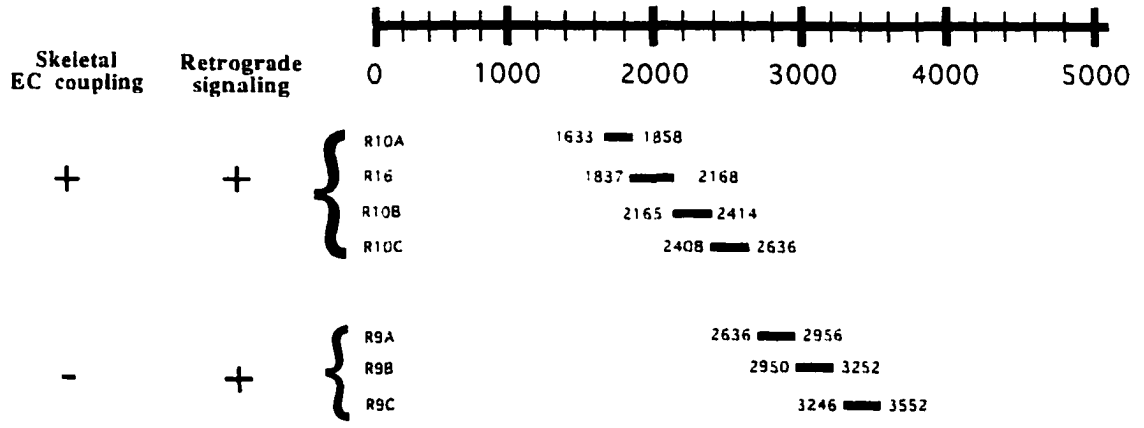

Figure 2.3 Structure of the Ryanodine Receptor.
A. Three-dimensional reconstruction based on EM images (from Wagenknect, et al., 1995).
B. Schematic representation of the RyR fragments used in two-hybrid experiments and their physiological effects when expressed in chimeric RyRs.

Taken together, all of the biochemical and physiological studies leave the question of whether direct functional coupling between DHPR and RyRs underlies skeletal-type EC coupling. The skeletal-cardiac chimeras identify regions of the DHPR and RyR that determine their ability to mediate skeletal-type EC coupling *in vivo*, but they do not answer the question of whether the important domains directly interact with each other. Experiments with isolated peptides, on the other hand, identify domains that

are important for binding and activation *in vitro*, but do not address the question of whether the interactions occur *in vivo*. The two-hybrid experiments reported here were designed in attempt to reconcile the various observations with a different technique.

RESULTS

αID x βID

The known interaction between the α_1 subunit interaction domain (αID) and the β subunit interaction domain (βID) was used as a control for yeast two-hybrid experiments. Figure 2.4 demonstrates that the control interaction activated both the *HIS3* and *lacZ* reporter genes.

s53 x sR16

Preliminary results suggest that sR16 may be the smallest part of RyR1 to confer skeletal-type EC coupling to an otherwise RyR2 chimera expressed in dyspedic myotubes (Proenza and Nakai, unpublished observations). In the case of the DHPR, s53 is the smallest part that is critical for full skeletal-type EC coupling in chimeric DHPRs. Based on these two observations, the hypothesis that s53 and sR16 interact was tested.

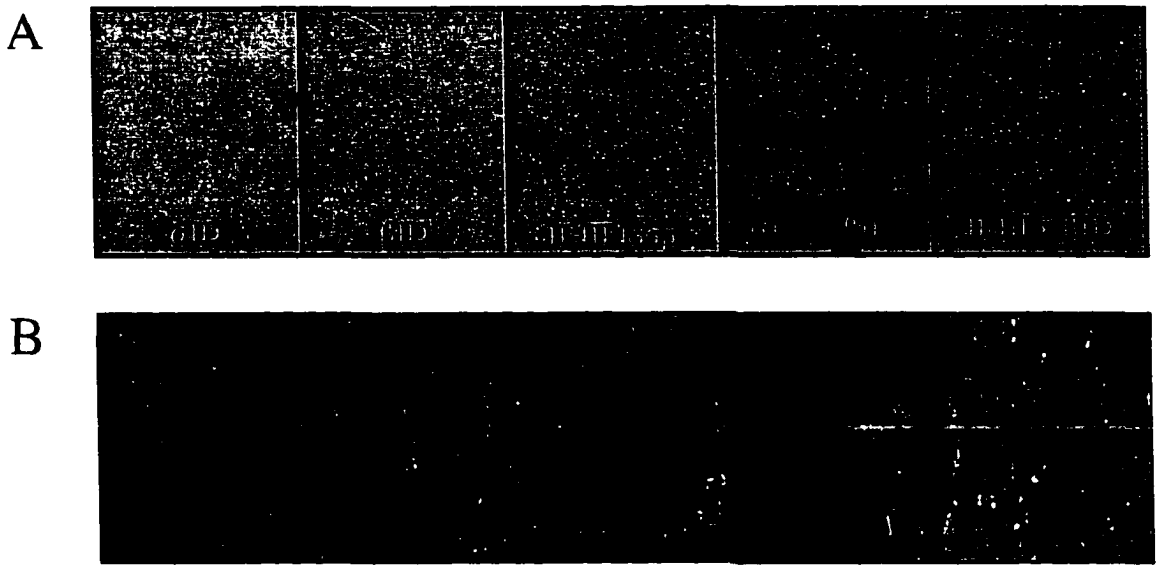

Figure 2.4 Control interaction between αID and βID.
A. Nutritional selection assay. Yeast HF7c was transformed with the indicated plasmids and plated on SD/-Trp/-Leu/-His minimal media. Growth indicates interaction between the test segments aID and bID.
B. b-galactosidase filter assay. Yeast HF7c transformed with the indicated plasmids. Blue color (arrowhead) indicated interaction.

As assayed by nutritional selection, s53 and sR16 interacted consistently in two-hybrid experiments in strain HF7c (n = 17; Fig. 2.5A). This interaction persisted when the plasmids containing the test segments were reversed. However, no interaction between s53 and sR16 was detected in β-galactosidase filter assays (n = 15; Fig. 2.5B). *HIS3* gene expression was also assayed in strain Y190 in the presence of 10 or 25 mM 3-AT. In these experiments, a weak interaction was seen between s53 and sR16 (data not shown).

As discussed in chapter 1, the *lacZ* reporter gene in yeast strain HF7c is under the control of a weaker promoter than is the *HIS3* reporter gene. Therefore, to test for

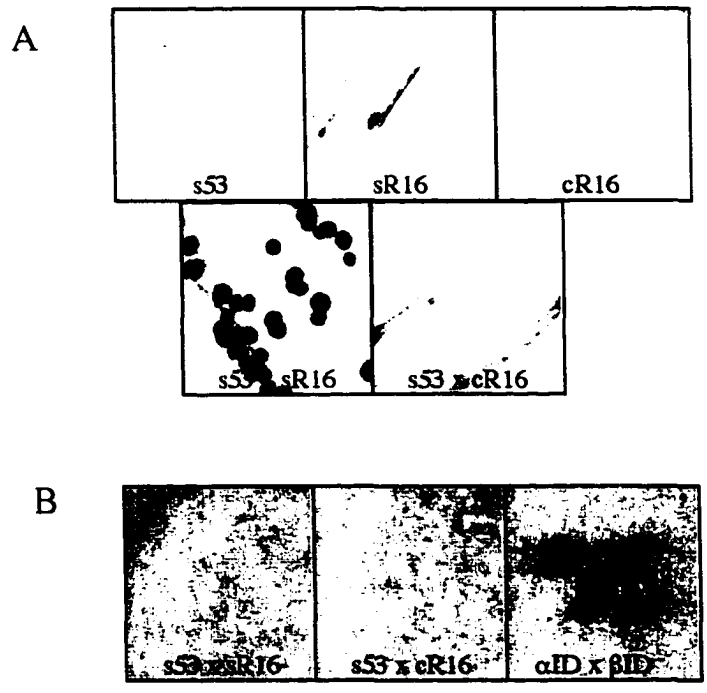

Figure 2.5 Tests for interaction between s53 of the DHPR and sR16 of the RyR.
A. Nutritional selection assay. Yeast strain HF7c was transformed with the indicated plasmids and plated on SD/-Trp-/-Leu/-His nutritional selection media. Colony growth on the s53 x sR16 plate indicates interaction. Lines on the sR16 and s53 x cR16 plates are from the initial streaking and do not indicate interaction.
B. β-galactosidase filter assay. No interaction was observed in the between the test plasmids. The αID x βID control interaction is shown for comparison.

interactions that may have been sub-threshold in β-galactosidase filter assays, the β-galactosidase activity was reassayed using a sensitive, semi-quantitative chemiluminescent substrate in an attempt to confirm the interaction detected in the growth assays. As shown in figure 2.6A, no significant β-galactosidase activity was detected between s53 and sR16 in yeast strain HF7c. The experiment was repeated in yeast strain Y190 in which the *lacZ* reporter gene is driven by the same promoter that controls *HIS3* expression in the HF7c strain. No significant β-galactosidase activity between s53 and sR16 in strain HF7c was detected (Fig. 2.6B).

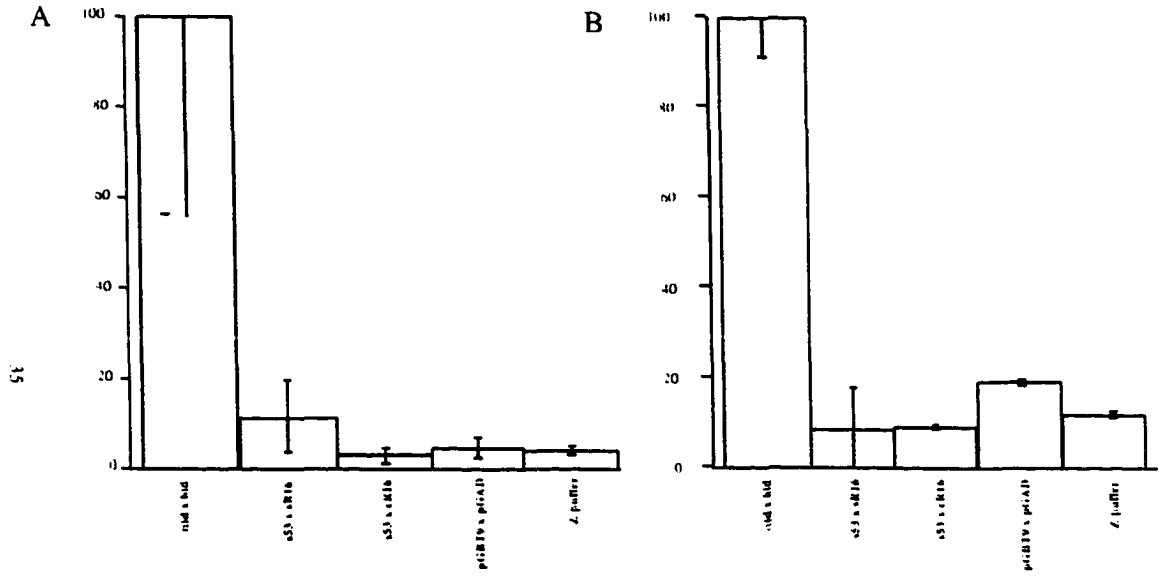

Figure 2.6 Chemiluminescent b-galactosidase assay of s53 x sR16.
Yeast strain HF7c (A) or Y190 (B) was transformed with the indicated plasmids and assayed for b-galactosidase activity. Activity is expressed as a percentage of the aid x bid control interaction. No significant difference between the test interactions and the negative controls was found.

Tests of interaction between II-III loop pieces and ryanodine receptor pieces

Next, other physiologically important regions of the RyR and DHPR were tested for interaction. Segment R10 (1633-2635) of RyR1 is important for skeletal type excitation-contraction coupling; additionally, both R9 (residues 2636-3920) and R10 are able to transmit the retrograde signal to the DHPR (Nakai et al., 1996). Preliminary data also suggests that R9 also organizes formation of DHPR tetrads (Protasi and Franzini-Armstrong, personal communication). Based on these results, R9 and R10 were tested for interaction with the DHPR II-III loop. Because these regions are so large — about

1000 amino acids each — it was necessary to subdivide them for expression in the two hybrid system where the maximum size of a test peptide is about 350 residues.

Various parts of the DHPR II-III loop and of segments R9 and R10 of RyR1 were tested for interaction using nutritional selection and β-galactosidase assays. In nutritional selection assays, no interaction was observed for any combination of test segments (data not shown). The same result was obtained in chemiluminescent β-galactosidase assays conducted in yeast strains HF7c and Y190 (fig 2.7AB).

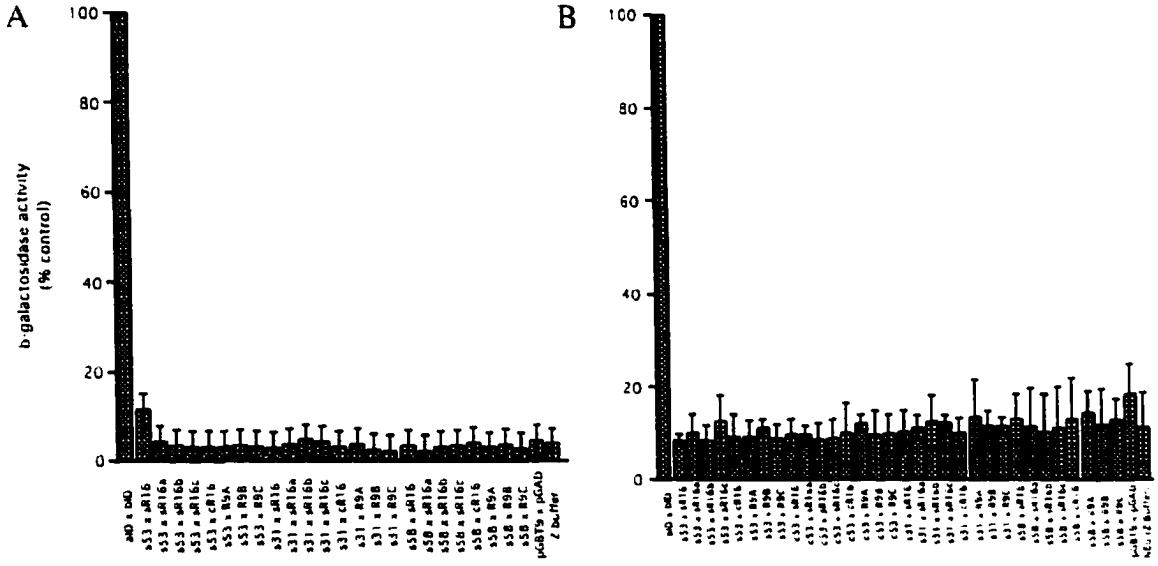

Figure 2.7 Chemiluminescent b-galactosidase assay of DHPR II-III loop segments x RyR segments.
Yeast strain HF7c (A) or Y190 (B) was transformed with the indicated plasmids and assayed for b-galactosidase activity. Activity is expressed as a percentage of the aid x bid control interaction. No significant difference between the test interactions and the negative controls was found.

Intra-loop interactions

Peptide A (DHPR residues 671-690) activates RyR activity and ryanodine binding (Lu et al., 1994; El-Hayek et al., 1995; El-Hayek and Ikemoto, 1998) and binds directly to ryanodine receptors (Leong and MacLennan, 1998). However chimeras do not substantiate the importance of peptide A; CSk31, the chimera that includes peptide A, does not confers skeletal-type EC coupling (Nakai et al., 1998). These results fit a model in which s53 controls peptide A activation of the RyR. In order to test this model, s53 and s31 were assayed for interaction in two-hybrid experiments. s53 and s31 did not interact in either nutritional selection or β-galactosidase two hybrid assays (data not shown).

RyR epitope library

As another approach to detecting a direct interaction between the DHPR and the RyR, a RyR1 "epitope library" was constructed by randomly fragmenting RyR1 cDNA and ligating size-selected fragments into a GAL4 activation domain plasmid.

All of the DHPR cytoplasmic loops were tested for interaction with the RyR epitope library. The number of interacting colonies from these library screens was surprisingly low, making the results difficult to interpret since while *all* positives contained RyR fragments, there were not enough positives to discriminate between false positives and actual interactions based on repeated "hits." As summarized in Table 2.1.

no His3p and β-galactosidase positive colonies were found using the N-terminus, III-IV loop, or s53 as bait to screen the RyR epitope library. The I-II loop, II-III loop and C-terminus of the DHPR caught RyR fish in the epitope library, however no overlapping interacting regions were found.

bait	sequence identity (RyR1 residues)
N-terminus	ND
I-II	#6: 616-872
s53	ND
II-III	#2: RyR 1419-1643 #3: RyR 433-594 #6: RyR 3727-3801
III-IV loop	ND
C-term	#7: RyR 4802-4990 #15: RyR 2991-3178

Table 2.1 Summary of DHPR cytoplasmic loops x RyR epitope library.

DISCUSSION

RyR Epitope Library

Although the epitope library approach has been applied successfully in two-hybrid assays (Kornau et al., 1995), it was of limited use in my hands. Since the epitope library was shown to contain sequences encoding physiologically important regions of RyR1 up to 400 residues in length (figure 1.2B), the lack of reactivity probably does not reflect a lack of representation in the library. Rather, the large size of the RyR may lower

the probability that binding domains are composed of contiguous primary amino acid sequences. Thus, the limited size of the two-hybrid test segments might be shorter than the primary sequence comprising a binding domain. Alternatively, correct tertiary structure of the test segments is probably not achieved when they are expressed as GAL4-AD fusion proteins. However, these two possible explanations are not corroborated by the observation that the DHPR II-III and III-IV loops interacted with short segments (<120 residues) of RyR1 *in vitro* (Leong and MacLennan, 1998; Leong and MacLennan, 1998).

II-III loop x RyR

No strong interactions were detected between any part of the DHPR II-III loop and any part of the R9 or R10 regions of RyR1. However, the consistent growth observed in nutritional selection assays of s53 x sR16 is suggestive of an interaction between these domains. If these two regions are essential for skeletal-type EC coupling in chimeric receptors, as our preliminary data suggests, then it is difficult to ignore the hint from the two-hybrid experiments that these important regions can bind one another. Yet, the two-hybrid data are inconclusive because no interaction was detected using the *lacZ* reporter gene in either HF7c or in Y190. Since the *lacZ* gene of Y190 has a promoter identical to that of the *HIS3* gene of HF7c, β-galactosidase assays in Y190 would be expected to yield the same results as nutritional selection assays in HF7c. This, however,

was not the observation. It is possible that a real interaction exists but is subthreshold for detection in even the most sensitive β-galactosidase assay because *lacZ* gene expression is not additive in the same manner as is *HIS3* gene expression: whereas a trickle of expression from the *HIS3* reporter gene would produce colonies — although they would grow extremely slowly — the same trickle of *lacZ* expression would produce single molecules of the enzyme which could be degraded before the next ones were formed. For this reason, the potential interaction between s53 and sR16 detected by *HIS3* gene expression in HF7c cannot be discounted. But, the yeast two-hybrid system cannot provide any more information about this potential interaction. Another major technique for detecting interaction between segments of proteins is *in vitro* binding assays. However, since many laboratories have failed to demonstrate direct interactions between the s53 region of the II-III loop and the s53 region of RyR1, it is not expected that affinity chromatography of the fragments would produce different results than those obtained by other laboratories. Consequently, electrophysiological experiments are currently underway to definitively establish whether sR16 is indeed a determinant of skeletal-type EC coupling.

Summary

In summary, no strong interaction between the DHPR and RyR was detected in two-hybrid experiments. This may mean that the two molecules don't interact, that they

interact with an affinity that is below the threshold of two-hybrid detection, or that they interact in a conformation-dependent manner that is not duplicated in the yeast expression system.

CHAPTER 3
FORMATION OF TRIAD JUNCTIONS: WHAT PROTEINS ARE INVOLVED?

INTRODUCTION

DHPRs and RyRs are arranged in curious patterns in skeletal muscle triad junctions. These patterns require the presence of at least two proteins in addition to DHPRs and RyRs in triad junctions, and predict the nature of some of the protein interactions that must occur at triad junctions:

(i) Membrane junctions form between T-tubules and the SR in both dysgenic and dyspedic myocytes. On the basis of this observation, it is clear that at least one other protein must be involved in coupling the membrane systems.

(ii) Arrays of ryanodine receptors form on the SR membrane, constituting the "feet" that bridge 20 nm gap between the T-tubule and the SR (Block et al., 1988). Isolated RyRs also form ordered arrays (Franzini-Armstrong and Protasi, 1997). Thus, interactions between and among RyRs must occur.

(iii) DHPRs appear in freeze-fractures of the T-tubule membrane as regularly spaced groups of four particles called junctional tetrads (Fig. 3.1A; Block et al., 1988; Takekura et al., 1994). Tetrads are not observed in dyspedic myocytes and tetrads probably do not form in cardiac muscle (Sun et al., 1995; Franzini-Armstrong, personal communication). Comparison of the T-tubular and SR membrane faces shows that DHPR tetrads are arranged relative to the RyR array such that only every other RyR is matched with a tetrad (Fig. 3.1B; Franzini-Armstrong and Kish, 1995). During development, RyR arrays form first, followed by tetrad assembly (Protasi et al., 1998). These observations suggest that interaction directed by RyRs and specific to the skeletal isoforms of both DHPRs and RyRs is likely to be responsible for tetrad formation. Recent work suggests that it is the R9 portion of RyR1 that is capable of inducing tetrad formation (Protasi and Franzini-Armstrong, personal communication).

(iv) Tetrads pop-up across the surface of an RyR array in register with the alternating pattern, but with initial gaps in the arrangement (Protasi et al., 1998). This argues for the existence of another protein that coordinates the alternating arrangement. This protein is distinct from RyRs and DHPRs, and is probably also distinct from the protein or proteins that form the membrane junctions in dyspedic and dysgenic myotubes.

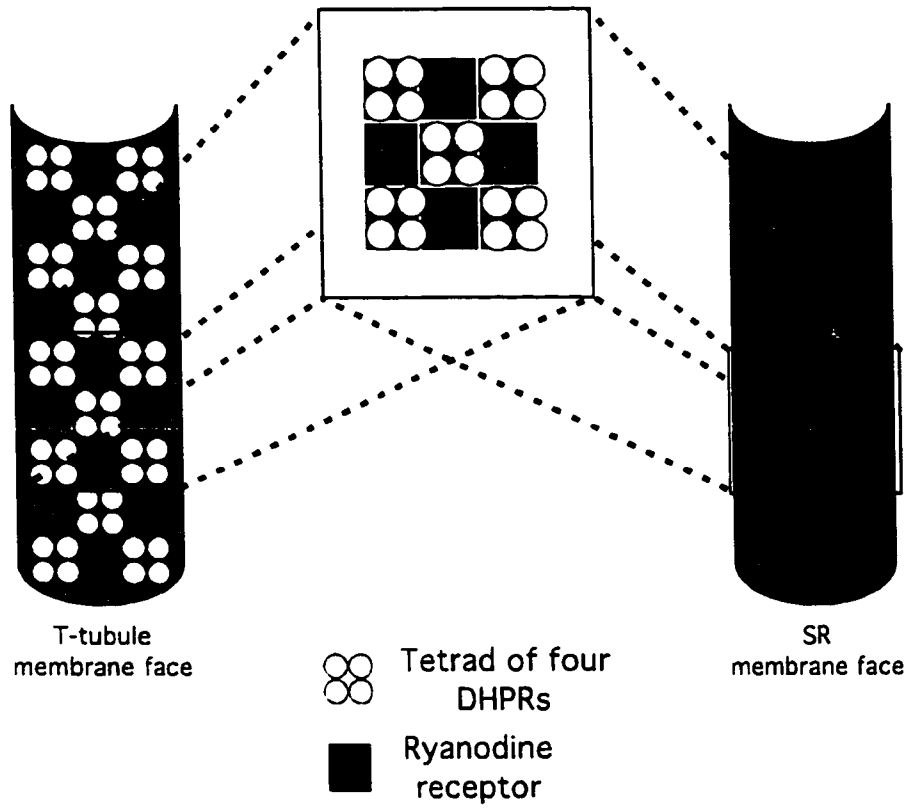

Figure 3.1 Schematic illustration of the arrangement of DHPRs and RyRs at the triad junction.
Representations of T-tubule membrane face (left) and SR membrane face (right) illustrating tetrads and arrays of ryanodine receptors. The center panel represents superimposition of the tetrad and ryanodine receptor patterns.

One candidate protein that may play a role in the formation of triad junctions is junctin. Junctin is a 210 residue SR membrane protein that is expressed exclusively in junctional SR of heart and skeletal muscle (Jones et al., 1995). Junctin is predicted to have a single transmembrane domain which separates a highly charged SR luminal domain from a 22 amino acid cytoplasmic domain. Junctin forms complexes with other SR proteins including the RyR, calsequestrin, and triadin (Zhang et al., 1997). The

function of junctin is not known, however preliminary evidence suggests that junctin could play a role in stapling the SR membrane to the plasma membrane since triad junction-like membrane alignments form between the plasma membrane and ER of CHO cells transiently overexpressing junctin (Franzini-Armstrong, personal communication). These observations lead to the hypothesis that the cytoplasmic domain of junctin may bind to a common membrane or cytoskeletal protein (i.e., one that is found in CHO cells in addition to muscle cells).

In order to identify proteins that may play a role in the formation and function of triad junctions, a skeletal muscle cDNA library was screened in two-hybrid assays using parts of DHPRs, RyRs, and junctin as bait.

RESULTS

DHPR cytoplasmic domains x skeletal muscle cDNA library

To test for interactions of the DHPR with known or novel proteins of the triad junction, a skeletal muscle cDNA library was screened using the five putative cytoplasmic domains of DHPR as bait. No strong interactors were found for the N-terminus, I-II, II-III, or III-IV loops (data not shown). As discussed in chapter 4, the DHPR C-terminus was extremely reactive in two-hybrid library screens.

An important observation was made in these experiments: while the entire I-II loop did not yield any positive colonies in library screens, αID (the portion of the I-II loop that interacts with the β subunit) was highly reactive in library screens. Similarly, the entire I-II loop did not react strongly with βID (the interacting domain of the β subunit) whereas, αID did. These results suggested that in some cases smaller test segments may interact with higher affinity in two-hybrid assays.

Based on this hypothesis and based on the fact that the II-III loop is a physiologically important domain of the DHPR, additional library screens were conducted to search for interaction partners of s53, s58, and s31, smaller fragments of the II-III loop.

s53 x library

As illustrated in figure 2.2, chimeric DHPRs have been used to establish that the s53 region of the II-III loop is important both for skeletal-type EC coupling (Tanabe et al., 1990), and for transmitting the retrograde signal to the RyR (Nakai et al., 1996; Grabner et al., 1999). In order to test the hypothesis that the physiological effects of s53 result from interaction with other muscle proteins — including, but not limited to, the RyR —s53 was used as bait to troll for interaction partners in two-hybrid library screens. The results of these experiments are summarized in Table 3.1.

Bait	growth	β-gal filter lift	sequenced clones	BLASTN	BLASTP with ORF)
s53	++ (120 colonies)	+ (24 hours)	36	human h19 gene **F**	44 residue ORF no hit
			50	troponin I **B**	114 residue ORF similar to mobilization protein A and to gramicidin synthetase
			60	troponin T **F**	80 residue ORF similar to hypothetical prokaryotic protein
s53	+ (7 colonies)	- (24 hours)	C2	creatine kinase **B**	32 residue ORF no hit
			C4	ATP synthetase lipid binding protein **F**	ATP synthetase lipid binding protein

Table 3.1 s53 library screening restults. **F** indicates forward sequence, **B** indicates backward sequence.

In the first experiment, eighteen His3p- and β-galactosidase-positive clones were isolated. To check for the presence of the RyR, these colonies were tested for hybridization to a DIG-labeled RyR probe: none were positive (Fig. 3.2A). Next, the library inserts were amplified from 10 of the clones by PCR (Fig 3.2B). Inserts were sequenced and analyzed for similarity to sequences in the non-redundant DNA and

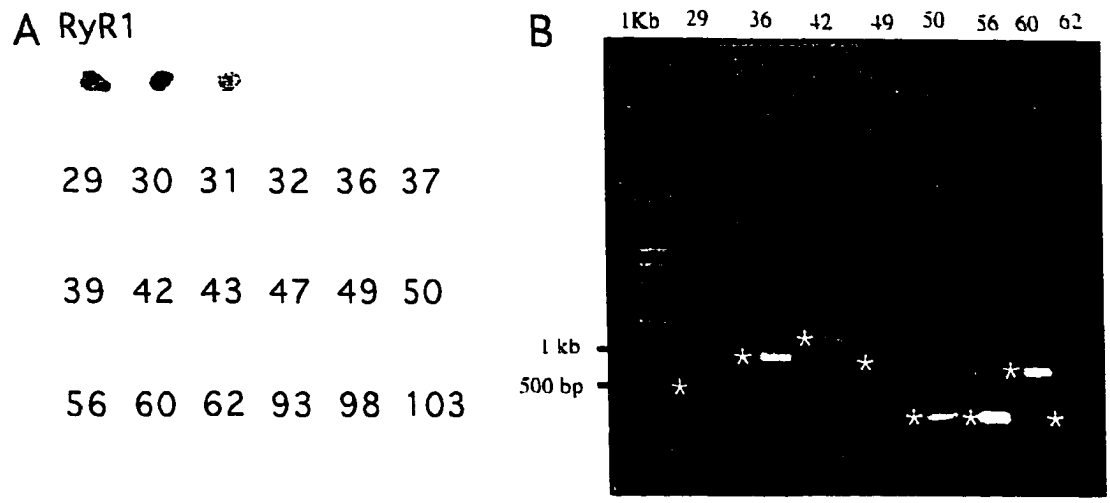

Figure 3.2 s53 x library
A. DIG-RyR probe does not hybridize to positive colonies from s53 library screening. *Upper row:* dilution series of pCI-neo-RyR1 (90 ng, 9 ng, 900 pg, 9 pg). *Lower rows:* PCR products amplified from His3p and b-galactosidase-positive colonies from s53 x library screen #2. None of the colonies hybridized strongly with the digoxigenin-labeled probe.
B. s53 x library PCR products. Inserts from the indicated colonies were amplified by PCR and the marked bands (*) were purified for sequencing

protein databases using the BLAST-n, -x, and -p algorithms. The results of these searches are summarized in Table 3.1.

Clone s53-36 contained the human h19 gene, a maternal imprinting gene that is highly expressed in skeletal muscle. Although h19 encodes five small open reading frames, it is thought to function as an mRNA rather than as a translated product (Brannan et al., 1990). A BLAST search of the ORF that was in-frame with GAL4-AD gave no hit in database searches.

Clones s53-50 and -60 contained substantial inserts (several hundred base pairs each) from troponin I and troponin T, respectively. However, both of these inserts were

backwards with respect to the GAL4 activation domain and so most likely would not have been translated into troponin fusion proteins. Reverse translation of the gene fragments reveal ORFs expressed in-frame with GAL4-AD. The ORF of clone 50 encoded 114 residues which were similar to both the *E. coli* mobility protein B, mobB, and to *Bacillus brevis* gramicidin S synthetase 2. Clone 60 contained a 80 residue ORF that produced no database hits.

Clone s53-4 contained the gene for the ATP synthetase lipid binding protein subunit C expressed in-frame with GAL4-AD. Subunit c is the H^+-translocating subunit of F0, the nonenzymatic membrane component of the mitochondrial ATPase.

Clone s53-2 contained a backward fragment of the human muscle creatine kinase gene and clone s53-7 carried a backward fragment of the polyubiquitin gene. No database hits were generated for the ORFs encoded by either of these sequences.

The ORFs of clones 36, 50, 60, and C4 were aligned using the Clustal algorithm, but no consensus sequence emerged (data not shown).

s58 x library

s58 is a smaller region of s53 that confers weak skeletal-type EC coupling to chimeric cardiac-skeletal DHPRs (Nakai et al., 1998). One library screen was conducted, but His3p- and β-galactosidase-positive clones arose.

s31 x library

s31 is a region of the II-III loop which is not important in determining EC coupling in chimeric DHPRs but which contains peptide A, a segment that can activate RyRs *in vitro*. In order to test the hypothesis that the peptide A has its effects by interacting with other proteins of the triad junction, s31 was used as bait to screen the skeletal muscle cDNA library. The only $His^+/\beta\text{-}gal^+$ colony with an ORF contained an insert encoding sLIM, a skeletal muscle-specific LIM-domain containing protein of unknown function (Morgan and Madgwich, 1996). To test this intriguing interaction further, direct interaction assays of s31 x sLIM were performed. However, no interaction was detected in these experiments (Fig. 3.3).

sLIM x library

Even though sLIM did not interact with s31, it was used as bait in two-hybrid library screens in order to identify other proteins from the skeletal muscle cDNA library with which it could interact. Two His3p- and β-galactosidase-positive clones were isolated. However, both clones contained inserts that were backward with respect to the GAL4-AD sequence: sLIM x lib #1 contained a backward segment of the titin gene, and clone #2 contained a backward segment of creatine kinase DNA. No significant ORFs were encoded by either insert (data not shown).

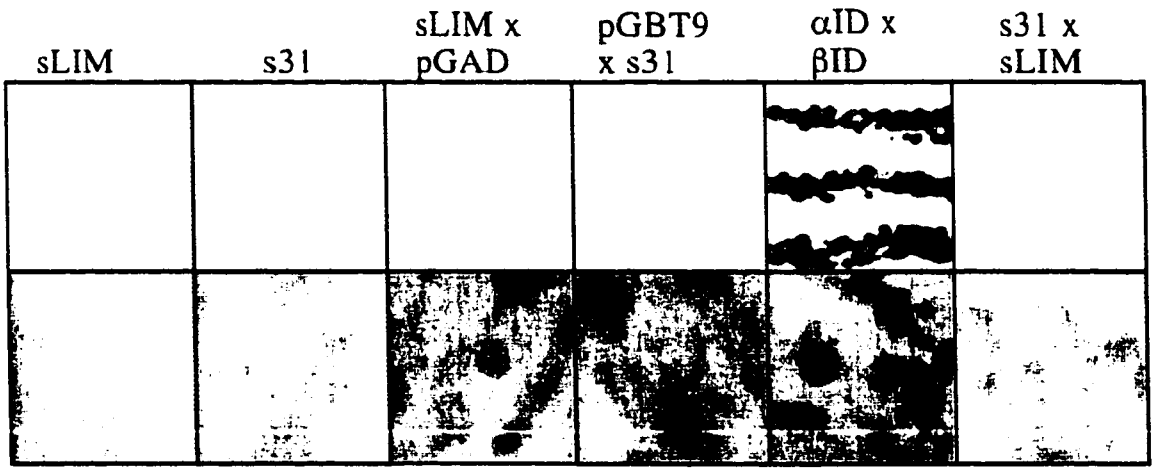

Figure 3.3 sLIM does not interact with s31 in yeast two-hybrid assays.
Nutritional selection *(top row)* and β-galactosidase *(bottom row)* assays of yeast strain HF7c transformed with the indicated plasmids. Growth *(top)* or blue color *(bottom)* indicates an interaction between the controls aID and bID. No interaction is detected between s31 and sLIM in either assay.

Ryanodine receptor x library

In order to find proteins that interact with RyR1 and that may play a role in excitation-contraction coupling, the skeletal muscle cDNA library was screened with parts of RyR1 that have been shown to be important for skeletal EC coupling in chimeric ryanodine receptors (Fig. 2.3).

R9 is a segment of RyR1 which is important for transmitting the retrograde signal back to the dihydropyridine receptor (Nakai et al., 1998; Grabner et al., 1999) and which may direct the formation of DHPR tetrads (Protasi, personal communication). In order to use R9 as bait in yeast two-hybrid library screens, it was divided into four segments, three of which, R9A, B and C, were used as baits in cDNA library screens (Table 3.2).

R10 is a region of RyR1 that is important both for skeletal-type EC coupling and for transmission of the retrograde signal back to the DHPR (Nakai et al., 1998). A total of 7 fragments of R10 were used as bait in two-hybrid library screens. Sequence data were obtained for interactors of three of these fragments, R10A, R10C, and R16 (Table 3.3).

R9A x Library

Three His3p- and β-galactosidase-positive clones were isolated from a library screen using R9A as bait. Plasmids from these colonies were isolated and their inserts were amplified by PCR.

As summarized in Table 3.2, clones R9-A2 and -A3 contained 3' untranslated regions from, respectively, the human protein synthesis initiation factor 4B gene and the unr protein gene. Neither encoded a significant ORF.

R9B x Library

Only one clone, R9-B2, was positive for both *HIS3* and *lacZ* reporter genes. It contained a backward fragment of the alpha actin gene. The ORF expressed in-frame with GAL4-AD encoded 15 residues, including eight sequential phenylalanines. There were no database hits for this sequence.

bait	growth	β-gal filter lift	clones	BLASTX	BLASTP with ORF (expect = 10)
sR9A	+ (8 colonies)	+ (3 colonies, 16 hours)	A2	5' untranslated region of protein translation intiation factor 4Bgene **F**	39 residue ORF no hit
			A3	5' untranslated region of unr protein gene **F**	4 residue ORF no hit
sR9B	+ (6 colonies)	+ (1 colony, 16 hours)	B2	skeletal muscle alpha actin **B**	15 residue ORF poly-Phe (8) no hit
sR9C	+ (18 colonies)	+ (3 colonies, 16 hours)	C2	ferritin heavy chain F	proline-rich domain with several hits (see text)
			C13	blood coagulation factor VIII gene **B**	poly-CV domain similar to haptoglobin-related protein
			C17	skeletal muscle alpha actin **F**	23 residue ORF no hit

Table 3.2 R9 library screening results. F indicates forward sequence, **B** indicates backward sequence.

R9C x Library

Three His3p- and β-galactosidase-positive colonies were isolated and their inserts amplified. Clone R9-C2 contained bases 22-862 of the gene for ferritin heavy chain expressed out-of-frame with the GAL4-AD. This segment included 184 bases of 5'

untranslated region which encoded a 58 residue ORF. This sequence was proline-rich. Consequently, it had many matches in the database, including the β_{1a} subunit of the DHPR.

Clone C13 contained a backward segment of the blood coagulation factor VIII gene. The ORF expressed in-frame with GAL4-AD had a poly-CV region with similarity to the human haptoglobulin-related protein.

Clone C17 contained bases 26-685 of the human adult skeletal muscle alpha actin gene expressed out-of-frame with the GAL4-AD. The initial part of the fragment, the 5' untranslated region of the alpha actin gene, encoded a 23 residue ORF with no hits in the database.

R10A x Library

Ten His3p- and β-galactosidase-positive clones were isolated and inserts from six of these were amplified and sequenced (Table 3.3). Clone R10A3 contained the gene for β-tropomyosin, but the coding sequence was not in-frame with GAL4-AD. There was an in-frame 23 amino acid ORF, but it produced no database hits. Clone R10A-4 contained a backward fragment of the cytochrome c oxidase gene with no ORF. Similarly, clone R10A5 contained the gene for NADH dehydrogenase subunit 2, but without an in-frame ORF. Clone R10A-6 contained an out-of-frame segment of gene for proteosome subunit Y with a 40 residue ORF which produced no match in BLAST searches. Finally, clone

Bait	growth	β-gal filter lift	clones	BLASTX	BLASTP with ORF (expect <10)
R10A	++ (16 colonies)	++ (10 colonies)	A3	β tropomyosin **F**	23 residue ORF no hit
			A4	cytochrome c oxidase **B**	4 residue ORF no hit
			A5	NADH dehydrogenase subunit 2 **F**	7 residue ORF no hit
			A6	proteosome subunit Y **F**	40 residue ORF no hit
			A9	ubiquitin fusion degradation protein 1 homolog **F**	~30 residue ORF no hit
			A10	no match in database	5 residue ORF no hit
R10C	+ (12 colonies)	+ (4 colonies)	C1	DNA repair helicase **F**	29 residue ORF no hit
			C4	eukaryotic translation initiation factor-4E **F**	residues 9-129 of eIF-4E
			C5	cytochrome b **B**	72 residue ORF no hit

Table 3.3 R10 library screening potential interactors. **F** indicates forward sequence, **B** indicates backward sequence

R10A-9 contained the gene for a homologue of the ubiquitin fusion degradation protein-1, including 5' untranslated sequence and out-of-frame coding sequence. A 30 residue ORF in-frame with GAL4-AD produced no hits.

R10C x Library

Four clones were positive for both His3p and β-galactosidase. PCR amplification of the inserts gave bands for clones R10C-1, -4, and -5. R10C-1 contained part of the DNA repair helicase gene out-of-frame with GAL4-AD. R10C4 was a segment of the eukaryotic translation initiation factor 4E (eTI4E) gene encoding 120 residues of the eTIF-4E gene product in-frame with GAL4-AD. Clone C5 was a segment of the cytochrome b gene inserted backwards with respect to the GAL4 activation domain, but encoding a 72 residue ORF. This sequence produced no matches in the database.

R16 x library

Since R16 is a region of the ryanodine receptor that may be important for skeletal-type EC coupling (Proenza and Nakai, unpublished), the muscle cDNA library was screened for proteins that may interact with R16. A total of 7 inserts from three separate library screens were sequenced. One clone, which encoded carbonic anhydrase III, had an open reading frame contiguous with the GAL4 activation domain (Table 3.4).

Junctin x library

Junctin is a single transmembrane protein of unknown function which is exclusively expressed in junctional sarcoplasmic reticulum Jones et al., 1995 and which has been shown to form a complex with triadin, calsequestrin, and ryanodine receptors Zhang et al., 1997. Preliminary data suggests that junctin causes junction-like

arrangements to form between the plasma membrane and the endoplasmic reticulum of CHO cells (Franzini-Armstrong, personal communication). Table 3.5 summarizes results of library screens using junctin and "cytojunctin" as bait.

Bait	*growth*	*β-gal filter lift*	*clones*	*BLASTX*	*BLASTP with*
sR16	+ (17 colonies)	- (16 hours)	2.1	cytochrome b **B**	poly-Phe (10) no hit
			2.2	cytochrome c oxidase, subunit III **B**	2 residue ORF no hit
			2.3	carbonic anhydrase III **F**	in-frame carbonic anhydrase with 36 bp 5' UTR
			3.2	cytochrome oxidase c **F**	1 residue ORF no hit
			3.8	synaptobrevin **B**	1 residue ORF no hit
			3.11	triadin **B**	52 residue ORF similar to NADH ubiquinone oxidoreductase chain 2, and to cytochrome c oxidase subunit III
			3.12	myoglobin **F**	myoglobin with 24 bp 5' UTR

Table 3.4 sR16 library screening results. **F** indicates forward sequence, **B** indicates backward sequence.

Junctin x Library

Inserts from three His3p- and β-galactosidase-positive clones were amplified and sequenced. J2 was a small (13 bp) insert with no match in the database. J4 was the gene for a translationally controlled tumor protein inserted backwards with respect to GAL4-AD It did not encode an in-frame ORF. J5 contained sequence encoding residue 12 at least through residue 74 of the arsenite translocating ATPase gene, hASNA-1, a member of the superfamily of ATP-binding proteins with a distinct nucleotide binding motif (Kurdi-Haidar et al., 1998).

Cytojunctin x library

Cytojunctin is a clone expressing only the putative cytoplasmic domain of junctin. Inserts from seven interacting colonies from two separate library screens were sequenced (Table 3.5). Sequence data for these clones was poor. Consequently, it was difficult to get a meaningful database match for any but clones CJ1.7 and CJ2.1, which contained backward sequences for NADH dehydrogenase and ribosomal protein L29, respectively. The ORFs from all clones were compared to the protein database. Several contained poly-phenlyalanine regions, but no database matches were found for any of these sequences.

Bait	growth	β-gal filter lift	clones	BLASTN/X	BLASTP with ORF
Junctin	+ (13 colonies)	+ (5 colonies)	J2	no hit (13 bp insert)	no ORF
			J4	translationally controlled tumor protein **B**	no ORF
			J5	arsenite translocating ATPase residues 12-74 **F**	arsenite translocating ATPase residues 12-74
Cytojunctin	+ (8 colonies)	+ (8 colonies)	CJ1.1	weak similarity to ladinin **F**	11 residue ORF no hit
			CJ1.2	no hit	poly-Phe (10 res) no hit
			CJ1.3	no hit	6 res ORF no hit
			CJ1.4	no hit	poly-Phe (noisy) no hit
			CJ1.5	no hit (no insert?)	no hit
			CJ1.6	no hit	no hit
			CJ1.7	NADH dehydrogenase **B**	7 residue ORF (3 Phe's) no hit
			CJ2.1	ribosomal protein L29 gene **B**	no hit

Table 3.5 Junctin library screening results. F indicates forward sequence. **B** indicates backward sequence.

FKBP x library

FKBP (FK506-binding protein) is a peptidyl proline isomerase that is closely associated with ryanodine receptors with a stoichiometry of 1 FKBP per RyR subunit (Jayaraman et al., 1992). FKBP may couple gating of adjacent ryanodine receptors

(Marx et al., 1998). To search for interaction partners of FKBP and to identify its binding site on RyR1, FKBP was used as bait in two-hybrid library screens.

FKBP x library

In two separate library screens, no His3p-, β-galactosidase-positive interactors were isolated for FKBP (data not shown).

FKBP x RyR epitope library

In three separate screens of the ryanodine receptor epitope library, only one interacting colony was identified and sequenced: it encoded RyR residues 3522-3741 (data not shown).

DISCUSSION

Yeast Two-Hybrid Negative Results

No strong interactors were detected in yeast two-hybrid library screens using the N-terminus, or the I-II, II-III, or III-IV loops of the DHPR or for regions R9A and R10A of RyR1 as bait. This, of course, does not mean that these regions of the DHPR or RyR do not participate in protein-protein interactions, only that none were found in these experiments. In fact, the cytoplasmic domains of the DHPR *must* interact with other muscle proteins in order to localize the channel. Indeed, despite the fact that the I-II loop of the DHPR is known to bind to the β subunit, this interaction was not detected in library screens using the entire I-II loop as bait. One possible explanation, as discussed above, is that larger test segments may not react as strongly as smaller pieces. The same phenomenon was also seen in the case of the DHPR II-III loop which did not yield positive interactions until subdivided.

Likely-False Positives

The "likely-false positive" group of $His3^+$/ β-gal^+ clones fell into several categories which made them improbable candidates for a physiological role in triad junctions. The first category consists of clones which contained little or no ORF. These

clones were most likely to give positive results through a variety of unorthodox mechanisms of reporter gene activation, as discussed in chapter 1. The second category of likely-false positives is composed of colonies that encoded ORFs and that probably yielded positive results in the normal manner, i.e., by interacting with the bait fusion protein to reconstitute the GAL4 transcription factor. In these cases, factors such as the subcellular localization of the encoded protein suggested that these interactors were false positives because they were unlikely to play a role in the formation or function of triad junctions. One possible mode of gene activation for these interactors is via a protein binding motif that mimics an actual interactor. However, obtaining circumstantial evidence for such a mechanism requires the presence of the motif in multiple hits. Moreover, prevalent interactors are likely to include false positives that satisfy an unknown set of requirements for artifactual gene activation.

UTRs and components of the mitochondrial oxidative phosphorylation complexes were common interactors in two-hybrid library screens using triad junction proteins as bait.

The sense and anti-sense nucleotide sequences of parts of the NADH-ubiquinone oxidoreductase complex and of the cytochrome c oxidase complex were common interaction partners of many of the baits used in library screens. Parts of these sequences were isolated from library screens multiple times using a variety of triad junction protein segments as bait. The fact that many of these interactors did not encode an ORF in-frame

with the GAL4-AD suggests that a mechanism other than functional reconstitution of the GAL4 transcription factor was responsible for reporter gene expression in these cases. Their recurrent appearance as positive interactors suggests artifactual reporter gene activation. Similarly, other interactors were observed to contain 5' and 3' UTRs of various genes as the GAL4-AD plasmid inserts. In the cases where no ORF was encoded by these gene fragments, they were probably false positives. However, it is possible that the transcripts could have been spliced in the yeast to produce in-frame fusion proteins of the inserted genes.

The R10C region of RyR1 may interact with eukaryotic translation initiation factor 4E.

The 5' end of mRNAs are capped by a methyl-guanosine. The cap stabilizes mRNA, allows efficient processing in the nucleus, promotes transport to the cytoplasm, and facilitates ribosome binding to the mRNA. The eukaryotic translation initiation factor 4E (eIF4E) is the rate-limiting, cap-binding subunit of eIF4F, an RNA helicase. It is therefore probable that the interaction detected between R10C and eIF4E is a false positive. However, several theoretical alternatives exist. First, it is possible that R10C interacts with a protein that has a domain in common with eIF4E. Second, a complex of endogenous yeast proteins, according to the models presented in chapter 1, could provide a link between R10C and eIF4E. This idea is supported by the recent observation that the target of rapamycin (TOR) signal transduction pathway regulates the eIF complex

(Berset et al., 1998). This is alluring because it suggests that R10c could be connected indirectly to eIF4E via a complex that includes FKBP. Such a complex could provide a pathway for post-transcriptional control of gene expression in response to cellular stressors and may indicate a possible binding site for FKBP on the RyR.

h19

Another strange, but recurrent, interactor was the h19 gene which was isolated using s53 as bait. Although the ORF encoded by the h19 gene did not produce any database hits at E=10, increasing the expect value to 100 showed that the ORF was similar to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This is of special interest for several reasons that will be discussed further in chapter 4: (i) GAPDH has been demonstrated to bind DHPRs and RyRs in triad junction preparations (Brandt et al., 1990). (ii) Other glycolytic enzymes were potential interactors of the DHPR C-terminus (chapter 4). (iii) h19 was also found to be an interactor of the DHPR C-terminus.

Dubious Positives

This group of interactors contains proteins that are of questionable significance for the structure and function of triad junctions.

Junctin may interact with the arsenite translocating ATPase

Full-length junctin gave a strong positive signal for interaction with the human arsenite stimulated ATPase (hASNA-1). This ATPase is a member of a superfamily of ATP-binding proteins which includes the *E. coli arsA* gene product. hASNA-1 is found in the cytoplasm and nucleus of both cardiac and skeletal muscle (Kurdi-Haidar et al., 1998), but its function is unknown. It is possible that junctin actually interacts with the known sarco-endo-plasmic reticulum Ca^{2+} ATPase (SERCA) via a domain that is similar to the hASNA-1 gene product.

s53 may interact with ATP synthetase lipid binding protein subunit C

The ATP synthetase lipid binding protein subunit C was a potential interactor of the s53 region of the DHPR II-III loop. The established subcellular location for this protein is the mitochondrial inner membrane, making it an unlikely interactor for a cytoplasmic domain of the DHPR. However, since this subunit contains a loop in the mitochondrial cytoplasm that is known to participate in protein-protein interactions (Fillingame, 1997) it is theoretically possible that the ATPase subunit C could interact with s53 via a complex of proteins such as transporters or porin that transverse the outer mitochondrial membrane.

Several recent reports make this suggestion plausible (if unlikely). First, the mitochondrial adenine nucleotide translocase, ANT, has been identified as a component

of SR preparations (Yamaguchi and Kasai, 1998). ANT transverses the mitochondrial membrane and is known to interact with the voltage-dependent anion channel (VDAC; Wallace, 1999). Second, a body of data is emerging that suggests a physical connection between mitochondria and ER/SR (Rizzuto et al., 1998). Thus, it is possible to build a model by which SR are linked to the plasma membrane via association with mitochondrial proteins.

Slightly Less Dubious Positives

Carbonic anhydrase III is a potential interactor of the sR16 region of RyR1.

Carbonic anhydrase III (CAIII) is highly expressed in skeletal muscle (~8% of soluble protein), and is unique among the carbonic anhydrases because it also possesses a phosphatase activity that is regulated by glutathiolation (Cabiscol and Levine, 1996). The physiological function of CAIII is unknown, but this finding suggests that it may also function as a protein phosphatase in response to oxidative stress. CAIII is a traditionally viewed as cytosolic enzyme; thus, CAIII could be available to interact with the RyR in skeletal muscle cells. This may be one mechanism by which oxidative stress affects EC coupling and RyR function. Alternatively, some evidence suggests the association of CAIII with the SR which has been hypothesized to play a role in producing or buffering the protons that are exchanged for Ca^{2+} across the SR membrane (Wetzel and Gros, 1998).

The R9B region of RyR1 and the cytoplasmic domain of junctin may interact with phenylalanine repeat motif

A total of 6 interactors were isolated that contained stretches of up to 10 consecutive phenylalanines expressed in-frame with GAL4-AD, usually directly adjacent to the GAL4-AD sequence. The large number of such positives raises the question of whether this may represent an actual physiological interaction of several important triad junction proteins with a common motif. Since BLAST searches did not reveal any human proteins with multiple phenyalanines, this motif could represent a novel junctional protein. Extending the BLAST search to all organisms revealed that some carbonic anhydrases contain 10 consecutive phenylalanines. The human CAIII discussed above is not among these, but the coincidence raises the (remote) possibility of a unique carbonic anhydrase isoform participating in EC coupling interactions.

Positives of Intrigue

sLIM may interact with s31

sLIM is a skeletal muscle-specific LIM-domain containing protein of unknown function (Morgan and Madgwich, 1996). Because sLIM is localized to triads and contains four-and-a-half protein-binding LIM domains (Morgan and Madgwich, 1996), it seemed plausible not only that sLIM could be a physiological interaction partner of the DHPR, but that it could play a role in organizing multiple proteins at the triad junction.

Despite the fact that an interaction between sLIM and s31 was not confirmed by direct interaction two-hybrid tests, sLIM remains a good candidate for organizing protein-protein interactions at the triad junction (whether or not it interacts with DHPRs and RyRs) and could be tested by more extensive two-hybrid library screens as well as by *in vitro* binding studies.

The R9C region of RyR1 may interact with a proline-rich domain, possibly in the DHPR β subunit.

Clone R9C2 encoded a 58 residue ORF which contained 13 prolines, including 4 consensus SH3 binding domains (PXXPrp). Proline-rich domains are common in protein-protein interaction domains that mediate low-affinity, high-specificity binding based on proline's unique N-substituted peptide backbone rather than on canonical high affinity side-chain interactions (Nguyen et al., 1998):

> The recognition strategy of SH3 and WW domains allows for high specificity binding that need not be of high affinity. In vivo, binding to proline peptides is highly selective, despite sub-optimal shape complementarity, because there are no other natural sequences that can satisfy the minimal ligand backbone requirements. The resultant weak but specific interactions are ideal for intracellular signaling domains. These modules must recognize ligands with high enough selectivity to maintain proper information flow but with low enough affinity to allow for sensitive and dynamic modulation in response to changing signals. In contrast, the high-affinity and high-specificity interactions that result from typical lock and key recognition are ill-suited for such a function. The

ability to recognize proline in this way may explain why proline-rich motifs are so commonly used in regulatory interactions.

The ORF encoded by clone R9C2 had no exact hit in the protein database, but had many hits of high similarity. Based on the frequency of proline-rich domains, this is not surprising. Notably, however, one of these hits was the β_{1a} subunit of the DHPR which is compelling enough to deserve further discussion and investigation.

As diagrammed in figure 3.4A, five domains have been described in β subunits. Domains II and IV are highly conserved among the β's, whereas domains I, III, and V are

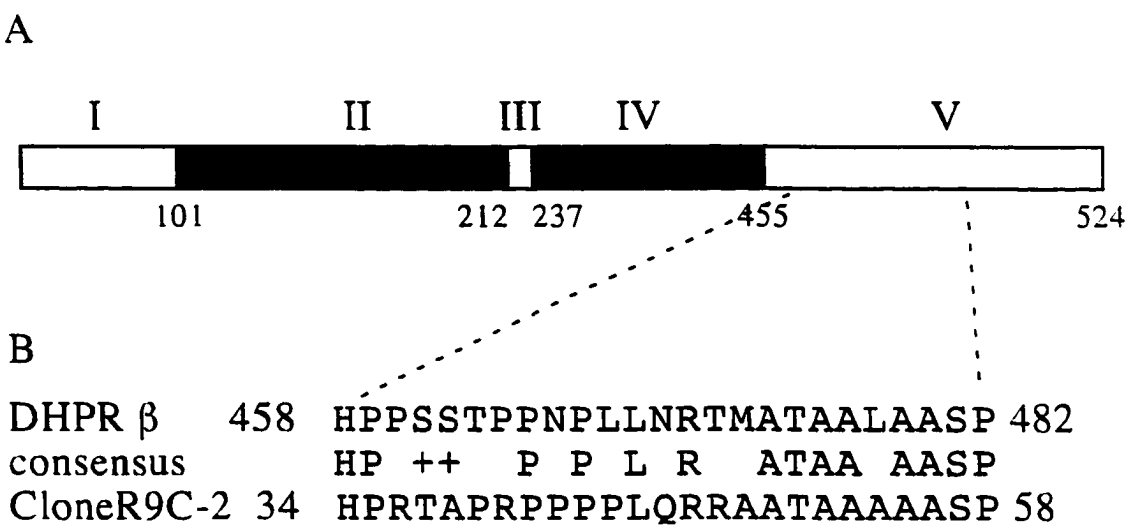

Figure 3.4 DHPR beta subunit.
A. Domain structure of the beta subunit. Five domains, separated by alpha helices have been identified (I-V; Ruth et al., 1989). The grey boxes (domains I, III and V) are divergent among beta subunit isoforms.
B. Alignment of clone R9C with β1A.

rather divergent and undergo alternative splicing. Thus domains I, III, and V have been suggested as possible determinants of the β-specific effects on α subunits. Indeed, domain V of β_4 (a neuronal β isoform) associates with the C-terminus of α_{1a} (a neuronal α isoform) to affect channel inactivation kinetics (Walker et al., 1998), and domain V of β_{1a} (the skeletal muscle β isoform) is required for complete restoration of EC coupling in β-null myotubes (Beurg et al., 1999).

As shown in figure 3.4B, clone R9C-2 was similar to residues 458-482 in domain V of the $\beta_{1\alpha}$ subunit of the DHPR. DHPR β subunits are hydrophilic peripheral membrane proteins that associate with α_1 subunits via the previously described high affinity interaction between the conserved αID and βID motifs. This association has been shown to be important for targeting of both α_{1s} and β_{1a} to the membrane (Neuhuber et al., 1998). There are 4 classes of β subunits, each of which has different modulatory effects when co-expressed with α subunits. Since the α and β interaction domains are highly conserved among all isoforms, the interaction between αID and βID alone cannot be sufficient to account for all of the effects of the β subunits.

The identification of domain V of β_{1a} as a potential interactor of R9C thus suggests a model in which β_{1a} may bridge between α_{1s} and RyR1 in skeletal muscle (Fig. 3.5). The putative interaction between R9C and domain V of β_{1a} could be the structural

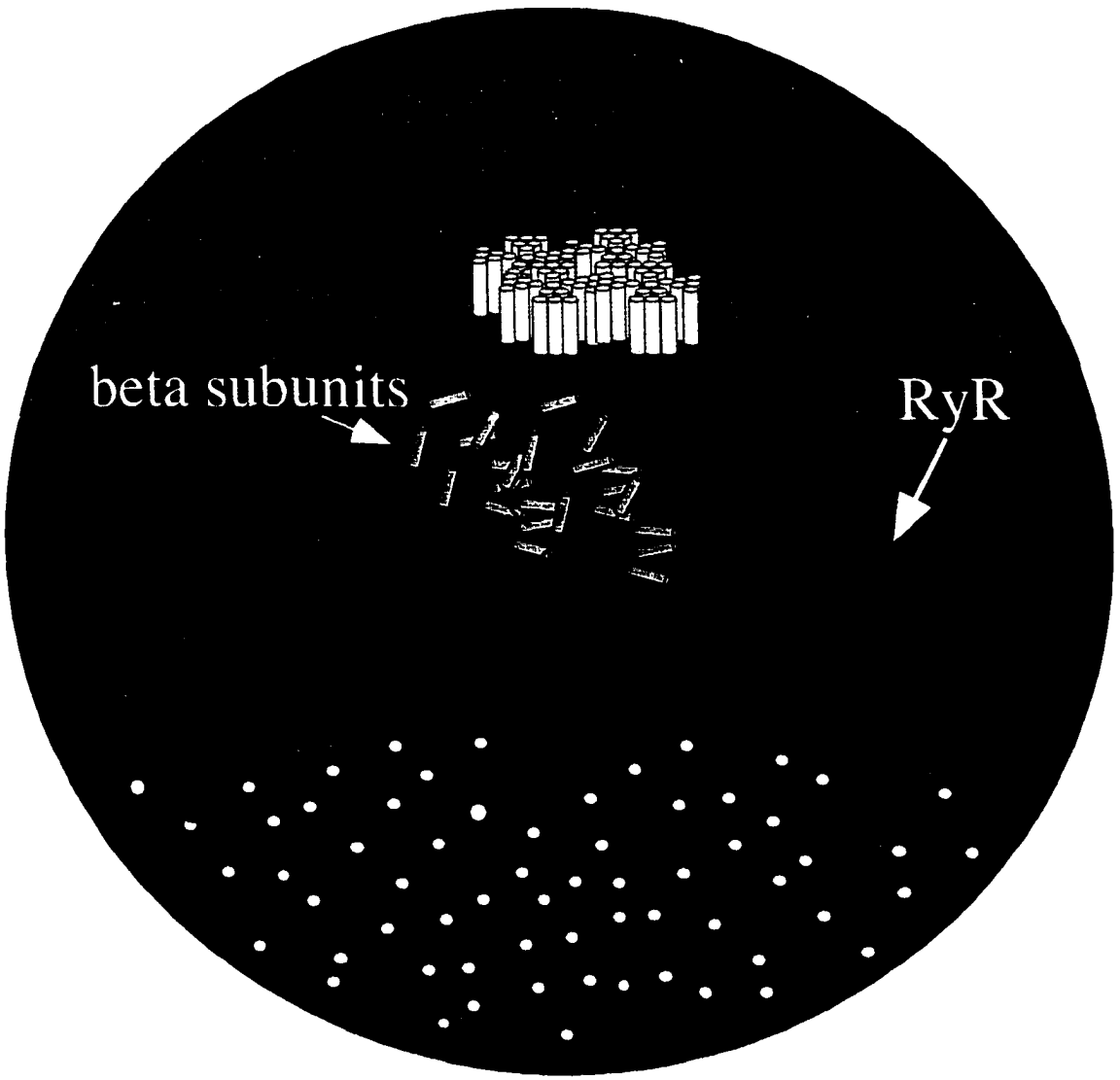

Figure 3.5 Model for DHPR beta subunit involvement in tetrad formation. The RyR directs formation of tetrads via the interaction of its R9C domain with the β1A subunit of the DHPR.

determinant of R9-directed tetrad formation (Protasi, personal communication) and retrograde signaling (Nakai et al., 1998). We plan to test this hypothesis according to the following strategy. (i) The possible interaction between R9C and domain V of β_{1a} suggested by the two-hybrid experiments must be verified. This will be done both by

two-hybrid direct interaction tests and by binding assays *in vitro*. (ii) Deletion and site-directed mutant RyRs will be expressed in dyspedic myotubes to test the prediction that disruption of R9C will interfere with tetrad formation and with retrograde signaling via a loss of signaling through β_{1a}.

CHAPTER 4
IDENTIFICATION OF A REACTIVE DOMAIN OF THE DHPR C-TERMINUS

INTRODUCTION

Although the C-terminus of the DHPR is large and cytoplasmic, little is known about the role it may play in channel function, triad junction formation, or EC coupling. Based on the following observations, we hypothesized that the DHPR C-terminus is a potential site for protein-protein interactions that may be involved in the formation and function of triad junctions. (i) The C-termini of other ion channels — including Shaker K^+ channels, NMDA receptors, Na^+ channels, and TRP Ca^{2+} channels — interact with cytoplasmic proteins containing PDZ domains to determine subcellular localization and channel clustering (reviewed in Kornau et al., 1997). (ii) The carboxyl terminals of Ca^{2+} channel α_1 subunits are the largest of their cytoplasmic domains and are highly divergent among the different isoforms, suggesting a possible role in isoform-dependent channel functions. (iii) α_{1s} lacking a C-terminal domain is not functional (Tanabe and Beam,

unpublished observations), suggesting that the C-terminus is an essential feature of the channel.

The cDNA for α_{1s} predicts a protein of 1873 amino acids; however, the predominant form of α_{1s} in mature skeletal muscle is truncated in the C-terminus at a site between amino acids 1685 and 1699 (De Jongh et al., 1991). A mutant truncated at amino acid 1662 retains normal function as a calcium channel and as the voltage sensor for EC coupling when expressed in dysgenic myotubes (Beam et al., 1992), and the cardiac C-terminus can be substituted for skeletal in chimeras that still support skeletal-type EC coupling (Tanabe et al., 1990). The conserved region of α_1 subunits that is adjacent to IVS6 appears to be crucial to channel function since deletion of α_{1c} at this region results in dramatically decreased channel expression (Wei et al., 1994), and since a truncation of α_{1s} at 1393, just past IVS6, does not produce a functional channel (Tanabe and Beam, unpublished observations). These results together suggest that a critical domain, perhaps involved in channel localization, may exist in the region between residues 1393 and 1662 of α_{1s}.

One candidate domain for such an interaction is a consensus PDZ binding motif located near the end of the truncated C-terminus at residues 1639-1642. PDZ domains are modules of 80-90 amino acid that mediate protein-protein interactions by at least three distinct mechanisms. First, PDZ domains can bind directly to specific recognition sequences at the C-terminus of transmembrane proteins. For example, PSD-95 interacts

with a C-terminal motif in NMDA receptor 2B subunits via this mechanism (Kornau et al., 1995). Second, PDZ domains can form heterotypic dimers with other PDZ domains, as is the case for the interaction of the PDZ domain in nitric oxide synthetase with the PDZ domains in PSD-95 and in syntrophin (Brenman et al., 1996). The third mode of interaction, in which the consensus binding motif is not at the C-terminus, is demonstrated by the interaction of the TRP Ca^{2+} channel with the PDZ domain of INAD in *Drosophila* photoreceptors (Shieh and Zhu, 1996) and by the ability of synthetic cyclic peptides lacking a terminal carboxylate group to interact with syntrophin PDZ domains (Gee et al., 1998).

In order to test the hypothesis that some part of the DHPR C-terminus — including the consensus PDZ binding motif — interacts with other proteins of the triad junction, segments of the C-terminus were used as bait in yeast two-hybrid screens of a skeletal muscle cDNA library.

RESULTS

The C-termini of α_{1s} and of α_{1c} are highly reactive

Four separate two-hybrid screens of the skeletal muscle cDNA library were performed using the full-length C-terminus as bait. In each of these experiments, tens of thousands of $His3p^+$ and β-gal^+ colonies were identified (Fig. 4.1A,B). The cardiac

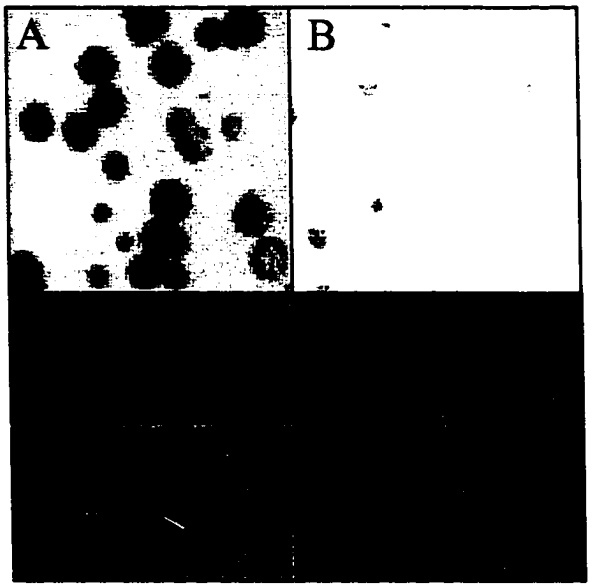

Figure 4.1. The C-terminals of both α1s and α1c are highly reactive in two hybrid library screens. A human skeletal muscle cDNA library was screened for interaction using the C-terminus of α1s and α1c as bait. (A,C) Nutritional selection assays of library screens using yeast strain HF7c carrying the C-terminal domain of α1s (A) or α1c (B) and plated on SD/-Trp/-Leu/-His minimal media. β-galactosidase filter assays of α1s (C) and α1c(D) library screens.

DHPR carboxyl terminus was similarly reactive in two-hybrid library screens (Fig. 4.1C,D). Neither the skeletal nor the cardiac C-terminus activated either the *HIS3* or the *lacZ* reporter gene alone or when co-transformed with the empty pGAD424 plasmid (data not shown).

Identification of the reactive domain of α1s

Because smaller test peptides reacted more specifically in control two-hybrid screens (chapters 2 and 3), and because the reactivity of the C-terminus was a potential artifact to be eliminated from further analysis of C-terminal interaction partners, the "sticky" domain was identified by screening the cDNA library with progressively smaller

parts of the C-terminus. As schematically illustrated in figure 4.2, segment E of the skeletal DHPR C-terminus (residues 1621-1647) was necessary and sufficient for the high reactivity.

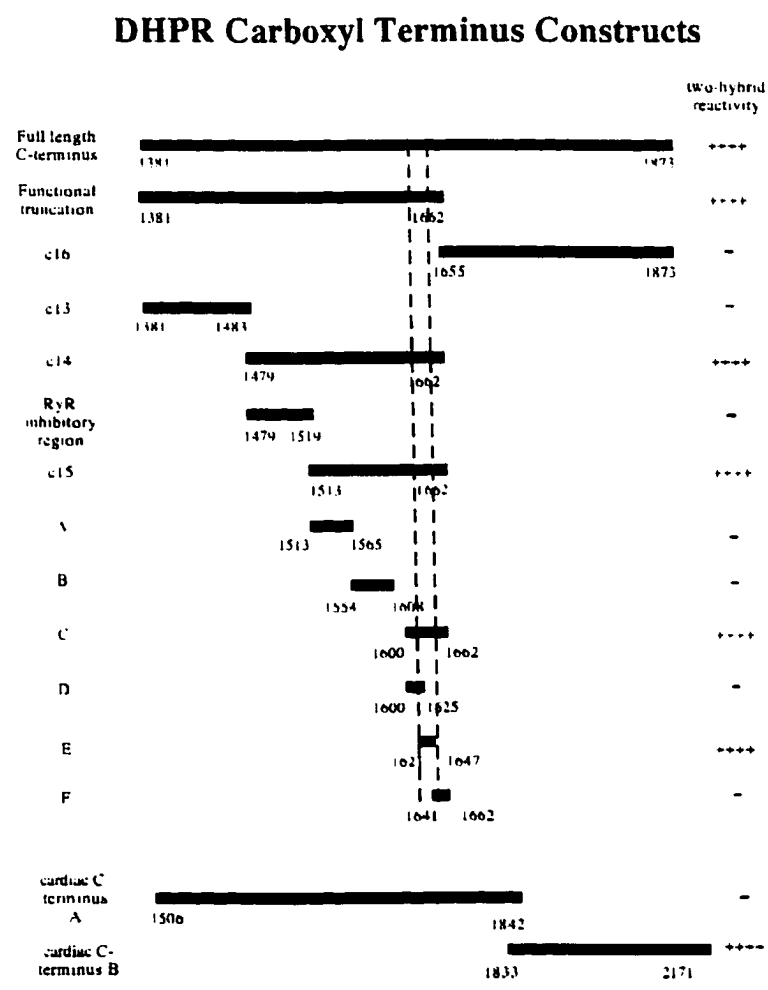

Figure 4.2 Identification of the reactive region of the DHPR C-terminus. A human skeletal muscle cDNA library was screened for interaction using segments of the C-terminals of α_{1s} and α_{1c} as bait. Segment E of α_{1s} and Segment B of α_{1c} were necessary and sufficient to confer the high reactivity.

In contrast to the full-length C-terminus, segment E alone, and in combination with the empty pGAD424 plasmid, activated the *lacZ* reporter gene (Fig. 4.3). Segment

E did not, however, activate the *HIS3* gene in the absence of library cDNA (data not shown).

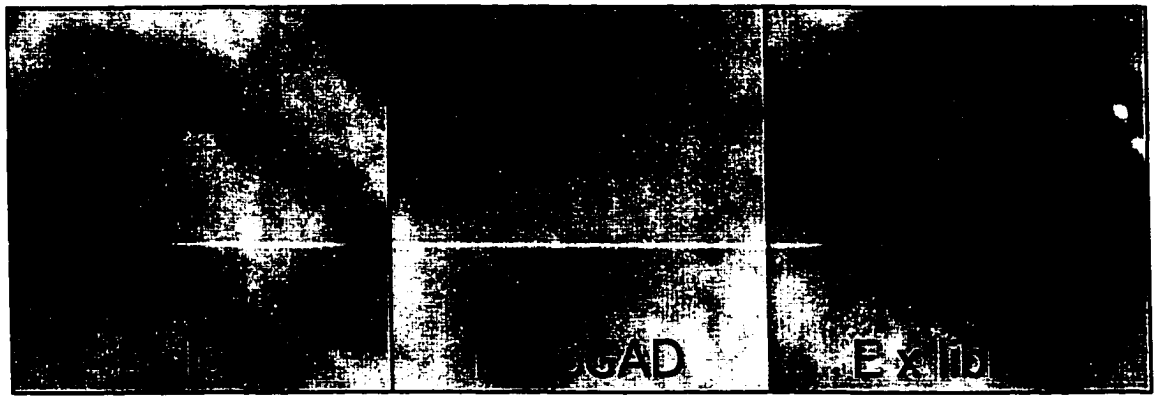

Figure 4.3 Segment E of the skeletal DHPR C-terminus can alone activate transcription of the β-galactosidase gene. E alone, E x pGAD, and E x library demonstrate high levels of β-galactosidase activity in a filter assay.

Identification of possible interaction partners

Table 4.1 summarizes the sequencing data obtained for some inserts from representative positive colonies from library screens in which the reactive domain of the C-terminus was used as bait. The ORFs of these interactors shared little overall similarity to one another when aligned (data not shown), but some of the individual interactors had weak similarity to proline-rich motifs such as the SH3 domain (PXXPXr), the Homer domain (PPXXFr), and the WW domain (PPXY).

Table 4.1 C-Terminus sequenced interactors. **F** indicates forward sequence, **B** indicates backward sequence.

Bait	*growth*	*β-gal filter*	*clones*	*BLASTN*	*BLASTP with ORF*
full length (1381-1873)	++++	++	C-43	human h19 gene **F**	indeterminate ORF no hit
			C-63	human h19 gene **F**	16 residue ORF (6 prolines) no hit
c15 (1513-1662)	++++	++	c15.2	aldolase A **F** (with 194 bp 5' UTR)	22 residue ORF no hit
			c15.4	skeletal myosin light chain **B**	36 residue ORF no hit
			c15.6	muscle specific enolase **F** (with 40 bp 5' UTR)	57 residue ORF similar to human retinoic acid receptor
Segment C (1600-1662)	++++	++	C1	cytochrome b **B**	2 residue ORF no hit
			C2	insulin receptor kinase **B**	71 residue ORF similar to human muscarinic AChR
			C4	cytochrome b **B**	2 residue ORF no hit
			C6	medium chain acyl coA dehydrogenase **B**	5 residue ORF no hit
			C7	myosin light chain **F**	58 residue ORF similar to HOX-D3 homeobox protein
			C9	cytochrome b **B**	11 residue ORF poly-Phe (8) no hit
			C10	NADH ubiquinone oxidoreductase chain 4 **F**	in-frame NADH ubiquinone oxidoreductase
			C12	titin **B**	20 residue ORF no hit

Table 4.1 (continued)

Segment E (1621-1662)	++++	++	E5	NADH ubiquinone oxidoreductase chain 1 **F**	23 residue ORF (proline-rich) similar PTB-associated splicing factor
			E7	perioxisomal assembly factor-1 **F**	30 residue ORF similar to putative protein kinase (tobacco)
			E9	NADH dehydrogenase **B**	2 residue ORF no hit
			E11	carbonic anhydrase **B**	7 residue ORF no hit
			E12	nuclear core complex protein **B**	indeterminate ORF no hit

Neutralization of three acidic residues does not alter the reactivity of E

The sequence of the reactive domain of the α_{1s} C-terminus is shown in figure 4.4. In order to test the hypothesis that E's reactivity was a function of its acidity, a mutant was constructed in which three of the glutamate residues (positions 1632, 1634, and 1636) in segment E were changed to glutamines. As shown in figure 4.5, the E→Q mutant retained the high reactivity of unmodified segment E.

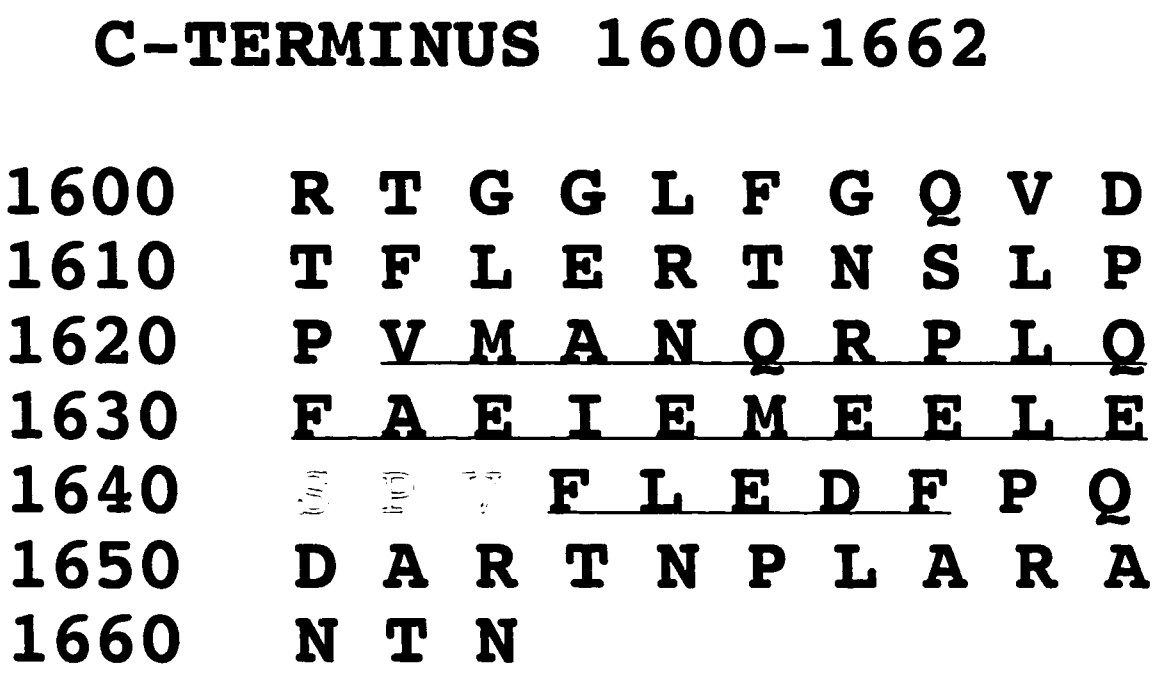

Figure 4.4 Sequence of the reactive region of the DHPR C-terminus. Underline indicates segment E, residues 1621-1647. The consensus PDZ binding motif is outlined.

V1642D eliminates reactivity

Since a consensus PDZ binding motif occurs within segment E, we hypothesized that it may be important for both physiological and artifactual interactions of segment E. To test this hypothesis, the PDZ motif was disrupted with a targeted mutation patterned on that shown to eliminate binding of the photoreceptor TRP Ca^{2+} channel to the PDZ scaffolding protein, INAD (Shieh and Zhu, 1996). As shown in figure 4.6, the V1642D mutation decreased the reactivity of segment E in library screens by about 50-fold.

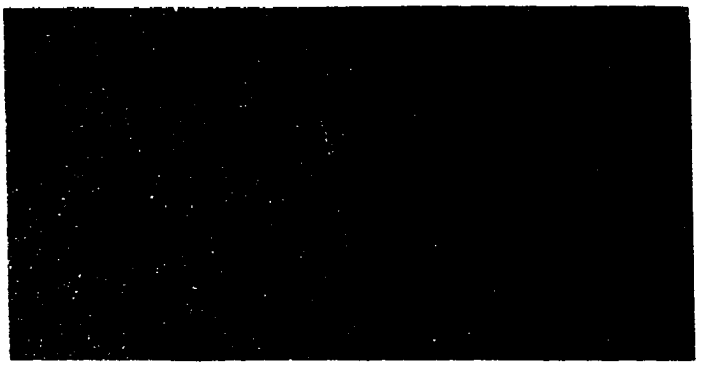

Fig 4.5 E-Q mutant retains the high reactivity of in two hybrid library screens. A. Nutritional selection assay of yeast cotransformed with the E-Q mutated segment E (see text) along with a skeletal muscle cDNA library, and plated on SD/-Trp/-Leu/-His nutritional selection media. (B) β-galactosidase filter assay of cotransformed yeast.

Electrophysiological assays of a truncated DHPR

To determine if the E region of the C-terminus plays a critical role in targeting or function of α_{1s}, a cDNA encoding a channel truncated at residue 1620 (just before segment E) was constructed and inserted into a mammalian expression vector in-frame with a modified green fluorescent protein (GFP) gene (Grabner et al., 1998). This plasmid, δ1620, was expressed in dysgenic myotubes as described in chapter 1. Cells showing green fluorescence were analyzed 18-48 hours post-injection.

The ability of δ1620 to support EC coupling was assayed by whether electrical stimulation could elicit contraction of injected dysgenic myotubes (Tanabe et al., 1988). Definite electrically-evoked contractions were observed in three of 21 cells injected with δ1620 (data not shown). In parallel experiments in dysgenic myotubes from the same

primary cultures injected with wild-type GFP-α_{1s}, five of 28 cells were observed to have electrically-induced contractions (data not shown). These comparisons of the EC coupling function of δ1620 to that of wild type α_{1s} are problematic so far because only a low percentage of the α_{1s}-expressing cells contracted. This is probably indicative of poor myotube health. In initial experiments, it was the impression of one observer (KGB) that the contraction frequency in d1620-injected myotubes was lower than that previously observed for α_{1s}.

In addition to EC coupling, δ1620 was compared to wild-type α_{1s} in its ability to produce Ca^{2+} current (Fig. 4.7A,B). Average Ca^{2+} current density for δ1620 was 4.62 +/- 0.45 pA/pF (mean +/- S.E.M, n=7). In the few successful parallel measurements, α_{1s} current density was 3.57 +/- 0.68 pA/pF (n=3).

In summary, the electrophysiological analysis indicates that δ1620 can function as a voltage-sensor for EC coupling and as a Ca^{2+} channel. However, it is not yet possible to tell if δ1620 functions quantitatively less effectively than the wild-type α_{1s}. To determine if this is the case will require more extensive comparisons of δ1620 and α_{1s}, including analysis of charge movement and fluorescent measurement of intracellular Ca^{2+} release.

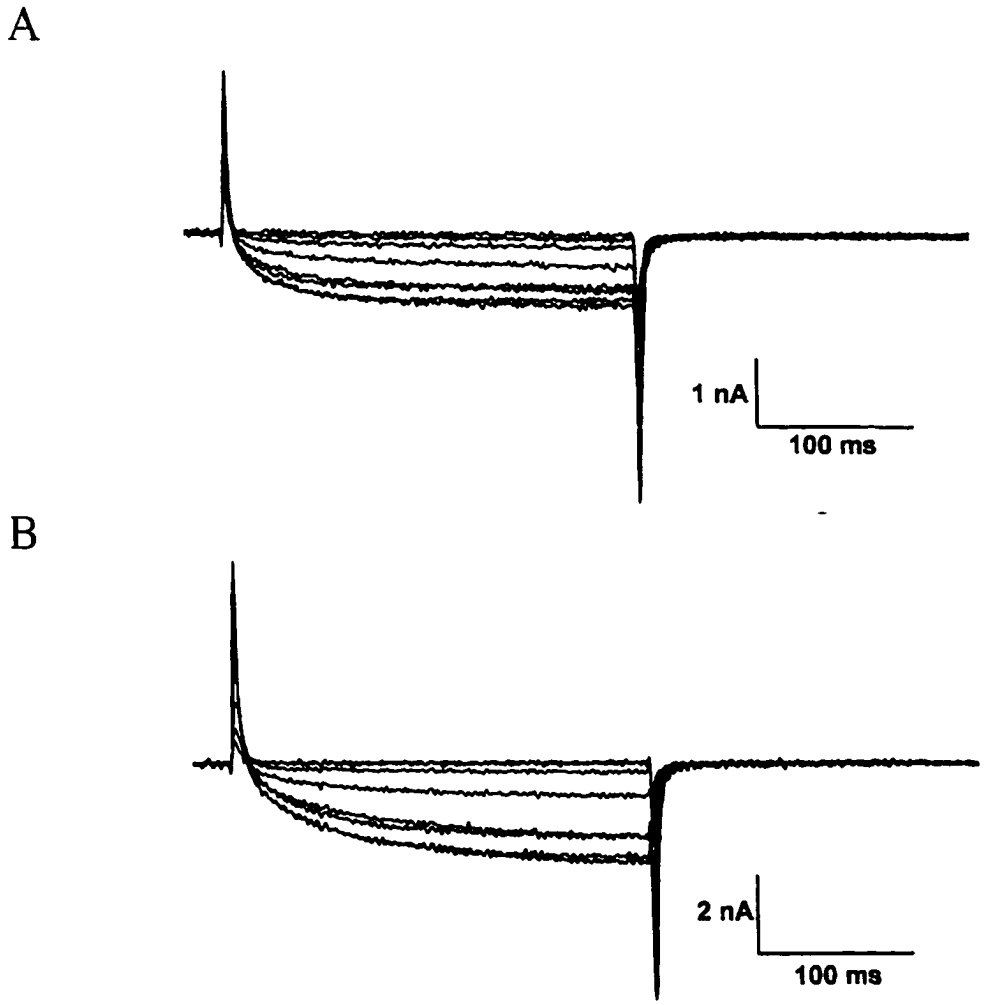

Figure 4.7 Whole cell calcium currents elicited by α1s and by Δ1620 are similar. Representative current traces were recorded from dysgenic myotubes expressing α1s (A) or Δ1620 (B) 24 hours post-injection. Peak current density for α1s was 3.57 +/- 0.68 pA/pF (mean +/- SEM; n=3). Current density for Δ1620 was 4.63 +/- 0.45 pA/pF (n=7).

Subcellular distribution of δ1620

Figure 4.8 shows representative confocal images of GFP fluorescence in two cells expressing wild type α_{1s} (Fig.4.8A,B) and in two cells expressing δ1620 (Fig.4.8C,D). For each construct, one image was gathered that showed the presence of punctate dots

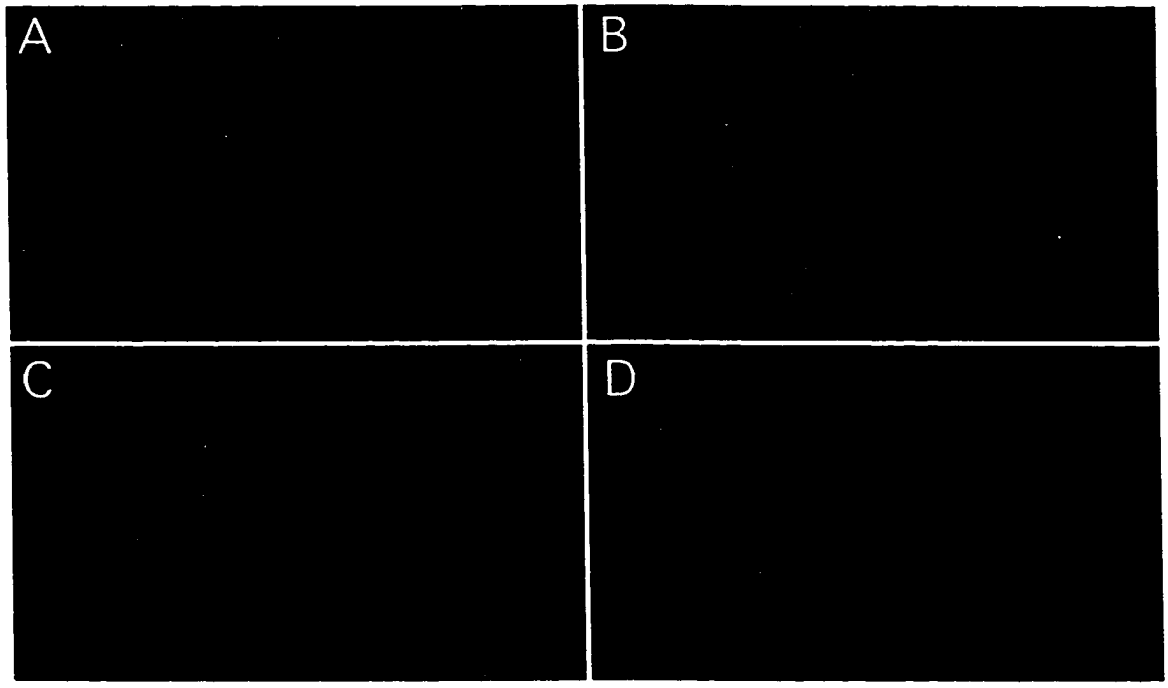

Figure 4.8 Subcellular distribution of α1s and Δ1620 are similar. Representative dysgenic myotubes expressing α1s (A, B) or Δ1620 (C, D) 24 hours post-injection.

indicating channel clustering (Fig. 4.8A,C), and another image was gathered to show membrane localization of the channels (Fig 4.8B,D).

DISCUSSION

Segment E alone can activate *lacZ* ...

Segment E alone (either directly or via an intermediary yeast) activates expression of β-galactosidase in absence of a GAL4-AD fusion protein. This activity was not observed for the full-length C-terminus, suggesting that some part of the full-length C-terminus prevents transcriptional activation. Alignment of segment E with the GAL4-AD

peptide (fig 4.9) reveals that both sequences contain an acidic domain. Since acidic proteins are common transcriptional activators, the acidic domain of E is one possible explanation for its ability to activate *lacZ*. However, this hypothesis is not supported by the observation that the E→Q mutant, in which three of the acidic residues in E were neutralized, did not alter E's ability to activate *lacZ*. Furthermore, this hypothesis does not explain why the V1642D mutation eliminates the reactivity of segment E . An alternate explanation is that E's exceptional reactivity may depend on an interaction with an endogenous PDZ protein in yeast.

Another important question about E's reactivity remains: why does segment E activate only *lacZ* and not *HIS3* ? The ability of E to act as an activation domain to recruit the polymerase should be independent of its binding to the promoter, which, in either case, ought to exist since it resides within the GAL4-BD that is supposed to bind to the consensus GAL4 binding sites in both promoters. It can only be suggested that the segment E fusion protein somehow affects the association of its GAL4-BD with the two promoters differently based on their genetic context.

It is important to reiterate that the full-length C-terminus did not independently activate the *lacZ* gene and that segment E did not have equivalent actions on the two reporter genes. These observations underscore the importance of thorough controls for two-hybrid experiments,

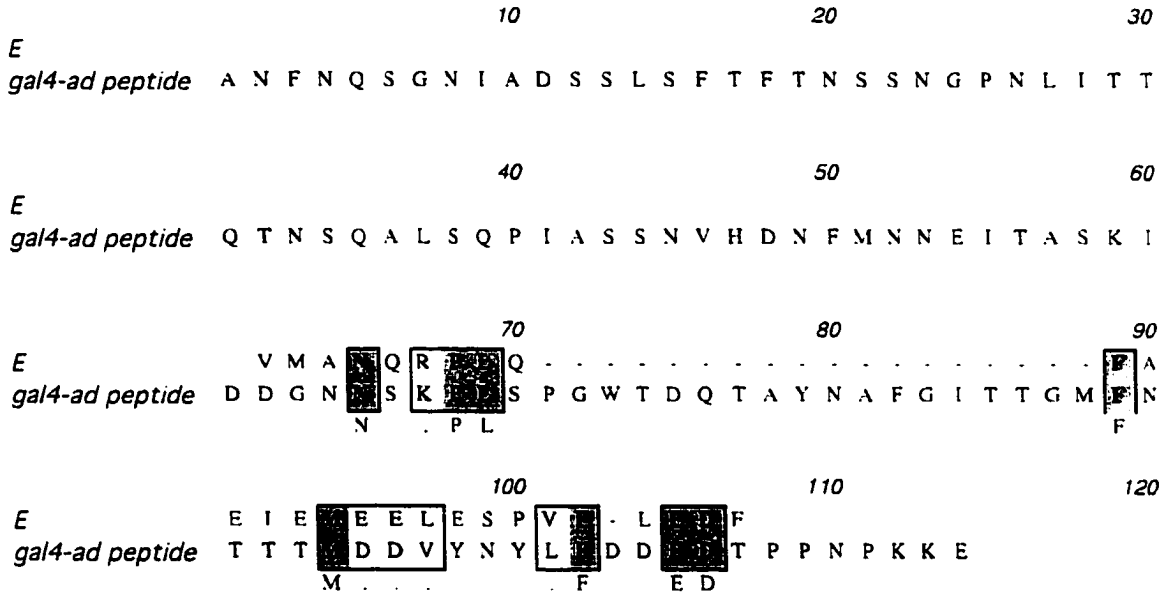

Figure 4.9 Amino acid sequence alignment of C-terminal segment E and the GAL4-AD peptide.

... but, could there be a "real" interactor hiding in the blue?

While there was a qualitative difference between the β-galactosidase activity of segment E alone compared to that of E cotransformed with the empty GAL4-AD plasmid or with the cDNA library, the saturating level of β-galactosidase activity precluded determination of a quantitative assessment of whether there was an additional component of reporter gene activation in the presence of the cDNA library that would suggest an

interaction of segment E with a library protein. However, the *HIS3* reporter gene required the presence of library inserts in the GAL4-AD vector for transcriptional activation. This suggests that there is a component of E's reactivity in library screens that activates transcription via the normal mechanism of reconstitution of the binding and activation domains of GAL4 via a protein-protein interaction.

αID was the only other test fragment to exhibit high reactivity in library screens; moreover, αID alone also weakly activated the *lacZ* reporter gene. It is an interesting coincidence that αID is a known protein interaction domain and that, as suggested by the data presented in chapter 3, its binding partner, the β subunit, may be a multivalent interactor which participates in interactions with proline-rich motifs. Taken together these observations hint at the possibility of common mechanism for activation of the GAL4 promoter by direct or indirect involvement of proline-rich proteins.

Speculations about possible interactors of E

Since *HIS3* gene activation depends on an interaction with a library protein, it is worthwhile to consider some of the possible interactors of E. First, since E contains that alluring PDZ binding motif, and since the reactivity of segment E is dependent on V1642, it is possible that E does interact with a PDZ protein in muscle. However, the lack of a strong phenotype for the δ1620 mutant argues against an essential role for such an interaction, if any exists. Furthermore, none of the sequenced interactors of segment E

contained a the signature PDZ sequence (GLGF). It is worth noting that the DHPR C-terminus contains the sequence GGLFG (residues 1602-1606) which bears enough similarity to the PDZ signature sequence to suggest an interaction within one C-terminus or between C-terminal domains of adjacent DHPRs in a tetrad.

In the absence of a common motif among the C-terminal interactors, other commonalties were sought. Based on the observation that the interactors included the glycolytic enzymes enolase and aldolase (Table 4.1), and that the ORF of the double-interactor h19 bore some similarity to GAPDH, it was hypothesized that the C-terminus may interact with glycolytic enzymes. The glycolytic enzymes pyruvate kinase, enolase, phosphoglyceromutase, GAPDH, phosphoglycerate kinase and aldolase have been reported to be bound to SR of skeletal and cardiac muscle (Xu and Becker, 1998). GAPDH and aldolase are reported to interact with DHPRs and RyRs (Brandt et al., 1990). Aldolase has been observed to coimmunoprecipitate with RyR (Allen, personal communication). Furthermore, not just the presence, but the specific localization of GAPDH, aldolase, and glycerol-3-phosphate dehydrogenase (GPDH) to the sarcomere is essential for flight muscle function in *Drosophila* (Wojtas et al., 1997).

These observations lead to a speculative model in which the DHPR C-terminus interacts with a complex of glycolytic enzymes in muscle (Fig 4.10). In yeast, the participation of endogenous glycolytic enzymes in such a complex could explain the extreme reactivity of segment E as a multivalent association that includes a

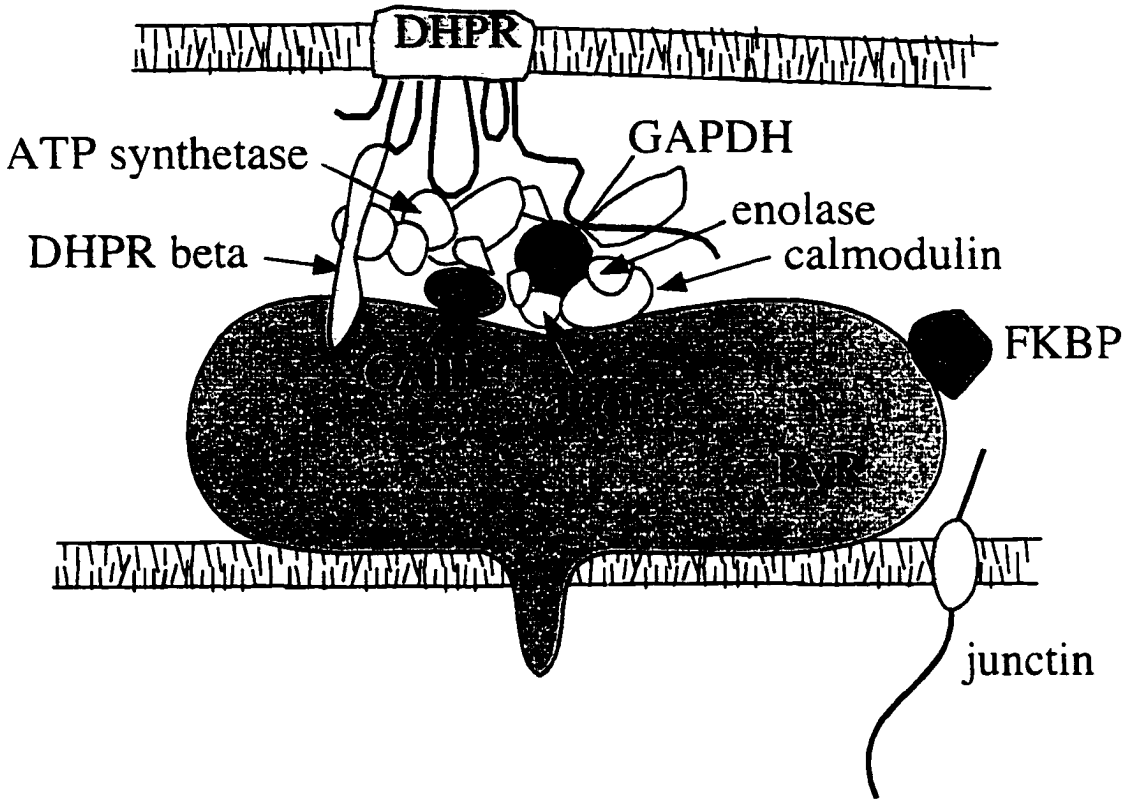

Figure 4.10 The Couplozyme model. Indicated proteins include known triad junction proteins and proteins that interacted with junctional proteins in yeast two-hybrid experiments. Unlabelled proteins indicate unknown interactors.

transcriptional activator(s). One prediction of this model is that glycolytic enzymes would be highly reactive in two-hybrid library screens. Oddly, given the vast interactions of glycolytic enzymes, there are few reports in the literature of two-hybrid experiments either using or detecting glycolytic enzymes. Why not? Could it be that the many proteins that interact with glycolytic enzymes — calmodulin, actin, the erythrocyte anion transporter (band 3), laminin, and glucose transporters, to name a few — are not

interesting enough to suggest two-hybrid experiments? Or could it be that these proteins are not tenable in the yeast two-hybrid system because they exhibit independent activation of reporter gene transcription?

If glycolytic enzymes are, in fact, associated with DHPRs in muscle, what is the physiological function of the association? Based on the observation that glycolytic enzyme localization is required for flight muscle function in *Drosophila* (Wojtas et al., 1997), it is possible that enzyme localization may be similarly obligate in mammalian muscle. Muscle is extremely metabolically active and so it intuitively makes sense to have microdomains of glycolysis. Indeed, this type of localization of glycolytic enzymes may be common at subcellular locations with high metabolic activity. For instance, there is a prominent glycolytic enzyme presence at post-synaptic density and in the erythrocyte membrane where binding of glycolytic enzymes to band 3, the erythrocyte anion transporter, is known to regulate their enzymatic activity.

In light of an emerging "paradigm for the qualitative change in the activity of an enzyme within a multienzyme complex," (Engel et al., 1998), a complex of glycolytic enzymes — the "couplozyme" — could contribute to the structure and function of triad junctions.

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