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ORIGINAL RESEARCH

Mass spectrometric analysis and mutagenesis predict involvement of multiple cysteines in redox regulation of the skeletal muscle ryanodine receptor ion channel complex

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Evgeniy V Petrotchenko[1,2,4]
Naohiro Yamaguchi[1,3,4]
Daniel A Pasek[1]
Christoph H Borchers[1,2]
Gerhard Meissner[1]

[1]Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, USA; [2]University of Victoria, Genome BC Proteomics Centre, Victoria, British Columbia, Canada; [3]Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, SC, USA
[4]Contributed equally to the work

Abstract: The tetrameric skeletal muscle ryanodine receptor ion channel complex (RyR1) contains a large number of free cysteines that are potential targets for redox-active molecules. Here, we report the mass spectrometric analysis of free thiols in RyR1 using the lipophilic, thiol-specific probe monobromobimane (MBB). In the presence of reduced glutathione, MBB labeled 14 cysteines per RyR1 subunit in tryptic peptides in five of five experiments. Forty-six additional MBB-labeled cysteines per RyR1 subunit were detected with lower frequency in tryptic peptides, bringing the total number of MBB-labeled cysteines to 60 per RyR1 subunit. A combination of fluorescence detection and mass spectrometry of RyR1, labeled in the presence of reduced and oxidized glutathione, identified two redox-sensitive cysteines (C1040 and C1303). Regulation of RyR activity by reduced and oxidized glutathione was investigated in skeletal muscle mutant RyR1s in which 18 cysteines were substituted with serine or alanine, using a [^{3}H]ryanodine ligand binding assay. Three single-site RyR1 mutants (C1781S, C2436S, and C2606S) and two multisite mutants with five and seven substituted cysteines exhibited a reduced redox response compared with wild-type RyR1. The results suggest that multiple cysteines determine the redox state and activity of RyR1.

Keywords: mass spectrometry, mutagenesis, ryanodine receptor, redox modification of cysteines

Introduction

Ryanodine receptor ion channels (RyRs) control diverse cellular functions by releasing Ca^{2+} from an intracellular compartment, ie, the sarcoplasmic reticulum. Skeletal muscle type 1 (RyR1) and cardiac muscle type 2 (RyR2) ryanodine receptors play a pivotal role by releasing the Ca^{2+} ions necessary for muscle contraction. The RyRs are redox-sensitive Ca^{2+} channels[1–4] composed of four RyR 560 kDa peptide subunits, 12 kDa tacrolimus binding proteins, and multiple associated proteins for a total molecular weight of >2500 kDa.[5,6] The tetrameric RyR1 has 404 cysteines, with 100 cysteines per 565 kD subunit, and one per tacrolimus binding protein, respectively. RyR1 redox state and activity are dependent on oxygen tension,[7] glutathione redox potential,[7,8] effector molecules (Ca^{2+}, Mg^{2+}) that control RyR1 channel activity,[9] and reactive oxygen molecules that are produced in working muscle at a low basal rate.[1] Aberrant S-nitrosylation of RyRs has been implicated in a malignant hyperthermia mouse model[10] and in dystrophic muscle.[11,12]

A number of RyR1 redox-reactive cysteines have been identified. Site-directed mutagenesis showed that at physiologic oxygen concentration, nitric oxide specifically

Correspondence: Gerhard Meissner
Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599-7260, USA
Tel +1 919 966 5021
Fax +1 919 966 2852
Email meissner@med.unc.edu

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S-nitrosylates one cysteine in RyR1 (Cys3635).[13] Mass spectrometric analysis identified seven hyperreactive RyR1 cysteines that were selectively labeled by 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin under conditions that favored channel closing.[14] Nine RyR1 cysteines were shown to be endogenously S-nitrosylated or S-glutathionylated.[15] Another three residues were modified by exogenous reactive oxygen and nitrogen molecules. However, the contribution of individual cysteines toward the redox regulation of RyR1 is not known, with the exception of RyR1-C3635.[13]

In the present study, we used a previously developed procedure[16] and mutagenesis to identify RyR1 cysteines involved in redox regulation of RyR1. Free thiols in skeletal muscle sarcoplasmic reticulum membranes enriched in RyR1 were labeled with the lipophilic, thiol-selective, and fluorescent reagent monobromobimane (MBB) in the presence of reduced and oxidized glutathione. Tryptic proteolysis of MBB-labeled, purified RyR1 protein and mass spectrometric analysis of MBB-labeled peptides identified 60 free thiols per RyR1 subunit in the presence of reduced glutathione and two thiols that likely formed disulfides in the presence of oxidized glutathione. Site-directed mutagenesis revealed three cysteines that are involved in the functional redox modulation of RyR1 by reduced and oxidized glutathione.

Materials and methods

Preparation of sarcoplasmic reticulum membranes

Rabbit skeletal muscle sarcoplasmic reticulum membranes enriched in RyR1 were isolated in the presence of protease inhibitors, as described elsewhere.[17]

MBB-labeling, purification, and digestion of RyR1

Labeling of sarcoplasmic reticulum membranes with MBB and digestion of purified MBB-labeled RyR1 with trypsin have been described.[16] Briefly, rabbit skeletal muscle sarcoplasmic reticulum membranes enriched in RyR1 were reacted for 150 minutes at 30°C with 5 mM reduced or oxidized glutathione in buffer A, ie, 0.15 M NaCl, 20 mM Na-PIPES, pH 7 buffer containing 100 µM EGTA, 100 µM Ca^{2+} (approximately 10 µM free Ca^{2+}), and protease inhibitors. Membranes were sedimented, resuspended in buffer A containing 50 µM reduced or oxidized glutathione, respectively, at a protein concentration of 3 mg/mL, reacted for one hour at 24°C in the dark with an excess of MBB (1 mM), and solubilized by the addition of two volumes of 1.5 M NaCl, 20 mM Na-PIPES, pH 7.0 buffer containing 2.2% CHAPS, 7.5 mg/mL soybean phosphatidylcholine, and protease inhibitors. MBB-labeled RyR1 s were purified as 30S protein complexes on sucrose gradients in the presence of 1% CHAPS but in the absence of phospholipids. To determine the position of MBB-labeled RyR1 on gradients, sarcoplasmic reticulum membranes were reacted with the RyR-specific probe [^{3}H]ryanodine (3 nM), solubilized with CHAPS, placed on a separate gradient, and centrifuged. Fractions containing MBB-labeled RyR1 were digested with immobilized trypsin for 72 hours at 24°C. Completion of digestion was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by silver staining.

Mass spectrometric analyses of MBB-labeled RyR1 peptides

Tryptic peptides were separated by reverse-phase high-pressure liquid chromatography (HPLC) and analyzed on a 4700 Proteomics Analyzer matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)/TOF mass spectrometry (MS; Applied Biosystems, Foster City, CA, USA) as described.[16] To assign the MBB-modified peptide peaks in the MALDI MS spectra, the MS-Fit program (Protein Prospector; MS Facility, UCSF, Berkely, CA) was used. Additionally, spectra were screened for the presence of ion signals at 190.07 ± 0.1 Da apart using a software program.[16] Peaks matching the theoretically predicted masses for MBB-modified peptides were selected for MS/MS analysis by MALDI-TOF/TOF. The fragment ion spectra were manually matched to the predicted peptide fragmentation spectra generated by MS-Product (Protein Prospector).

Construction and expression of mutant cDNAs

Single and multiple base changes of rabbit RyR1 cDNA were performed by PfuTurbo® (Stratagene, La Jolla, CA). cDNA fragments containing the mutation sites were cloned into vectors and used for polymerase-based chain reaction using mutagenic oligonucleotides and the QuikChange site-directed mutagenesis kit (Stratagene), as described previously.[18] The complete mutated fragments were confirmed by DNA sequencing. The fragments with mutations were cloned back into the original positions by standard cloning techniques. Finally, full-length RyR1 cDNAs with the mutations were cloned into a pCMV5 (*ClaI/XbaI*) expression vector. The cDNA of RyR2 was provided by Dr Junichi Nakai, of Saitama University, Japan. Nucleotide numbering was as described elsewhere.[19]

Expression of wild-type and mutant RyR1 proteins in HEK 293 cells

Wild-type RyR1,[18] RyR2,[20] and RyR1 mutant cDNAs were transiently expressed in HEK293 cells. Cells were transfected with FuGENE 6 (Roche Applied Science, Branford, CT) according to the manufacturer's instructions. Cells were maintained at 37°C and 5% CO_2 in high-glucose Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and plated the day before transfection. For each 10 cm tissue culture dish, 3.5 µg of cDNA was used. Cells were harvested 48 hours after transfection. Crude membrane fractions were prepared in the presence of protease inhibitors as described previously.[18]

[^{3}H]Ryanodine binding

The highly specific plant alkaloid ryanodine is widely used as a probe for RyR channel activity because of its preferential binding to the open channel.[21] [^{3}H]Ryanodine binding experiments were performed with crude membrane fractions from HEK293 cells. Unless otherwise indicated, membranes were incubated in the presence of protease inhibitors with 3 nM [^{3}H]ryanodine in 20 mM imidazole, pH 7.0, 250 mM KCl, 25 µM free Ca^{2+} (75 µM Ca^{2+} and 50 µM EGTA), and 5 mM reduced or oxidized glutathione. Nonspecific binding was determined using a 1000–2000-fold excess of unlabeled ryanodine. After 20 hours at 24°C, samples were diluted with seven volumes of ice-cold water and placed on Whatman GF/B filters preincubated with 2% polyethyleneimine in water. Filters were washed with three 5 mL ice-cold 100 mM KCl, 1 mM K-PIPES, pH 7.0 solution. The radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

Data analysis

Results are given as mean ± standard error of the mean. Differences between recombinant wild-type RyR1 and mutants were analyzed with the Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results and discussion

Identification of cysteinyl residues that react with MBB

To identify free thiols in the presence of reduced and oxidized glutathione, sarcoplasmic reticulum membranes isolated from rabbit skeletal muscle were reacted with 1 mM MBB after treatment with 5 mM reduced or oxidized glutathione, respectively. Analysis of the labeled and purified RyR1 indicated that MBB-labeled numbers in the reduced receptor were 37.5 ± 1.0 thiols/per RyR1 subunit (n = 5). Exposure of sarcoplasmic reticulum membranes to oxidized glutathione reduced the

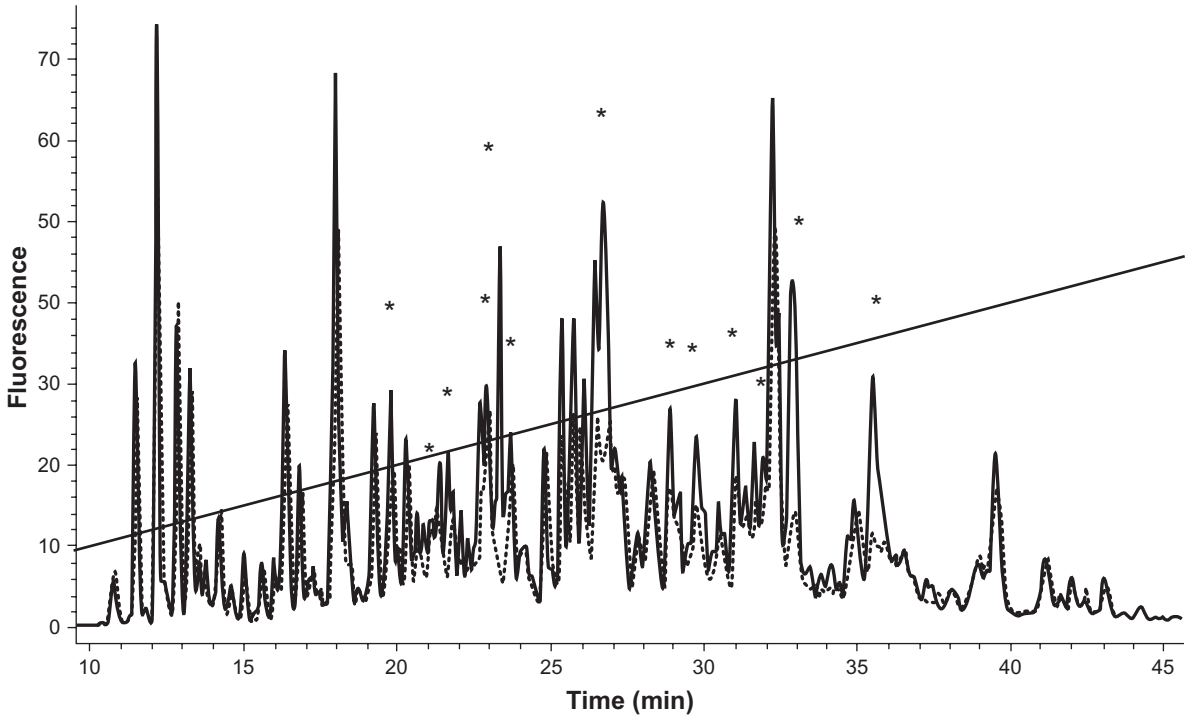

Figure 1 HPLC fluorescence diagram of tryptic digests of RyR1 labeled with monobromobimane under reducing (solid line) and oxidizing (dotted line) conditions.
Note: *Noticeable differences in peak intensity under reduced and oxidized conditions. HPLC fractions at 24 minutes (Figure 2) and 27 minutes (Figure 3) were analyzed using MALDI-MS and MALDI tandem MS analyses.
Abbreviations: HPLC, high-pressure liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; RyR1, skeletal muscle type 1.

number of MBB-labeled thiols/RyR1 subunit to 30.8 ± 2.7 (n = 5), in reasonable agreement with a previous study.[7]

The purified MBB-labeled RyR1 protein was digested with immobilized trypsin, and the resulting peptides were separated by reverse-phase HPLC with fluorescence detection. Figure 1 shows two representative superimposed HPLC fluorescence chromatograms of RyR1 tryptic peptides obtained from two samples treated with 5 mM reduced glutathione (solid lines) or oxidized glutathione (dotted lines) and then labeled with MBB. Increased fluorescence signals (labeled with *) were observed in the sample that was labeled under reducing conditions (solid lines) compared with that under oxidizing conditions (dotted lines). Fractions exhibiting fluorescence signals were lyophilized and subsequently analyzed by mass spectrometric analysis using MALDI-MS and MS/MS.[16] Table 1 summarizes the MS results obtained under reducing conditions. Of the 100 cysteines in each RyR1 subunit, 60 MBB-modified cysteines were observed with varying frequency. Under reducing conditions in five experiments, 14 MBB-modified cysteines were detected in all five experiments, 10 in four experiments, 13 in three experiments, eight in two experiments, and 15 in one experiment. Forty cysteines either did not react with MBB or were not detected as MBB-modified cysteines by MS. With some exceptions, the frequency of detecting MBB-labeled peptides was high in peptides of less than 20 amino acids, and low as peptide length exceeded 20 amino acids. Thus, the frequency of detecting MBB-modified cysteines likely depended, at least in part, on the length of the tryptic peptides. In two previous studies, in which seven[14] and 12[15] redox-modified cysteines were identified by MS analysis, peptide size may also have had a limiting role, because all modified cysteines reported were present in peptides that had 20 or less amino acids. To decrease peptide size, tryptic peptides were treated in one experiment with immobilized V8 protease.

Table 1 Identification of MBB-labeled cysteines in RyR1 under reducing conditions.

RyR1 aa	aa/ peptide[a]	mBB-Cys[b]	RyR1 aa	aa/ peptide[a]	mBB-Cys[b]
36	11	5	146	25	1
315	7	5+1	346	25	1
811	17	5+1	845	25	1+1
1040	8	5+1	854	25	1+1
1591	9	5+1	1447	30	1
1647	10	5+1	1947	18	1
2232	7	5+1	2310	19	1
2233	7	5	2651	3	1
2326	14	5+1	2656	23	1
2363	10	5	2704	24	1
2436	12	5+1	3170	12	1
3044	9	5+1	3839	18	1
3635	7	5	3892	18	1+1
3786	14	5+1	4876	17	1
			4882	17	1
24	14	4+1			
253	14	4	47	31	0+1
937	15	4	65	31	0
1303	20	4	66	31	0
2021	15	4+1	206	42	0
2237	7	4	208	42	0
2611	13	4	209	42	0
3193	11	4	230	42	0+1
3240	21	4	490	19	0
5018	12	4	537	16	0+1
393	10	3	566	31	0
604	17	3	746	75	0+1
607	17	3	747	75	0
609	17	3	762	30	0
906	16	3+1	1151	59	0+1
1674	9	3+1	1192	12	0
2305	19	3+1	1217	28	0+1
2555	12	3+1	1489	20	0
2606	13	3+1	1492	20	0
2702	24	3	1518	26	0
3402	8	3+1	1630	23	0
3650	10	3	1686	28	0
5027	12	3+1	1781	38	0
			2042	34	0
120	10	2+1	2158	23	0
305	17	2+1	3014	23	0
1269	28	2+1	3067	20	0+1
1724	17	2	3165	44	0
1940	18	2+1	3216	26	0
2240	7	2	3278	21	0+1
2565	11	2	3304	24	0
3525	21	2+1	3733	25	0
			3918	21	0
			3973	25	0
			4114	30	0+1
			4238	65	0+1
			4645	84	0
			4657	84	0
			4663	84	0
			4958	41	0+1
			4961	41	0

Notes: [a]Amino acids per tryptic peptide; [b]monobromobimane-labeled cysteine detected in tryptic peptides (number of experiments out of five experiments). The second number indicates monobromobimane-labeled cysteines detected in V8 peptides (n = 1).
Abbreviation: RyR1, skeletal muscle type 1.

Digestion with V8 resulted in the tentative identification of 11 additional MBB-modified cysteines (Table 1).

In addition to revealing MBB-labeled peptides, LC-MALDI-MS analysis can provide information about the redox state of cysteines in RyR1 under reducing and oxidizing conditions.[16] To identify redox-sensitive cysteines, HPLC fractions of peptides obtained from RyR1 under reducing (Figure 1, solid line) and oxidizing (dotted line) conditions were compared by MALDI-MS. We identified two cysteines (C1040 and C1303) in two separate tryptic peptides (Figures 2 and 3) that showed under reducing conditions a significantly higher intensity in a peak doublet 190.07 ± 0.1 Da apart, which is characteristic for MBB-labeled peptides.[16] Significance was ascertained by statistical analysis of the logarithmic peak intensity ratios of mass spectra of samples treated with reduced glutathione and oxidized glutathione. The identity of the peptides was confirmed by MS/MS analysis. Evidence for a third redox-sensitive cysteine (C2326) was obtained previously.[16] Collectively, the data suggest that cysteines that react with MBB under reducing conditions may be present in a partially oxidized state. Replacement of reduced glutathione with oxidized glutathione shifted the redox state of cysteines from a free to an oxidized state but without fully oxidizing cysteines.

Redox modulation of RyR1 cysteine mutants by glutathione redox state

To gain a better understanding of the effects of a change in glutathione redox potential on RyR1 activity, we studied the redox behavior of single- and multiple-site RyR1 cysteine mutants. Full-length RyR1 single-site mutants were generated by substituting 18 cysteine residues with serine or alanine (Table 2). Single-site RyR1 mutants included three redox-sensitive cysteines identified by MS[16] (Figures 2 and 3), seven hyperreactive cysteine residues implicated in RyR1 function,[14] and six additional cysteines

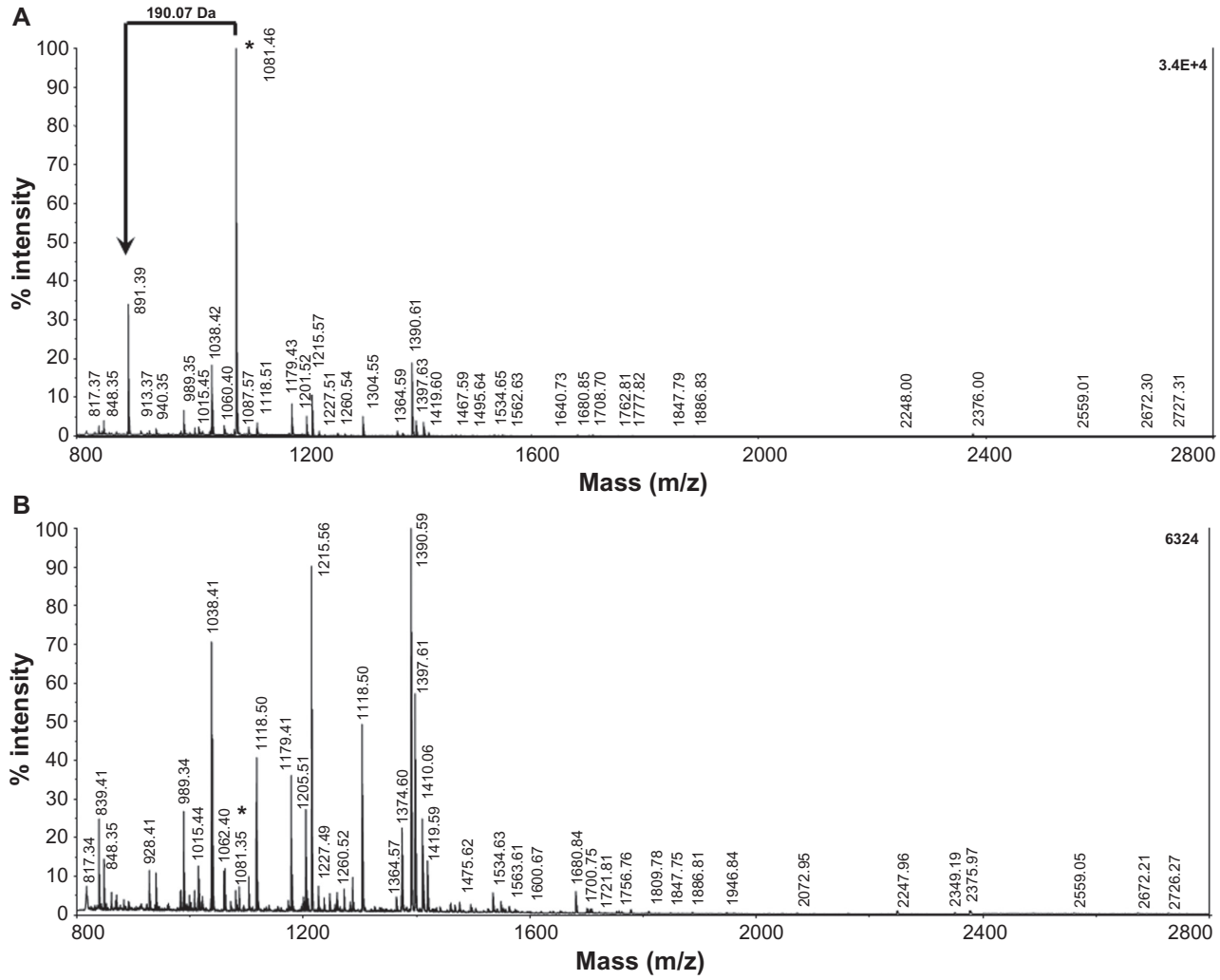

Figure 2 (*Continued*)

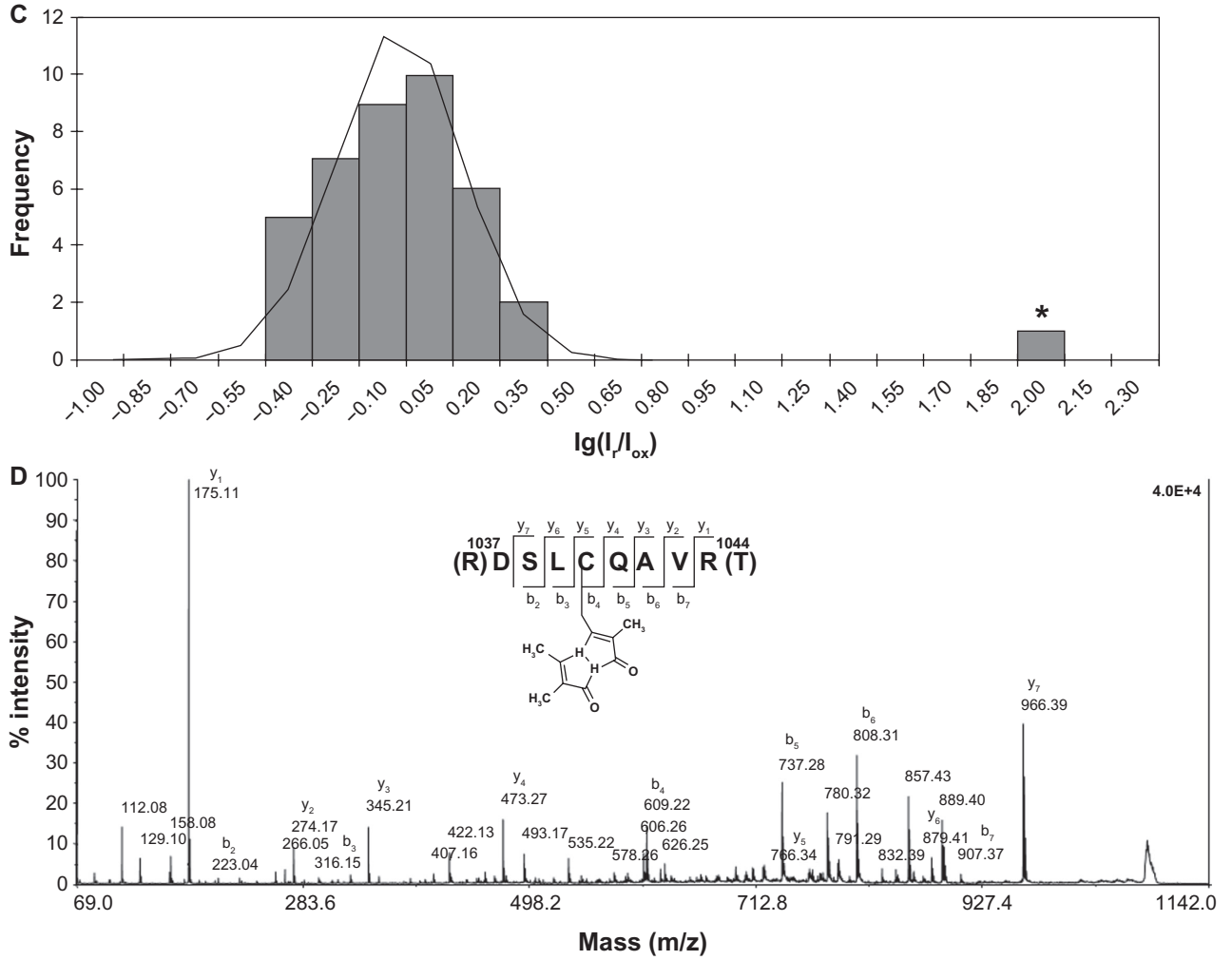

Figure 2 Identification of redox-sensitive Cys-1040 by mass spectrometry. **A, B**) MALDI mass spectra of the chromatographic fractions of trypsin digest of the monobromobimane-modified RyR1 in the presence of reduced and oxidized glutathione, respectively. Monobromobimane-modified peptides manifest in MALDI mass spectra as characteristic pairs of peaks 190 Da apart, due to partial photocleavage of the monobromobimane moiety. **C**) Statistical analysis of the logarithms of the MS peak intensity ratios of the reduced glutathione (Panel A) and oxidized glutathione (Panel B) samples. **D**) MS/MS analysis of the peptide containing the redox-sensitive monobromobimane-modified cysteine.

Note: *Value corresponding to the peptide containing the redox-sensitive cysteine 1040.

Abbreviations: MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; RyR1, skeletal muscle type 1.

not conserved in RyR2 (Table 2). Mutation of three cysteines (C1781, C2436, and C2606) resulted in a significantly reduced redox response compared with wild-type RyR1. None of the three cysteine mutants (C1040S, C1303S, and C2326S) identified as redox-sensitive cysteines by MS[16] (Figures 2 and 3) showed a significant change in activity in the presence of oxidized glutathione compared with reduced glutathione. We also found that among the seven hyperreactive cysteines previously implicated in RyR1 function,[14] only two (C2436S and C2606S) showed a significantly decreased redox response compared with wild-type RyR1. The reason for this discrepancy is not clear but could have been due to the fact that RyR1 redox state and activity are dependent on Ca^{2+}, which controls RyR1 channel activity.[9] The seven hyperreactive cysteines were identified under conditions that favored channel closing, whereas in the present study RyR1 channels were partially activated by Ca^{2+}.

Next, we sought to determine whether mutagenesis of several cysteines eliminates the redox response in RyR1. Five cysteines (RyR1–5Cys, Figure 4A) and seven cysteines (RyR1–7Cys, Figure 4B) were mutagenized simultaneously in RyR1. Mutations in RyR1–5Cys included the three redox-active cysteines (C1781, C2436, and C2606) identified in this study (Table 2). Mutations in RyR1–7Cys corresponded to the seven hyperreactive cysteines that have been suggested to play a role in redox sensing of RyR1.[14] The two multisite mutants exhibited a smaller redox response than the single-site mutants, but without

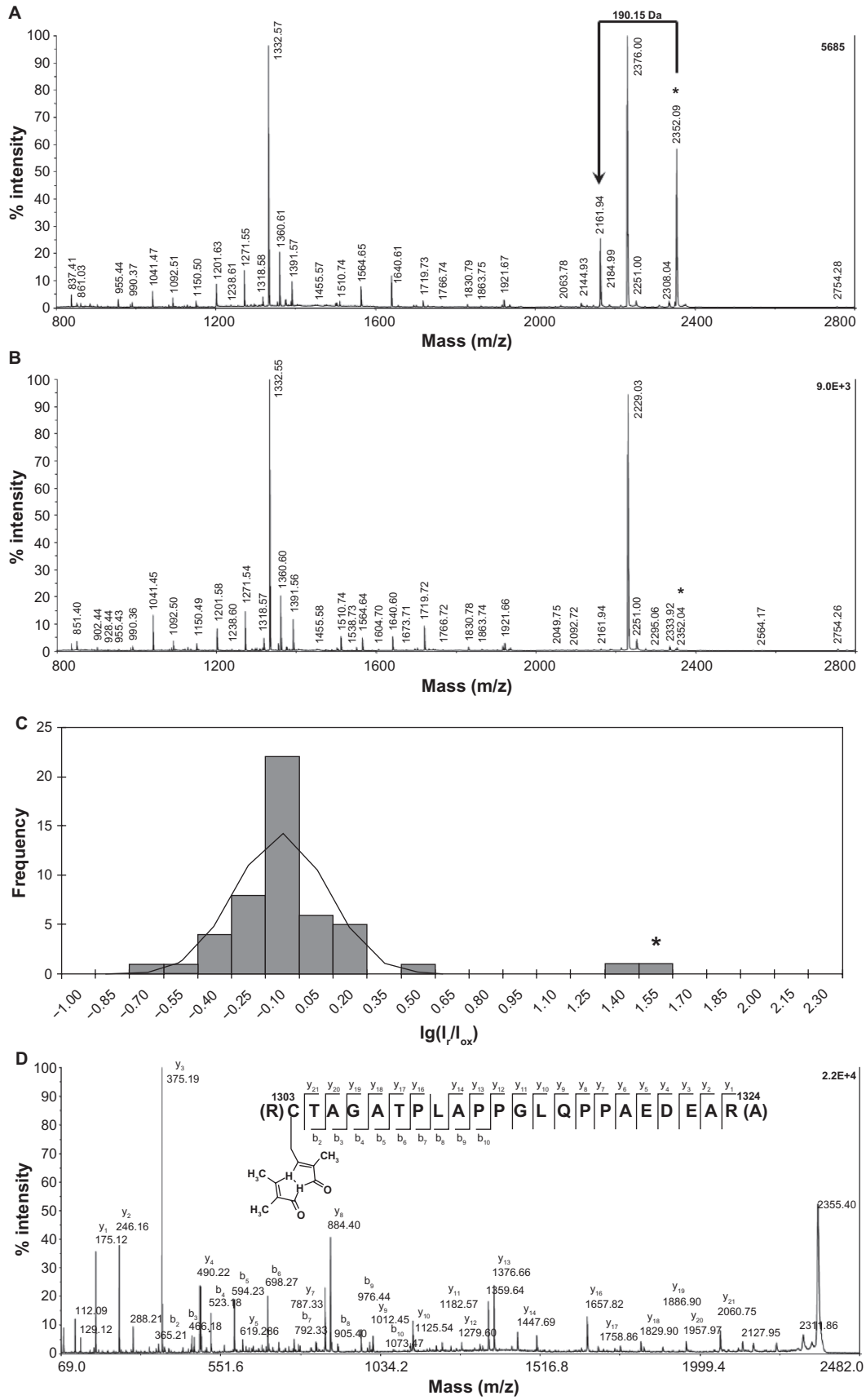

Figure 3 Identification of the redox-sensitive Cys-1303 by mass spectrometry. Panels are as in Figure 2.

Table 2 Effects of GSSG and GSH on recombinant WT-RyR1, WT-RyR2 and mutant RyR1 activities.

Mutants	GSSG/GSH[c]	RyR2 aa[d]	Detected as redox sensitive thiol by MS
WT-RyR1	3.27 ± 0.14(32)		
RyR1-C207S	3.00 ± 0.35(5)	S	
RyR1-C393S[a]	2.90 ± 0.11(7)	S	
RyR1-C854S	3.25 ± 0.44(4)	I	
RyR1-C1040S[b]	3.18 ± 0.29(7)	R	√
RyR1-C1303S[b]	3.15 ± 0.31(8)	K	√
RyR1-C1447S	3.20 ± 0.42(4)	N	
RyR1-C1648S	2.95 ± 0.22(5)	S	
RyR1-C1781S[a]	2.45 ± 0.16(5)*	S	
RyR1-C2232S	3.30 ± 0.30(4)		
RyR1-C2233S	3.15 ± 0.53(3)		
RyR1-C2326S	3.05 ± 0.44(5)	V	√
RyR1-C2436S[a,b]	2.35 ± 0.26(7)*		
RyR1-C2565S[b]	2.64 ± 0.26(7)		
RyR1-C2606S[a,b]	2.78 ± 0.26(8)*		
RyR1-C2611S[a,b]	3.28 ± 0.36(6)		
RyR1-C3170S	2.80 ± 0.28(7)	T	
RyR1-C3402S	3.36 ± 0.38(7)	A	
RyR1-C3635 A[b]	2.66 ± 0.54(6)		
RyR1–5cys	2.11 ± 0.15(5)*		
RyR1–7cys	1.74 ± 0.13(9)*		
WT-RyR2	1.07 ± 0.11(6)*		

Notes: [a]Cysteines mutated to serine in RyR1–5Cys; [b]cysteines mutated to serine or alanine (C3635) in RyR1–7 Cys; [c]GSSG/GSH ratio corresponds to [^{3}H]ryanodine binding ratio in presence of 5 mM GSSG and 5 mM GSH as described in the Methods section; [d]RyR2 amino acid, present as cysteine in RyR1. Data are the mean ± standard error of the mean of number of experiments in parentheses. *$P < 0.05$ compared with WT-RyR1 as determined by the paired Student's *t*-test.
Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; MS, mass spectrometry; RyR1, skeletal muscle type 1; RyR2, cardiac muscle type 2.

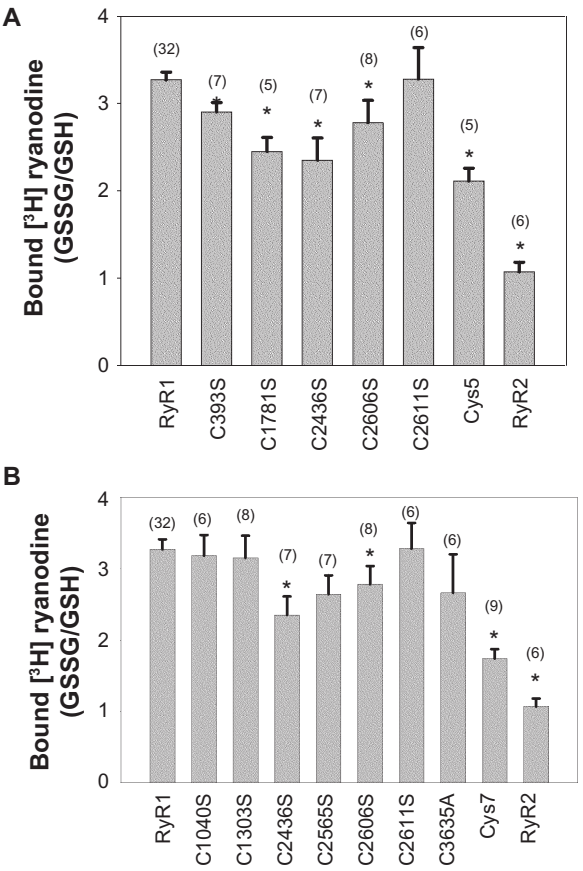

Figure 4 [^{3}H]Ryanodine binding of wild-type RyR1, wild-type RyR2, and RyR1 mutants in the presence of reduced and oxidized glutathione. The ratio of reduced to oxidized glutathione corresponds to the [^{3}H]ryanodine binding ratio in the presence of 5 mM oxidized glutathione and 5 mM reduced glutathione, and was determined as described in the Methods section. Data are the mean ± standard error of the mean of the number of experiments in parentheses of Table 2.
Note: *$P < 0.05$ compared with wild-type RyR1 as determined by the paired Student's *t*-test.
Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; RyR1, skeletal muscle type 1; RyR2, cardiac muscle type 2.

reaching the minimal response determined for recombinant RyR2. The results suggest that the 18 cysteine mutations tested in the present study alone or in combination are not sufficient to eliminate the channel's response to a change in glutathione redox potential.

In conclusion, the results of the present study add to our understanding of the complex regulation of RyR1 by redox-active species. MS experiments identified a large fraction of free RyR1 thiols under the reducing conditions used in the present study. Replacement of reduced glutathione with oxidized glutathione suggested that cysteines that reacted with MBB may be present in a partially oxidized state, and that a change in glutathione redox potential shifts the redox state of cysteines to a more oxidized state, but without fully oxidizing the cysteines. Mutagenesis identified three redox-active cysteines but also indicated that additional cysteines participate in the redox regulation of RyR1. Further studies are required to understand the redox regulation of RyR1 more completely.

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Disclosure

The authors report no conflicts of interest in this work.

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