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Research Article

Characterization of Grp78/BiP Protein Complexes in Skeletal Muscle Using Affinity Mass Spectrometry

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Activation of endoplasmic reticulum (ER) stress contributes to skeletal muscle adaptation and pathology in several diseases such as amyotrophic lateral sclerosis (ALS) and muscular dystrophy. Binding immunoglobulin protein, also known as 78 kDa glucose-regulated protein (Grp78/BiP), is a member of heat shock protein 70 kDa family and plays an important role in regulating ER stress by interacting with misfolded proteins. The purpose of this study was to identify proteins that interact with Grp78/BiP in skeletal muscle and investigate the biological function of protein-protein interactions. For this purpose, Grp78/BiP interacting proteins were enriched using a co-immunoprecipitation method with an anti-Grp78/BiP antibody and characterized by mass spectrometry. Mass spectrometry analysis showed that sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (SERCA1) was identified from Grp78/BiP interacting proteins. This protein-protein interaction was then validated with immunoblot analysis. We evaluated the biological function of SERCA1 and Grp78/BiP protein-protein interaction using an *in vitro* enzymatic activity assay for SERCA. Our results showed that sarcoplasmic reticulum (SR) Ca^{2+} ATPase function was reduced by 20% when Grp78/BiP was neutralized by antibody incubation. Our results for the first time identify the protein-protein interaction between Grp78/BiP and SERCA1 in skeletal muscle and demonstrate the role of Grp78/BiP in preserving SERCA1 function.

Keywords: SERCA1, Grp78/BiP, skeletal muscle, mass spectrometry.

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Introduction

Glucose-regulated 78kDa protein/immunoglobulin binding protein (Grp78/BiP) is one of the most abundant chaperones in the endoplasmic reticulum (ER) lumen and participates in various cellular processes including maintenance of ER membrane integrity, protein folding, intracellular calcium homeostasis, and activation of ER stress (1). Regulation of the ER stress pathway by Grp78/BiP is initiated by protein-protein interactions (2). Under normal conditions, Grp78/BiP binds to three ER stress sensors that are located on the ER membrane: protein kinase RNA activated-like ER kinase (PERK), inositol-requiring kinase 1-alpha (IRE1α), and activating transcription factor 6 (ATF6) (2). These protein-protein interactions prevent ER stress sensors from auto-phosphorylation and repress activation of the ER stress pathway under conditions of homeostasis (2). But upon accumulation of misfolded or unfolded proteins, Grp78/BiP preferentially binds to abnormal proteins and disturbs the protein-protein interactions with ER stress sensors, which results in auto-phosphorylation of ER stress sensors and activation of ER stress (2).

Activation of ER stress has been reported to contribute to skeletal muscle dysfunction in several diseases including amyotrophic lateral sclerosis (ALS) and muscular dystrophy (3, 4). Short-term or moderate activation of ER stress is critical to preserving cellular homeostasis by upregulating ER chaperones and heat shock proteins (HSPs) such as Grp78/BiP and Hsp70s (2). However, long-term or prolonged activation of ER stress is fatal and could initiate cell death pathways by upregulating ER stress-specific cell death factors such as C/EBP homologous protein (CHOP) and caspase-12 (2). Upregulation of ER stress markers, including Grp78/BiP, protein disulfide isomerase (PDI), and CHOP, was observed in skeletal muscle of a mouse

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model of ALS (3). Activation of ER stress was also observed in skeletal muscle in a mouse model of Duchenne muscular dystrophy (DMD) and repression of ER stress-specific cell death signal caspase-12 was shown to attenuate skeletal muscle pathology (4). These observations suggest that activation of ER stress is tightly associated with skeletal muscle function and that a thorough investigation of factors involved in ER stress could improve the understanding of the pathogenesis of muscle diseases. Considering the important role of Grp78/BiP in modulating the ER stress pathway and its strong binding capacity with ER proteins, it is of great interest to characterize proteins that could potentially interact with Grp78/BiP and investigate the biological function of these protein-protein interactions.

Investigation of protein-protein interactions and the associated biological function is essential to understanding disease pathogenesis as well as the identification and validation of novel drug targets (5). However, our current knowledge of protein-protein interactions for characterizing interactions and elucidating the biochemical processes disrupted is still limited due to the low throughput and poor reproducibility of traditional techniques such as the yeast two-hybrid approach (6). Mass spectrometry-based proteomic analysis has been emerging as a powerful technique to determine protein expression level, protein-translational modifications, and protein-protein interactions due to its sensitivity, high-capacity, and accuracy (7). When coupled with protein enrichment technique, this technology can be used to characterize protein complexes in biological samples. In this study, we optimized a method for coupling protein immunoprecipitation and tandem mass spectrometry to characterize novel protein-protein interactions of Grp78/BiP. We have since used this method to study Grp78/BiP protein complexes in mammal skeletal muscle. The purpose of this study was to identify novel protein-protein interactions of Grp78/BiP and the biological function of the interaction for one sample protein.

Methods

Ethics statement

All animal procedures were conducted under a protocol approved by the Institutional Animal Care & Use Committee (IACUC) of the University of Maryland, College Park.

Animals

Control C57BL/6 SJL hybrid mice were obtained from a breeding colony established at the University of Maryland (22). Mice were weaned at postnatal day 21 and genotyped. At time of use, animals were euthanized by CO_2 inhalation followed by cervical dislocation. Quadriceps muscle tissues isolated from 4 mice were harvested, quickly frozen in liquid nitrogen and stored in a -80^{o}C freezer.

Total protein extraction: Quadriceps muscle tissues were prepared as previously described (3). Briefly, muscle samples were homogenized on ice using a polytron at 50% maximal power for three 10 s bursts, separated by 30 s in ice-cold lysis buffer (20 mM HEPES, pH = 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 20% Glycerol) containing 1 mM DTT and protease inhibitor cocktail (cOmplete mini EDTA-free Protease Inhibitor Cocktail, Roche). After 20 min of incubation at 4^{o}C followed by centrifugation for 5 min at 20,000 x g, the supernatant was collected, quick frozen in liquid nitrogen and stored at -80^{o}C until required. A BCA protein assay kit (Thermo Scientific, Waltham, MA) was used to determine protein concentration in the samples.

Cross-linked magnetic co-immunoprecipitation (co-IP)

Protein samples prepared from quadriceps muscle tissues were used for the co-IP experiments. A protein co-IP kit was obtained (Thermo Scientific, Waltham, MA) and Grp78/BiP protein co-IP conducted according to the manufacturer's protocol. Briefly, protein magnetic beads were prepared and incubated with the anti-Grp78/BiP antibody (Abcam, Cambridge, MA) for 30 min at room temperature. After incubation, bead-antibody products were collected. For the purpose of cross-linking, diluted disuccinimidyl suberate (DSS) was added and incubated with bead-antibody for 30 min at room temperature. After cross-linking, the products were washed briefly and collected. Then, total protein samples were prepared and incubated with cross-linking products at 4^{o}C overnight. After protein sample incubation, Grp78/BiP protein complexes were washed off using elution buffer. After sample elution, the samples were neutralized by adding neutralization buffer and the sample pH was set to 7.0. The final elution contained Grp78/BiP and its interacting

proteins.

Mass spectrometry-based protein identification: An in-solution enzymatic digestion and bottom-up protein mass spectrometry strategy were used to identify proteins in Grp78/BiP co-IP products. Grp78/BiP co-IP products were reduced with 5 mM DTT and then alkylated with 55 mM iodoacetamide. Then, 50 µg Grp78/BiP co-IP products were incubated with trypsin (1 µg) at 37ºC overnight. Mass spectrometry-based protein identification and quantitation methods have been described previously (3). Briefly, after trypsin digestion, peptide products were collected and analyzed by nano-LC-MS/MS using LTQ Orbitrap mass spectrometer coupled to a Shimadzu 2D Nano HPLC system. Peptides were loaded with an auto-sampler into a Zorbax SB-C18 trap column (0.3 x 5.0 mm) (Agilent Technologies, Palo Alto, CA, USA) at 10 µL/min with solvent A (97.5% water, 2.5% ACN, 0.1% formic acid) for 10 min, then eluted and separated at 300 nL/min with a gradient of 0–35% solvent B (2.5% water, 97.5% ACN, 0.1% formic acid) in 30 min using a Zorbax SB-C18 nano-column (0.075 x 150 mm). The mass spectrometer was set to acquire a full scan at resolution 60,000 at m/z 400 followed by data dependent MS/MS analysis of top 10 peaks with more than one charge in the linear ion trap at unit mass resolution. The resulting LC-MS/MS data were searched against a mouse protein database generated from *UniProt* and a common contaminant database using Mascot (v2.3) and SEQUEST search engines through Proteome Discoverer (v1.4). Carbamidomethylation at Cys was set as fixed modification. Methionine oxidation and asparagine and glutamine deamidation were set as variable modification. Spectral counting with normalized total spectra was carried out using *Scaffold* software (Proteome Software, Inc). Protein probability >99% and at least one unique peptide with a probability score >95% was set as a minimum requirement for protein identification.

Western blots

To confirm protein expression levels in the sample, total protein samples isolated from quadriceps muscle tissues were prepared with 5 x loading buffer and then heated at 100ºC for 5 min. After being cooled down on the ice, protein samples were loaded onto bis-acrylamide gels and separated using polyacrylamide gel electrophoresis (PAGE). Samples were then transferred to PVDF membrane and blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline (pH 8.0) for 1 hour. The primary anti-Grp78/BiP antibody (1:1000, Abcam, Cambridge, MA) was added and incubated with membranes at 4ºC overnight. After primary antibody incubation, membranes were washed briefly with Tris-buffered saline (0.1% Tween 20). An anti-rabbit secondary antibody (1:1000, Cell Signaling Technology, Danvers, MA) was added and incubated with membranes for 1 hour at room temperature. Secondary antibody signals were detected using SuperSignal West Dura Chemiluminescence Substrate (Thermo Scientific, Waltham, MA) or Clarity™ ECL western blotting substrate. The signal for the target protein of each sample was quantified by densitometry using Image Lab Software 5.0 (Bio-Rad, Hercules, CA) and protein levels expressed in arbitrary units (AU).

SR Ca^{2+} ATPase activity assay

Sarcoplasmic Reticulum (SR) Ca^{2+} ATPase activity was measured in isolated SR vesicles from rabbit skeletal muscle, which was a kind gift of R. Bloch, University of Maryland School of Medicine. The methods for measuring SR Ca^{2+} ATPase activity have been described previously and this was applied to SR vesicles (8). The SR Ca^{2+} ATPase reaction was in a reaction buffer containing 100 mM KCl, 20 mM HEPES, 10 mM NaN_3, 1 mM EGTA, 15 mM $MgCl_2$, 5 mM ATP, and 10 mM phosphoenolpyruvate (PEP). To measure the Ca^{2+} ATPase activity 18 U/mL each of lactate dehydrogenase (LDH), pyruvate kinase (PK), and 3.5 µM calcium ionophore A23187 were added to the assay buffer. SR Ca^{2+} ATPase activity was measured at different calcium concentrations: 0.1, 0.5, 1.0, 2.0, and 5.0 mM $CaCl_2$. SR Ca^{2+} ATPase activity reaches the maximal activity at 1.0 mM total Ca^{2+}, whereas higher calcium concentration, such as 5.0 mM total Ca^{2+}, inhibits SR Ca^{2+} ATPase activity. The reaction is driven by adding 0.4 µM NADH and the absorbance of NADH is measured at 340 nm every 30 s for 40 min. Specific SR Ca^{2+} ATPase activity is calculated using total ATPase activity at 1mM $CaCl_2$ minus basal ATPase activity, which is generated by adding 10 µM cyclopiazonic acid (CPA), a selective SERCA inhibitor. For testing SR Ca^{2+} ATPase activity, 7.5 µL of SR vesicles were added into SR Ca^{2+} ATPase activity buffer and the activity was measured at different calcium concentrations as described above. The method was performed in 96-well plates.

Statistical analysis

All results were expressed as means ± S.E. For protein validation using western blots of Grp78/BiP co-IP products and maximal SR Ca^{2+} ATPase activity determination in SR vesicles, a Student *t*-test was used. The statistical significance was set at $p<0.05$.

Results

Validation of anti-Grp78/BiP antibody for protein immunoprecipitation: The quality and specificity of the antibody is key to the accuracy and interpretation of protein immunoprecipitation experiments. To validate the quality and specificity of the anti-Grp78/BiP antibody used in this study, we designed a workflow to characterize the proteins obtained from our co-IP experiment (Figure 1 "Anti-Grp78/BiP antibody validation" section). In this workflow, we first evaluated proteins that were obtained from co-IP experiments with SDS-PAGE electrophoresis. Our results show that four protein bands were observed (Figure 2A, I-IV). The calculated masses of these bands are 230 kDa (I), 150 kDa (II), 78 kDa (III), and 50 kDa (IV). Based on the calculated masses, we expected that band III would be our target protein Grp78/BiP, which has a mass of ~78 kDa. The protein band III was sliced from the gel, processed with in-gel protease digestion, and analyzed with mass spectrometry. Our results showed that Grp78/BiP protein was identified from band III with 50% of sequence coverage (Figure 2B and 2C). We also observed that band II belonged to the antibody IgG (150 kDa), band IV belonged to the reduced heavy chain of the antibody (50 kDa), and band I belonged to Grp78/BiP-antibody complex (150 + 78 ≈ 230 kDa). Our mass spectrometry analysis confirmed that anti-Grp78/BiP antibody used in our study is specific and appropriate for the downstream protein-protein interaction study.

Identification of Grp78/BiP and SERCA1 protein-protein interaction in skeletal muscle with co-IP and mass spectrometry: Protein co-IP was conducted in quadriceps muscle tissues of mouse using anti-Grp78/BiP antibody. Protein samples yielded from Grp78/BiP co-IP experiments were digested with proteases and analyzed by mass spectrometry (Figure 1, "Affinity mass spectrometry"). As we expected, Grp78/BiP protein was

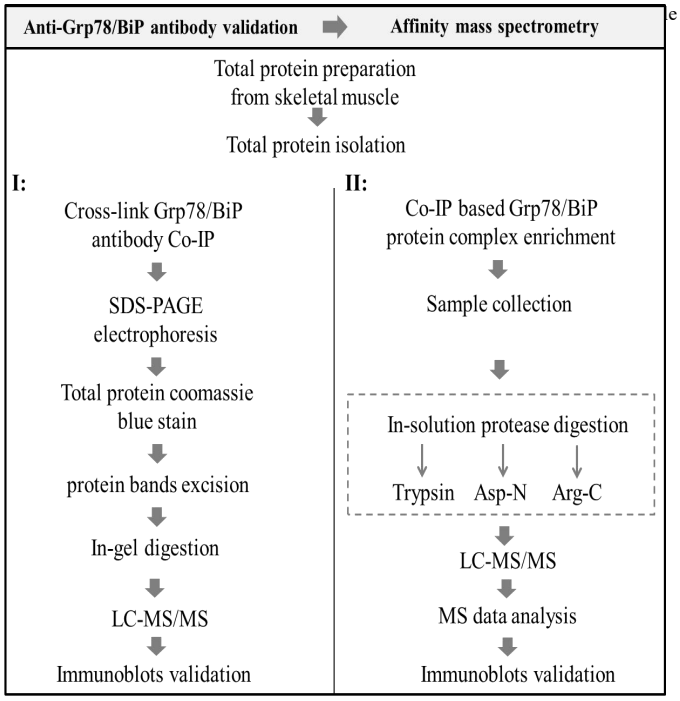

Figure 1: Workflow of identification of Grp78/BiP protein-protein interactions using co-immunoprecipitation (co-IP) and protein mass spectrometry. Skeletal muscle total protein lysates were prepared and Grp78/BiP and its binding proteins were enriched with anti-Grp78/BiP antibody and co-immunoprecipitation. Before being analyzed by mass spectrometry, co-IP protein products were digested with different proteases. Liquid chromatography-tandem mass spectrometry was used for the purpose of protein identification and western blots were used to validate protein mass spectrometry data.

identified by mass spectrometry analysis with 21% of sequence coverage (Figure 3A and 3B). In addition, SERCA1 was identified by mass spectrometry analysis with 11% of sequence coverage (Figure 3C). The fragmentation ion distribution of a peptide sequence VGEATETALTTLVEK of SERCA1 protein was demonstrated (Figure 3D). Our protein co-IP and mass spectrometry analysis showed that SERCA1 interacted with Grp78/BiP in skeletal muscle.

We further validated this protein-protein interaction by immunoblotting. For immunoblot analysis, quadriceps muscle tissues isolated from 4 mice were used and the product of Grp78/BiP co-IP was evaluated by anti-SERCA1 and anti-Grp78/BiP antibodies. Immunoblotting analysis showed that SERCA1 and Grp78/BiP were identified in the product of Grp78/BiP co-IP (Figure 3E). In summary, our affinity mass spectrometry and immunoblotting analyses identified the protein-protein interaction between SERCA1 and Grp78/BiP in skeletal muscle of mice.

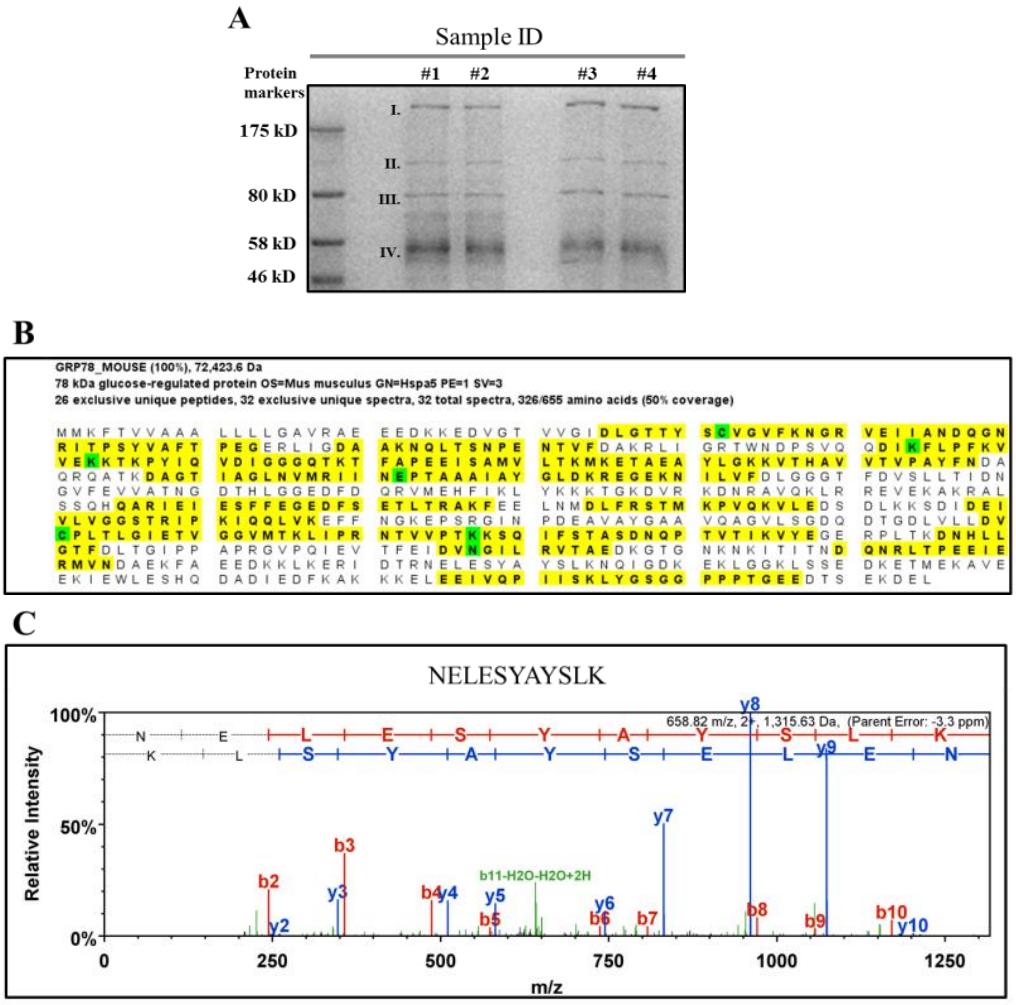

Figure 2: Mass spectrometry analysis of in-gel digestion of Grp78/BiP co-IP products. (A) SDS-PAGE gel electrophoresis using 30 µg of total muscle protein and coomassie blue staining. Grp78/BiP protein (~ 78 kDa, label III) and other protein bands were visualized using our imaging system. (B) Grp78/BiP sequence coverage (yellow highlights) identified by mass spectrometry from in-gel digestion of protein band III. Green highlights identify protein post-translational modifications. (C) A representative spectrum of a Grp78/BiP peptide NELESYAYSLK. *b* and *y* fragmentation ions are shown in the spectrum.

Investigation of the biological function of Grp78/BiP and SERCA1 protein-protein interaction with *in vitro* Ca^{2+} ATPase activity assay: To test the biological function of the Grp78/BiP and SERCA1 protein interaction, SR vesicle samples were prepared from rabbit skeletal muscles. Bioinformatic analyses showed that Grp78/BiP and SERCA1 are highly conserved among human and mouse with 99.9% and 96.6% sequence identity, respectively (Figure S1 and S2, supplemental information). Isolated rabbit SR vesicle proteins were analyzed by immunoblots and confirmed that Grp78/BiP protein was a component of the SR vesicle samples (Figure 4A and 4B).

SERCA1 is the most abundant protein on the ER membrane of muscle cells and responsible for calcium clearance to the ER lumen during skeletal muscle relaxation (8). Considering the important role of SERCA1 in regulating intracellular calcium levels, we hypothesized that the protein-protein interaction between Grp78/BiP and SERCA1 would be associated with its SR Ca^{2+} ATPase activity. To test our hypothesis, we utilized an *in vitro* SR Ca^{2+} ATPase activity assay and evaluated SERCA1 protein function (9). Our results showed that there was a significant decrease (25.6%) of the maximal SERCA1 calcium ATPase activity when Grp78/BiP was neutralized by an anti-Grp78/BiP antibody (Figure 5A and 5B). To rule out the possibility of non-specific antibody effects, we used a control antibody IgG and the maximal SR Ca^{2+} ATPase activity did not show a significant difference between

non-antibody and control antibody incubation conditions. Thus, our results suggest that the protein-protein interaction between SERCA1 and Grp78/BiP is critical to preserving the Ca^{2+} -dependent ATPase activity of SERCA1 protein and that disruption of this protein-protein interaction decreased the maximal SR Ca^{2+} ATPase activity of SERCA1.

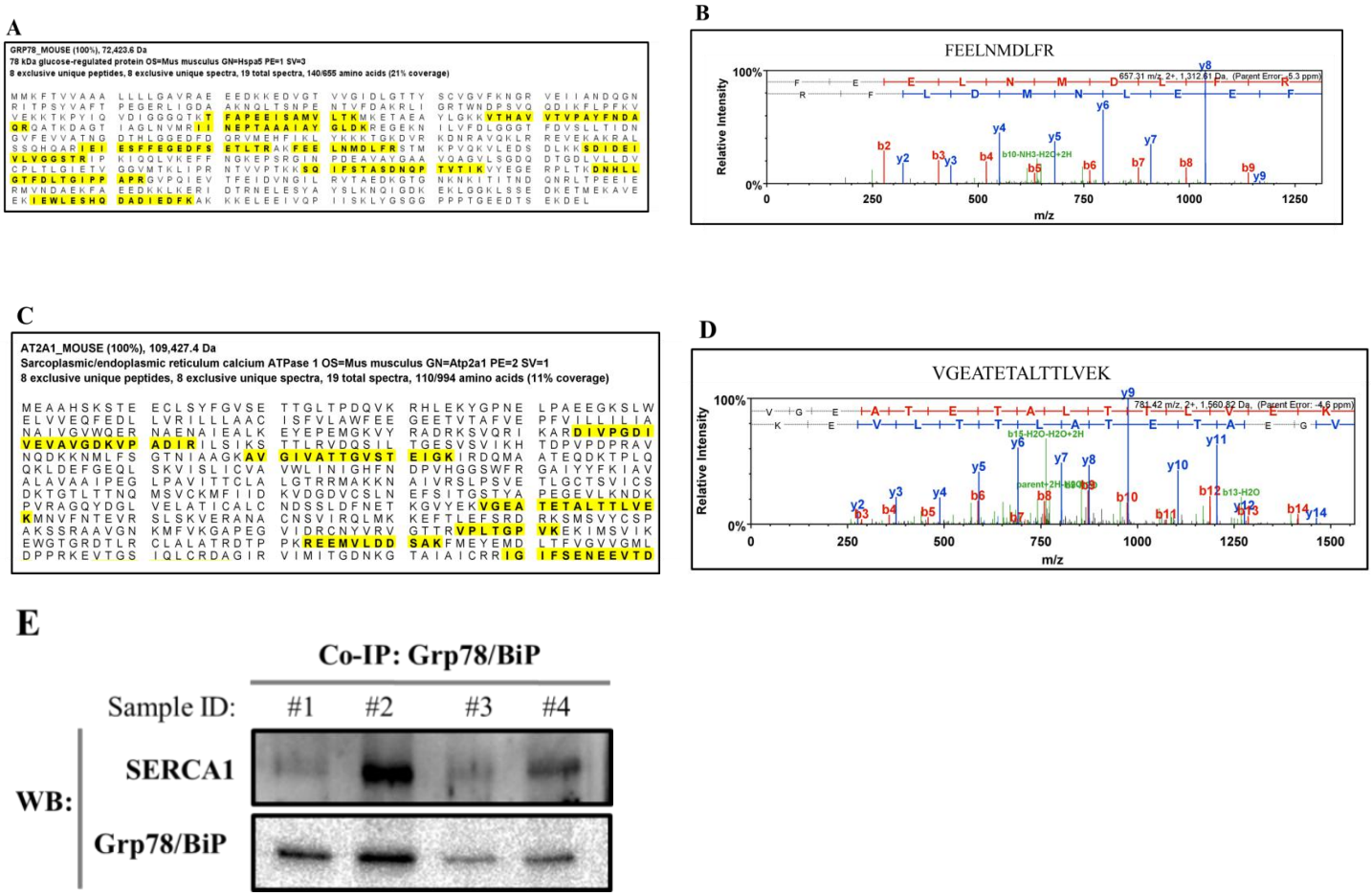

Figure 3: Mass spectrometry analysis of Grp78/BiP co-IP products. (A) Grp78/BiP sequence coverage (yellow highlights) identified by mass spectrometry from Grp78/BiP co-IP products. Green highlights indicate protein posttranslational modifications. (B) A representative spectrum of a Grp78/BiP peptide FEELNMDLFR. *b* and *y* fragmentation ions are shown in the spectrum. (C) SERCA1 sequence coverage (yellow highlights) identified by mass spectrometry from Grp78/BiP co-IP products. Green highlights identify protein post-translational modifications. (D) A representative spectrum of a Grp78/BiP peptide VGEATETALTTLVEK. *b* and *y* fragmentation ions are shown in the spectrum. (E) Western blot (WB) analysis of Grp78/BiP co-IP products. Both anti-SERCA1 and anti-Grp78/BiP antibodies confirmed the presence of these 2 proteins in the co-IP product.

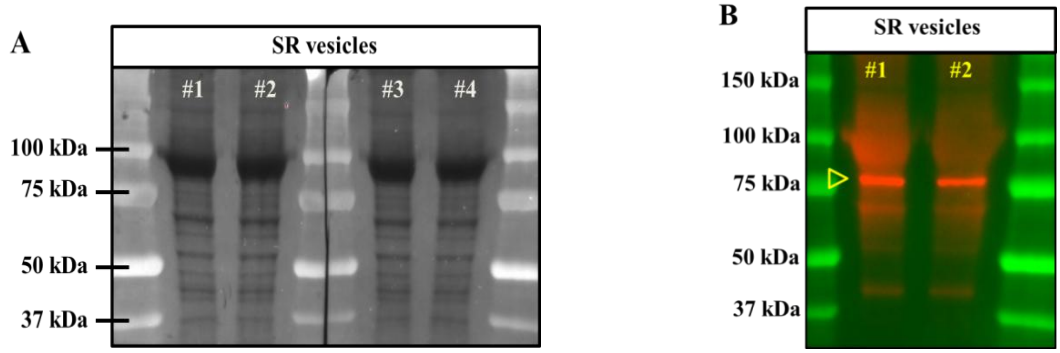

Figure 4: Identification of SERCA1 and Grp78/BiP proteins in SR vesicles. (A) SDS-PAGE gel electrophoresis and coomassie blue staining of SR vesicles. SERCA1 was shown to be the most abundant protein in the SR vesicles (~100kDa). (B) Western blot analysis of SR vesicle samples. The anti-Grp78/BiP antibody was used and Grp78/BiP protein bands (yellow open arrow and red bands) were shown.

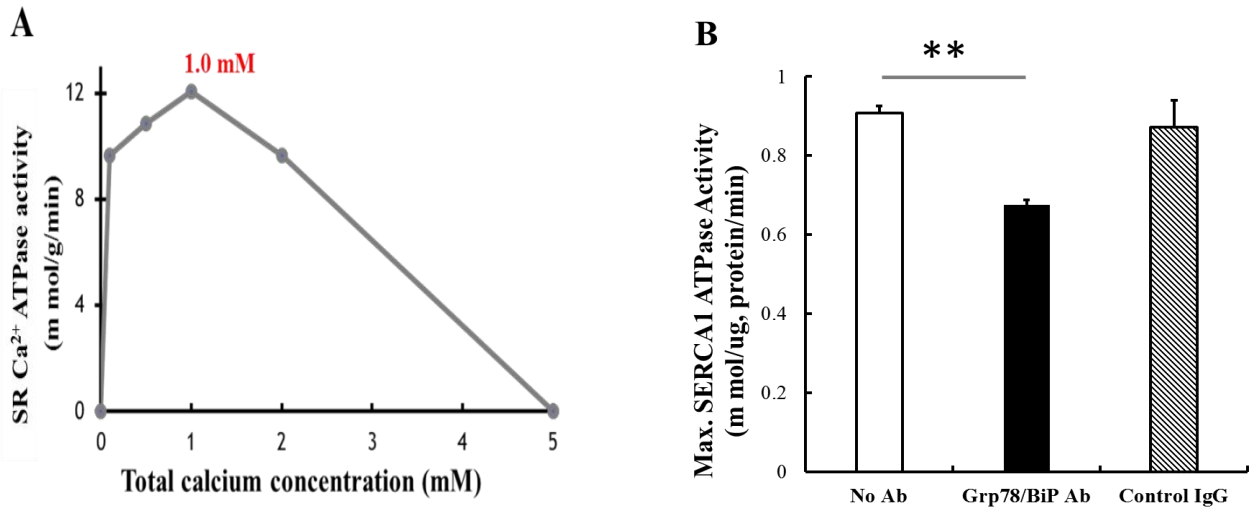

Figure 5: Decreased SR Ca^{2+} ATPase activity with Grp78/BiP antibody incubation in SR vesicles. (A) Maximal SR Ca^{2+} ATPase activity was shown at 1.0 mM free calcium concentration (red). (B) Maximal SR Ca^{2+} ATPase activity was determined without antibody present and in the presence of SERCA1 or a control IgG antibody and expressed as m mol/μg SR protein/min. Data represent mean ± S.E.. ** p<0.01 vs. No Ab condition.

Discussion

Characterization of protein-protein interactions by antibody-based affinity mass spectrometry: Proteins initiate many of their functions by protein-protein interactions (10). For example, proteins contain structural domains, which are specific to certain sequences and contribute to the function of a protein. Protein-protein interactions play an important role in a variety of cellular events such as intra- or extra-cellular signal transduction, protein quality control and transport across cell membranes (11). Therefore, there is great interest in determining specific protein-protein interactions of target proteins and evaluating the consequences of these interactions at a biochemical and molecular level. Understanding these molecular and biochemical dependencies is critical to the study of biological mechanisms and, where relevant, to understanding potential alterations in protein-protein interactions in pathological states. Identification of disease-specific alterations in protein-protein interactions may lead to the identification of novel drug targets and development of targeted drug therapies.

Mass spectrometry-based proteomics has been emerging as a powerful technique for protein research (12). In mass spectrometry analysis, proteins are first digested into small peptides using proteases (13). Digested proteins are then analyzed by mass spectrometry and protein sequences identified by alignment of observed spectra to known spectra of all proteins in the appropriate protein database (i.e. mouse vs. human) (13). This method provides supreme advantages over traditional analytical methods such as immunoblot and enzyme-linked immunosorbent assay (ELISA) due to its robust and high specificity (14). When coupled with different protein enrichment methods, mass spectrometry can be very useful in the investigation of protein quantitation, protein post-translational modifications, and protein-protein interactions (15). Antibody-based affinity mass spectrometry analysis is an example of the latter (16). In this approach, an immobilized antibody is used to capture the target protein complexes (16). After non-specific binding proteins are washed off, the protein complexes are collected and identified by mass spectrometry. In an antibody-based affinity mass spectrometry experiment, the most critical component is the quality and the specificity of the antibody (17). Poor antibody quality and specificity would result in a reduced number of target proteins detected and an increase in false positives for protein-binding partners (17). Keeping this in mind, we first evaluated the quality and specificity of anti-Grp78/BiP antibody and we showed that the most abundant protein identified in the product of protein co-IP was Grp78/BiP, suggesting that this antibody is specific and sensitive for Grp78/BiP.

Grp78/BiP-SERCA1 protein interactions in mammalian skeletal muscle: Grp78/BiP is a member of heat shock protein 70 kDa family (Hsp70s) and plays a critical role in

modulating numerous cellular functions such as protein synthesis, intracellular calcium homeostasis, unfolded protein response and ER stress (18). It is suggested that Grp78/BiP participates in various cellular functions by forming protein-protein interactions (2). For example, Grp78/BiP is reported to interact with proteins of the caspase family to initiate a cell death pathway in various stress conditions (2). Also, Grp78/BiP interacts with nuclear factor-kappa b (NFκB) and this protein-protein interaction is associated with pathological causes of neurodegenerative diseases (19). In the current study, we conducted experiments using an antibody-based affinity mass spectrometry strategy to characterize proteins that interacted with Grp78/BiP. Our analysis showed that SERCA1 protein was identified in the Grp78/BiP protein co-IP products, indicating the protein-protein interaction between Grp78/BiP and SERCA1. To confirm this protein interaction, we conducted immunoblotting analysis and the results are consistent with mass spectrometry analysis showing this protein-protein interaction in skeletal muscle of mice. To be noted, Grp78/BiP is a member of heat shock protein 70 kDa family (Hsp70s) and the interaction between Hsp70s and SERCA1 has been reported in previous studies. Tupling and colleagues (9) found that the 70 kDa heat shock protein interacted with SERCA1a in SR vesicles prepared from rat fast-twitch skeletal muscle (9). In another study, Dremina et al. (20) found that Hsp70 interacted with SERCA1 in C2C12 myocytes and that this protein-protein interaction protected SERCA1 from inactivation induced by Bcl-2, an apoptotic factor (20). In the current study, we show that Grp78/BiP interacted with SERCA1 in mouse skeletal muscle. Our current study is consistent with previous reports showing the protein-protein interactions between SERCA1 and Hsp70 family members.

Grp78/BiP-SERCA1 protein-protein interaction contributes to cellular function in mammalian skeletal muscle: SERCAs are SR/ER membrane-localized proteins that are essential to preserve intracellular calcium homeostasis in skeletal muscle (21, 22). Loss-of-function studies show the disruption of intracellular calcium homeostasis in skeletal muscle with SERCA protein deletion (23, 24). Reductions in SERCA protein expression or SR Ca^{2+} ATPase function has also been linked to several muscle disease models such as ALS and DMD (8, 22). SERCA proteins tightly maintain intracellular Ca^{2+} concentration by controlling resting intracellular Ca^{2+} level and by pumping intracellular Ca^{2+} back into the SR/ER lumen from cytoplasm during and following Ca^{2+} elevations with muscle contraction (21). To evaluate the biological function of Grp78/BiP and SERCA1 protein interaction, we conducted an *in vitro* assay to determine the maximal SR Ca^{2+} -dependent ATPase activity in different conditions: either with anti-Grp78/BiP incubation or with control IgG antibody incubation. Our results showed that there was a 25.6% of decrease in maximal SR Ca^{2+} -dependent ATPase activity when Grp78/BiP was neutralized using an anti-Grp78/BiP antibody. Incubation with control antibody (Rabbit IgG) did not affect the maximal SR Ca^{2+} -dependent ATPase activity, ruling out the non-specific effects of antibody incubation. Our results suggest that the protein-protein interaction between Grp78/BiP and SERCA1 is critical to preserving SERCA1 function and that interruption of this protein-protein interaction using antibody incubation resulted in a decrease in SR Ca^{2+} ATPase function in skeletal muscle.

The functional interdependence of Hsp70s and SERCAs in skeletal muscle tissues has been investigated in previous studies (9, 20). Tupling and colleagues (9) reported that Hsp70 protein could bind to SERCA1a and form a complex in adult fast-twitch skeletal muscle. They further evaluated this protein interaction and reported that Hsp70 could provide a protective function to SERC1a and prevent thermal inactivation of SR Ca^{2+} ATPase activity under certain heat stress conditions (9). In another study, Dremina and colleagues (20) showed that Hsp70 protein would protect SR Ca^{2+} ATPase function from inhibition by B-cell lymphoma 2 (Bcl-2) in C2C12 myoblasts and human embryonic kidney (HEK) 293 cells. In this study, although the interaction between Hsp70 and SERCA1 was not observed directly, it is can be expected that this protective effect is due to protein-protein interaction of Hsp70 with SERCA1. Grp78/BiP is a member of Hsp70 family of proteins and our finding is consistent with previous reports of functional dependencies and interactions between SERCA1 and Hsp70 family proteins. Evidence in the current study of the protein-protein interaction between these two families of proteins is an important new contribution to understanding SERCA1 function in skeletal muscle.

In summary, using antibody-based affinity mass

spectrometry analysis, we for the first time provide evidence of the protein-protein interaction between SERCA1 and Grp78/BiP in mammalian skeletal muscle tissues. In addition, we evaluated the biological function of this protein-protein interaction and we suggest that this protein interaction is critical to SERCA1 protein function. Disruption of this protein-protein interaction would result in impaired SR Ca^{2+}-dependent ATPase activity. Considering the important role of SERCA1 in regulating intracellular calcium, we suggested that Grp78/BiP plays an essential role in preserving calcium homeostasis in skeletal muscle.

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Conflict of Interest

None

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