Redox-dependent oligomerization through a leucine zipper motif is essential for MG53-mediated cell membrane repair

Moonsun Hwang,1 Jae-kyun Ko,1 Noah Weisleder,1 Hiroshi Takeshima,2 and Jianjie Ma1
1Departments of Physiology and Biophysics and Medicine, Robert Wood Johnson Medical School, Piscataway, New Jersey; and 2Department of Biological Chemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Kyoto, Japan

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Hwang M, Ko JK, Weisleder N, Takeshima H, Ma J. Redox-dependent oligomerization through a leucine zipper motif is essential for MG53-mediated cell membrane repair. Am J Physiol Cell Physiol 301: C106–C114, 2011. First published April 27, 2011; doi:10.1152/ajpcell.00382.2010.—We recently discovered that MG53, a muscle-specific tripartite motif (TRIM) family protein, functions as a sensor of oxidation to nucleate the assembly of cell membrane repair machinery. Our data showed that disulfide bond formation mediated by Cys242 is critical for MG53-mediated translocation of intracellular vesicles toward the injury sites. Here we test the hypothesis that leucine zipper motifs in the coiled-coil domain of MG53 constitute an additional mechanism that facilitates oligomerization of MG53 during cell membrane repair. Two leucine zipper motifs in the coiled-coil domain of MG53 constitute an additional mechanism that facilitates oligomerization of MG53 during cell membrane repair. Two leucine zipper motifs in the coiled-coil domain of MG53 (LZ1 - L176/L183/L190/ V197 and LZ2 - L205/L212/L219/L226) are highly conserved across the different animal species. Chemical cross-linking studies show that LZ1 is critical for MG53 homodimerization, whereas LZ2 is not. Mutations of the conserved leucines into alanines in LZ1, not in LZ2, diminish the redox-dependent oligomerization of MG53. Live cell imaging studies demonstrate that the movement of green fluorescent protein (GFP)-tagged MG53 mutants (GFP-LA1 and GFP-LA2) is partially compromised in response to mechanical damage of the cell membrane, and the GFP-LA1/2 double mutant is completely ineffective in translocation toward the injury sites. In addition to the leucine zipper-mediated intermolecular interaction, redox-dependent cross talk between MG53 appears to be an obligatory step for cell membrane repair, since in vivo modification of cysteine residues with alkylating reagents can prevent the movement of MG53 toward the injury sites. Our data show that oxidation of the thiol group of Cys242 and leucine zipper-mediated interaction among the MG53 molecules both contribute to the nucleation process for MG53-mediated cell membrane repair.

plasma membrane repair; cell membrane resealing; redox state; TRIM72; mitsugumin 53

REPAIR OF INJURY TO THE PLASMA membrane is an important aspect of normal cellular physiology, and disruption of this process can result in pathophysiology in many human diseases, including muscular dystrophy, cardiovascular disease, neurodegeneration, ischemic stroke, and traumatic injuries (2, 20). It is well known that cell membrane repair requires translocation of intracellular vesicles to the injury site that involves coordinated function of multiple intracellular components (10, 17). Recent studies have identified several molecular components involved in membrane repair, particularly those specific to cardiac and skeletal muscles (10). Bansal et al. (1) showed that dysferlin plays an important role in maintenance of sarcolemmal membrane integrity. Several mutations in the dysferlin gene have been linked to human muscular dystrophy (9). Recently, our laboratory discovered that MG53 is an essential component of the membrane repair machinery in skeletal and cardiac muscle, because MG53 ablation results in defective sarcolemmal membrane repair, progressive skeletal myopathy (3), and increased vulnerability of the heart to stress and ischemia-reperfusion-induced injury (5, 29).

As a tripartite motif (TRIM) protein family member, MG53 contains the prototypical TRIM domain at the amino-terminus consisting of the RING finger, B-box, and coiled-coil (CC) domain (18, 25). MG53 also contains a PRYSPRY domain at the carboxyl-terminus (3, 15). We found that MG53 can act as a sensor of oxidation to nucleate recruitment of intracellular vesicles to the injury site for membrane patch formation. MG53 can also interact with dysferlin to facilitate its membrane repair function, and altered interaction between MG53, dysferlin, and caveolin-3 is associated with membrane repair defects in muscular dystrophy (4).

While our previous data showed that disulfide bond formation at Cys242 is critical for MG53 oligomerization and the initiation of cell membrane repair (3), additional mechanisms must be involved to facilitate oligomerization of MG53 and assembly of the repair machinery (30). In this study, we examined the contribution of the CC domain to MG53 function in membrane repair. We present evidence that two leucine zipper motifs in the CC domain have differential functions for redox-dependent oligomerization of MG53 and are indispensable for nucleation of the cell membrane repair apparatus. We found that disruption of MG53 oligomerization through chemical modification of cysteine residues before membrane injury could disrupt the MG53-mediated membrane repair process. Since changes in cellular redox-state are associated with many human diseases, targeting the redox-dependent MG53 oligomerization could be a potential therapeutic avenue for prevention or treatment of tissue damage in human diseases.

MATERIALS AND METHODS

Expression vectors and cloning. The green fluorescent protein (GFP)-MG53 and FLAG-mRFP-MG53 expression vectors have been described and were originally characterized elsewhere (3). MG53 variants were generated by PCR-based site-directed mutagenesis method (14). Hemagglutinin (HA)-tagged MG53 wild type and mutants were cloned into pHM6 (Roche) and pIRESSneo3 (Clontech) at NotI and KpnI sites via PCR amplification. Mouse MG53 wild-type cDNA was cloned into Escherichia coli expression vector, pMAL-p2 (NEB) via PCR amplification at SalI and XhoI restriction sites. For the His6-MG53 expressing baculovirus, mouse MG53 cDNA was cloned into baculovirus vector, pAcHLT-C (Pharmingen) via PCR amplification at XhoI and NcoI sites.

Cell culture. Human embryonic kidney (HEK)293, HEK293T, and C2C12 cells were maintained in Dulbecco’s modified Eagle’s medium.
Preparation of protein extract and Western blotting. Western blotting was performed according to standard protocol (27). Whole cell extracts of culture cells were prepared in modified RIPA buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, and protease inhibitor cocktail (Sigma). Whole cell extracts of culture cells were mixed with equal volumes of 2/H11003 preRunions (New England Biolabs). For transient transfections, primary myoblasts or C2C12 cells were plated at 70% confluence in glass-bottom dishes (Biotech) and transfected using GeneGamer reagent (Stratagene) following the manufacturer’s manual. Cells were visualized by live cell confocal imaging at 24 h after transfection at times indicated for individual experiments. Differentiation of myoblasts into myotubes was induced at 90–95% confluence by replacement of growth medium with low-serum medium, consisting of DMEM supplemented with 2% horse serum (Cellgro). All cell lines were maintained in a humidified, 37°C incubator with 5% CO2.

RESULTS

MG53 forms a homodimer. Our previous study showed that changes in redox state can influence the oligomeric structure of MG53 expressed in adult skeletal muscle and differentiated C2C12 myotubes (3). Here we used either S99 insect cells to produce recombinant MG53 protein that contains a polyhistidine immunofinity tag (His6-MG53), or E. coli to produce MG53 fusion protein containing MBP at the amino-terminus. To test whether the redox-dependent oligomerization is an intrinsic property of MG53, as shown in Fig. 1A, higher-molecular-weight oligomers of MBP-MG53 fusion proteins were prevalent under oxidized conditions (in the absence of DTT), with increasing concentrations of DTT leading to progressive deoligomerization of MBP-MG53 into dimer (~200 μm) for FM4–64 fluorescence directly adjacent to the injury site. To allow for statistical analysis from different experiments, data are presented as fluorescence intensity relative to the value before injury (ΔF/Fo).

Dye exclusion experiments. For the dye exclusion experiments, isolated neonatal mg53−/− myoblasts were transfected with the GFP-tagged form of MG53 or mutants and differentiated into myotubes with 2% horse serum. Extracellular media were changed to BSS containing 2 mM Ca2+, and 2.5 μM FM4–64 dye (Molecular Probes) was added just before each damage experiment. To induce damage to myotubes, a 5×5 pixel area of the plasma membrane was irradiated at maximum power (Enterprise, 80 mW, 351/364 nm) for 5 s using a Zeiss-LSM 510 confocal microscope equipped with a ×63 water immersion lens (1.3 numerical aperture). Images were captured at 5-s intervals. To calculate the fluorescence intensity, we used an area of ~100 μm2 for GFP fluorescence directly adjacent to the injury site.

To allow for statistical analysis from different experiments, data are presented as fluorescence intensity relative to the value before injury (ΔF/Fo).
Previous studies showed that homodimer or homotrimer formation of other TRIM family proteins plays important roles in their cellular functions (8, 23, 26). Studies by other investigators used chemical cross-linking reagents to explore the various oligomerization properties of TRIM family proteins (13, 19). Here we followed their approach to test whether MG53 proteins form homodimers or trimers using MG53 fusion proteins linked to different affinity tags. HEK293T cells were cotransfected with FLAG-mRFP-MG53, with a molecular size of 80 kDa, and HA-MG53 with a molecular size of 54 kDa. After cross-linking with GA, proteins were precipitated with either anti-HA or anti-FLAG antibody and then probed with anti-HA-horseradish peroxidase (HRP). Figure 1, D and E, shows the predicted patterns of cross-linked protein bands in cases of dimerization and trimerization, respectively. If MG53 formed dimers, anti-HA would precipitate a 133-kDa dimer and a 53-kDa monomer, and anti-FLAG would precipitate protein bands of the same size, as shown in Fig. 1C. On the other hand, if MG53 were to form trimers, anti-HA would precipitate homotrimers of 160 kDa plus heterotrimers of 196 kDa and 216 kDa, which did not appear in Fig. 1C. In addition, anti-FLAG would precipitate two heterotrimers and a monomer, which are also absent in Fig. 1C. Note that anti-FLAG could precipitate FLAG-tagged homodimers or trimers, but they would not be detected with the anti-HA antibody. Clearly, our data shown in Fig. 1C are consistent with MG53 forming a dimeric structure under these experimental conditions. Since the apparent molecular size for the homo- and heterodimers of the tagged MG53 proteins is slightly larger than the predicted molecular size (Fig. 1C), it is possible that other cellular factors could form heteromeric structure with MG53 and contribute to the different MG53 oligomerization patterns observed in Fig. 1C. Future studies to resolve if a binding protein exists in the oligomeric complex of MG53 should reveal more insights into the mechanism for MG53-mediated cell membrane repair.

A leucine zipper motif is critical for homodimer formation of MG53. In many TRIM family proteins, the coiled-coil (CC) domain is known to mediate homo-oligomerization (25). To test the role of the CC domain in MG53 oligomerization, we generated a CC deletion mutant of MG53 (HA-H9004CC) and HA-CC constructs (Fig. 2A). As shown in Fig. 2B, with the full-length HA-MG53, cross-linking with GA led to a dimeric protein band of ~120 kDa (left). The HA-CC protein showed dimer and higher oligomers upon treatment with GA (middle), whereas the HA-ΔCC deletion construct did not show an oligomerization pattern following identical treatment with GA (right). This result demonstrated that the CC domain is critical for MG53 oligomerization.

Fig. 1. MG53 forms homodimers. A: purified recombinant maltose-binding protein (MBP)-MG53 from Escherichia coli showed deoligomerization at various concentrations of DTT (0, 0.01, 0.1, 1.0, 10, and 100 mM) on 3–15% gradient acrylamide gel. MBP-MG53 proteins were detected by Western blotting with anti-MG53 monoclonal antibody. Molecular mass of MBP-MG53 is 96 kDa. m, Monomer. B: purified recombinant His6-tagged MG53 from Sf9 showed oligomerization in the absence of DTT on 3–15% gradient acrylamide gel. Purified MG53 proteins were detected by colloidal blue staining. Molecular mass of MG53 is 53 kDa. C: FLAG-mRFP-MG53 and hemagglutinin (HA)-tagged forms of MG53 wild type were transiently transfected in human embryonic kidney (HEK293T) cells together. The cell lysates were cross-linked with glutaraldehyde (GA), immunoprecipitated with anti-HA (top) or anti-FLAG (bottom), and detected with anti-HA-horseradish peroxidase (HRP). D and E: the predicted migration patterns of proteins in these experiments. The fusion proteins are depicted with FLAG tag (small black square), mRFP (big white square), HA tag (small oval), and MG53 (big oval). Because the cross-linking reactions could not cross-link all MG53 proteins, HA-tagged monomeric MG53 protein (m) was detected.
The CC domain of MG53 contains two conserved leucine zipper motifs (LZ1 and LZ2) as shown in Fig. 2A. To elucidate the role of LZ1 and LZ2 in MG53 function, three mutant constructs were generated by converting the conserved leucines into alanines: LA1 (A176/A183/A190), LA2 (A212/A219/A226), and double mutant LA1/2 (Fig. 2A). We generated stable HEK293 cell lines that express the HA-tagged form of these various leucine zipper mutants. The oligomeric structures of HA-LA1, HA-LA2, and HA-LA1/2 were probed through chemical cross-linking with different concentrations of GA. As shown in Fig. 2C, the HA-LA1 protein displayed compromised dimeric structure (left), whereas the HA-LA2 protein appeared to be normal (middle) compared with the wild-type HA-MG53 (Fig. 2B, left). The HA-LA1/2 double mutant was completely defective in protein dimerization in response to GA treatment (Fig. 2C, right). These results indicate that LZ1 motif is a major site for MG53 dimerization, while LZ2 is not as essential for the MG53 homodimerization.

Effects of leucine zipper mutations on MG53-mediated cell membrane repair. The functional impact of these LZ mutations on MG53-mediated cell membrane repair was examined using live cell imaging by confocal microscopy. We first examined the subcellular localization of the GFP-tagged MG53 mutants transiently expressed in C2C12 myoblasts. Consistent with our previous immunohistochemical staining of MG53 in skeletal muscle (3), GFP-tagged wild-type MG53 (GFP-WT) displayed targeting to the plasma membrane, intracellular vesicle localization, as well as distribution in the cytosol (Fig. 3A). Interestingly, mutation in either the LZ1 or the LZ2 motifs led to alteration of the subcellular distribution of GFP-LA1, GFP-LA2, or GFP-LA1/2, since all three mutants display predominantly cytosolic distribution when transiently expressed in C2C12 cells (Fig. 3A). In more than 90% of the cells observed under confocal microscopy, we consistently found that the mutant proteins displayed cytosolic distributions (n = 32 for LA1, 22 for LA2, and 17 for LA1/2, experiments tested with C2C12 cells), which is in sharp contrast to the membrane-tethering property of the wild-type GFP-MG53 protein. While a full understanding of the role of LZ1 and LZ2 in targeting MG53 to membrane surfaces would require extensive additional studies, live cell imaging demonstrated compromised movement of these MG53 mutants in response to acute damage to the plasma membrane. As shown in Fig. 3B, penetration of a microelectrode into the C2C12 cells caused rapid transloca-
tion of GFP-wild type (GFP-WT) toward the acute injury site (top), consistent with our published studies (3, 4). While both GFP-LA1 and GFP-LA2 could sense the mechanical damage in C2C12 cells and move to injury sites, their efficiencies of translocation were significantly compromised (Fig. 3 C). Moreover, the GFP-LA1/2 double mutant was completely defective in cell membrane repair as it could not traffic to injury sites. These results indicate that LZ-mediated MG53 intermolecular interactions are essential for MG53-mediated vesicle translocation toward the acute membrane injury site.

To furthermore dissect the role of leucine zipper motifs in membrane repair function, we performed the FM4–64 dye exclusion assay using mg53/H11002/H11002 myotubes as a homologous reconstitution system (1). The various GFP-tagged MG53 constructs were transfected into the mg53/H11002/H11002 myotubes, and the membranes were damaged with UV laser in the presence of FM4–64 dye. As shown in Fig. 4, expression of GFP-WT could effectively prevent entry of FM4–64 dye into the mg53/H11002/H11002 myotube following UV irradiation, which is consistent with our previous study that showed restoration of membrane repair defects with expression of the wild-type MG53. Clearly, the LA1/2 mutant is ineffective in restoration of membrane repair defects, as more FM4–64 dye entry into the mg53/H11002/H11002 myotubes was observed following UV irradiation (Fig. 4A). Interestingly, transient expression of GFP-LA2 could restore membrane repair function to similar degree as GFP-WT, whereas transient of GFP-LA1 produced intermediate membrane repair defects between GFP-WT and GFP-LA1/2 (Fig. 4B). These results are consistent with our observation shown in Fig. 3 and further suggest that the two LZ motifs likely play differential functions in the membrane repair process mediated by MG53.

The leucine zipper motif modulates the redox-dependent oligomerization of MG53. Our previous studies identified Cys242 as a critical residue in the redox-dependent nucleation process for MG53-mediated membrane repair (3). Here we found that the disulfide bond formation through Cys242 is not required for MG53 homodimerization, because normal dimers could be observed with HA-C242A using the GA cross-linking reagent (Fig. 5A). To assay whether the LA1 and LA2 mutation affects the redox-dependent oligomerization of MG53, we transfected GFP- or HA-tagged MG53 mutants into HEK293T cells and performed Western blot analysis under reducing and nonreducing conditions. Consistent with our previous study, we found that GFP-WT and HA-WT MG53 protein formed dimers in the absence of DTT (Fig. 5, B and C, top), and addition of DTT could convert these dimers into monomers (bottom). GFP-C242A and HA-C242A were defective in dimer formation even in the absence of DTT. While GFP-LA2 and HA-LA2 maintained the redox-dependent protein dimerization similar to the GFP-WT and HA-WT proteins, GFP-LA1 and HA-LA1 displayed significant defects in dimer formation under oxidized conditions. Such defects were more obvious with the GFP-LA1/2 and HA-LA1/2 constructs. Together, these
results revealed that the LZ motif-mediated intermolecular interaction can modulate the efficiency of redox-dependent MG53 oligomerization.

In vivo chemical modification of cysteine residues leads to impairment of MG53-mediated cell membrane repair. To further test whether the redox-dependent MG53 oligomerization is an obligatory process for MG53-mediated cell membrane repair, we performed the following series of experiments. We used an alkylating reagent, N-ethylmaleimide (NEM), to modify the sulfhydryl groups in MG53 (11) by adding it to the cell lysis buffer before disruption of HEK293T cells. As shown in Fig. 6A, treatment with NEM completely blocked disulfide bond formation between MG53 in all constructs tested. Since NEM is a membrane-permeable reagent (12), we next treated cells with NEM at different time points to determine when disulfide bonds were formed before cell permeabilization. When NEM was added to the culture media 10 min before cell lysis, it completely blocked the disulfide bond formation between MG53 molecules (Fig. 6B, lane 3-4). Similar to Fig. 6A, the presence of NEM in the cell lysis buffer also prevented MG53 oligomerization (Fig. 6B, lane 5). However, when NEM was added after cell lysis, it did not affect the apparent
disulfide bridge between MG53 molecules (Fig. 6B, lane 6). These results provide direct evidence that the active sulfhydryl groups (−SH) of MG53 are free in intact cells and can form disulfide bridges following cell membrane disruption. We next examined the impact of NEM pretreatment on MG53-mediated cell membrane repair. For this purpose, we transiently transfected GFP-MG53 into C2C12 cells and performed microelectrode penetration experiments in the presence of 1 mM NEM. As shown in Fig. 6C, preincubation of HEK293T cells with NEM could prevent translocation of GFP-MG53 toward the acute injury sites. Thus, NEM treatment could mimic the effect of C242A mutation via its occupation of the critical sulfhydryl group, which could prevent oligomerization of MG53 and lead to compromised cell membrane repair.

**DISCUSSION**

In this study, we demonstrate that MG53 can form homooligomers via its conserved leucine zipper motifs in the coiled-coil domain. Although the two leucine zippers both contribute to the nucleation process during MG53-mediated cell membrane repair, they have differential functions in mediating the intermolecular interactions among the MG53 molecules. We also show that the active thiol groups of MG53 are accessible to modification by an alkylating reagent, and blocking activation of these sulfhydryl groups can prevent redox-dependent oligomerization of MG53. Overall, our data support the hypothesis that oxidation of the thiol group of Cys242 and leucine zipper mediated interaction among the MG53 subunits both contribute to the nucleation process for MG53-mediated cell membrane repair.
conserved cysteine residues were observed (data not shown). Therefore, this is a unique property of MG53 among the TRIM family proteins.

Although LZ2 motif (L205/L212/L219/L226) is not involved in homodimerization, LZ2 motif is required for MG53 repair function and its role is different from LZ1 in MG53 repair function. Indeed, double mutation of both LZ1 and LZ2 motifs leads to complete disruption of MG53 membrane repair function. Further study is warranted to better understand the role of LZ2 in MG53-mediated repair function. Our available data do not exclude the possibility that the LZ2 domain could be involved in heteromeric interaction of MG53 with other interacting partners, such as the anchoring protein that could tether MG53-containing vesicles to the acute injury sites (32).

On the basis of the results shown here, we propose a model for MG53 function during cell membrane repair (Fig. 6D). Under resting conditions with intact cell membrane structure, MG53 exists as a dimeric structure that involves leucine zipper-mediated intermolecular interaction. The leucine zipper-mediated MG53 protein interaction also allows for accessibility of active sulphhydril groups (e.g., Cys242), which are critical for formation of a larger oligomeric complex during the nucleation process of cell membrane repair. Acute injury of the plasma membrane creates transient changes in the redox-state near the injury site, where disulfide formation through Cys242 can provide the structural base for formation of an oligomeric structure that is essential for cell membrane repair. Future study is required to dissect the differential function of the two leucine zipper motifs in MG53, and whether heteromeric interaction with other binding partners contributes to the overall MG53-mediated membrane repair response.

Our studies also revealed that occupation of the active thiol (−SH) group of Cys242 with chemical reagent can influence the redox-dependent oligomerization of MG53 and impact its cell membrane repair function. This result is consistent with our previous study that showed transient exposure of cells with low concentration (2 μM) of thimerosal could enhance MG53-mediated cell membrane repair, whereas sustained exposure of cells with high concentrations of thimerosal could prevent MG53 function in membrane repair. Our recent studies also showed that preincubation of cardiomyocytes with 2 mM H₂O₂ could disrupt MG53 translocation to the acute membrane injury site (29), suggesting that prolonged incubation of membrane-permeable oxidants could oxidize all available Cys242 residues and render MG53 insensitive to oxidative signaling generated by membrane disruption and entry of the external environment.

Increased cellular oxidative stress is a common phenotype with many chronic human diseases, such as heart failure, muscle atrophy, diabetes, and neurodegeneration (7, 21). Overproduction of reactive oxidative species could potentially affect the oxidation state of MG53 and reduce its membrane repair capacity. Thus, targeting the intracellular redox state using antioxidant approaches could have beneficial effects in tissue repair and regeneration. Our identification of the differential function of the two leucine zipper domains in MG53 could provide an attractive target for molecular intervention to modulate endogenous cell membrane repair function in skeletal and cardiac muscle. This could prove to be useful for treatment of human diseases associated with reduced regenerative capacity and/or altered cardiovascular function.

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DISCLOSURES
J. Ma and N. Weisleder are cofounders for TRIM-edicine, Inc., a university spin-off biotechnology company that is developing recombiant MG53 protein as a therapeutic reagent for regenerative medicine.

REFERENCES