Polymerase Transcriptase Release Factor (PTRF) Anchors MG53 Protein to Cell Injury Site for Initiation of Membrane Repair

Plasma membrane repair is an essential process for maintenance of homeostasis at the cellular and tissue levels, whereas compromised repair capacity contributes to degenerative human diseases. Our recent studies show that MG53 is essential for muscle membrane repair, and defects in MG53 function are linked to muscular dystrophy and cardiac dysfunction. Here we report that polymerase I and transcript release factor (PTRF), a gene known to regulate caveolae membrane structure, is an indispensable component of the membrane repair machinery. PTRF acts as a docking protein for MG53 during membrane repair potentially by binding exposed membrane cholesterol at the injury site. Cells lacking expression of endogenous PTRF show defective trafficking of MG53 to membrane injury sites. A mutation in PTRF associated with human disease results in aberrant nuclear localization of PTRF and disrupts MG53 function in membrane resealing. Although RNAi silencing of PTRF leads to defective muscle membrane repair, overexpression of PTRF can rescue membrane repair defects in dystrophic muscle. Our data suggest that membrane-delimited interaction between MG53 and PTRF contributes to initiation of cell membrane repair, which can be an attractive target for treatment or prevention of tissue injury in human diseases.

Repair of acute damage to the plasma membrane is an important aspect of cellular physiology, and disruption of this process can contribute to pathophysiology in a number of human diseases including muscular dystrophy and heart failure (1–5). Several previous studies established the framework of the cell membrane repair response that involves translocation of intracellular vesicles to the injury site to form a membrane repair patch (6, 7). We recently discovered that MG53 protein is an essential component of the membrane repair machinery (8–11). We showed that MG53 ablation results in defective sarcolemmal membrane repair with progressive myopathy (11) and increased vulnerability of the heart to exercise stress and ischemia reperfusion-induced injury (9, 10). Because MG53 can discriminate between intact and injured membrane (10), a membrane-delimited signal would likely be involved in tethering MG53 to the injured site. Identification of such an anchoring molecule is essential for our understanding of the basic biology of membrane resealing, as well as for designing therapeutic means to improve membrane repair defects in human diseases.

Caveolae are specialized plasma membrane invaginations that play important roles in many cellular processes. Caveolins are major coating proteins of caveolae, and mutations in caveolins have been identified in various human diseases (12–15). Our previous study showed that MG53 can interact with caveolin-3, and altered interaction between MG53 and caveolin-3 is linked to membrane repair defects in muscular dystrophy (8). In addition to caveolins, the polymerase I and transcript release factor (PTRF), also known as cavin-1 (16), is enriched in caveolae and contributes to the stable formation of caveolae (16, 17). Mice lacking PTRF show defective caveolae structure accompanying a lipodystrophic phenotype (18). Several recent studies have shown that mutations in PTRF are also associated with human disorders including lipodystrophy, muscular dystrophy, and cardiac dysfunction (19–22); however, the molecular mechanisms underlying how mutations of PTRF lead to human diseases remain unclear.

Here we show that PTRF is an obligatory factor for MG53-mediated nucleation of the membrane repair response. PTRF anchors MG53 to the acute injury site by binding the cholesterol exposed during damage to the cell membrane (10). Our studies also reveal that increased expression of PTRF can rescue the membrane repair defects in dystrophic muscle fibers and support the idea that membrane-delimited signaling is an important component of the cell membrane repair machinery.

MATERIALS AND METHODS

Plasmids and Gene Transfection—Cloning of GFP-MG53, HA-MG53, and RFP-PTRF were described in detail in the supplemental material. C2C12 murine myoblast cell line, HepG2 human hepatocellular carcinoma cell line, H1299 lung cancer cell line, and HeLa human cervix carcinoma cell line were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Cells were transfected using Genejammer reagent per the manufacturer’s directions (Stratagene).

Western Blotting and Co-immunoprecipitation—A standard protocol was used for co-immunoprecipitation of MG53 and PTRF. See the supplemental material for detailed methods.

In Vivo Muscle Transfection and Membrane Repair Assay—For transfection of skeletal muscle with RFP-PTRF or pU6-

3 The abbreviations used are: PTRF, polymerase I and transcript release factor; FDB, flexor digitorum brevis; LDH, lactate dehydrogenase; PC, phosphatidylcholine; PS, phosphatidylserine; MβCD, methyl-β-cyclodextrin; MBP, maltose binding protein.
mRFP-shPTRF and their control vectors, 20 μg of plasmid DNA was injected into the flexor digitorum brevis (FDB) muscle following established protocols (23). Experiments were performed 14 days after electroporation to allow for recovery from any damage generated during experimental manipulations (11). Isolated FDB fibers were irradiated with UV laser, and the entry of FM1-43 fluorescent dye (2.5 μM) into the muscle fibers was captured with a Zeiss-LSM 510 confocal microscope.

**Live Cell Imaging**—Confocal microscopic imaging of GFP-MG53 translocation was performed following mechanical injury of the cell membrane or chemical treatment with saponin, as described previously (10, 11).

**RESULTS AND DISCUSSION**

Elevated membrane repair capacity is required in skeletal and cardiac muscles due to the contractile nature of these tissues under normal physiological conditions. Although MG53 is a muscle-specific protein, it may accelerate the conserved cell membrane repair mechanism in non-muscle cells to provide beneficial effects on the health of the targeted tissue as MG53-mediated membrane repair can be recapitulated in multiple non-muscle cell types (11). An exception was observed in HepG2 cells, a hepatocellular carcinoma cell line, which failed to show translocation of a green fluorescent protein-tagged MG53 (GFP-MG53) to sites of membrane disruption. An example is shown in Fig. 1A, where GFP-MG53 expressed in HeLa cells displayed rapid translocation toward the acute mechanical injury sites; in contrast, HepG2 cells showed no accumulation of GFP-MG53 at the injury sites (Fig. 1B). One explanation for loss of GFP-MG53 nucleation at injury sites is that HepG2 cells lack certain cellular factors essential for MG53 function.

Changes in plasma membrane structure, in particular the specialized invaginations of caveolae, are associated with membrane repair defects in human diseases (8, 14, 15). The PTRF (also known as cavin-1) (24) is present in large amounts in caveolae and contributes to the stable formation of caveolae (16, 18). Previous studies showed that PTRF is expressed in most tissues with the exception of liver (25). Western blots revealed abundant expression of PTRF protein in mouse kidney, lung, heart, and skeletal muscle, but not in liver tissue or HepG2 cells (Fig. 1C). To test whether PTRF contributes to MG53-mediated membrane repair, we transfected HepG2 cells with a red fluorescent protein-labeled PTRF (RFP-PTRF) and used saponin detergent to damage the plasma membrane. As shown in Fig. 1D, GFP-MG53 expressed in HepG2 cells was diffuse and could not target to the plasma membrane upon treatment with 0.005% saponin, whereas HepG2 cells with co-expression of RFP-PTRF and GFP-MG53 displayed concentration of GFP-MG53 and RFP-PTRF at the cell membrane following treatment with saponin (see also supplemental Movies S1–S4). This saponin-induced translocation of GFP-MG53 to the plasma membrane was confirmed by quantifying LDH release in HepG2 cells treated with mechanical injury and saponin (Fig. 1E).

**FIGURE 1.** MG53 membrane repair function requires PTRF. A, microelectrode penetration of HeLa cells expressing GFP-MG53 results in translocation of MG53 at the injury site. B, MG53 disperses and leaks out of HepG2 cells upon the same treatment as A. Arrowheads indicate the location of microelectrode penetration. C, Western blots show that PTRF is expressed in different mouse tissues, but not in liver and HepG2 cells. Transfection of RFP-PTRF in HepG2 cells produces protein expression (right panel). Tubulin serves as internal control (con). D, subcellular distribution of RFP, RFP-PTRF, and GFP-MG53 expressed in HepG2 cells, before and after treatment with 0.005% saponin. E, LDH release after glass microbead damage to HepG2 cells. Total LDH in the supernatant after mechanical damage minus the basal LDH level before damage was averaged from multiple experiments (mean ± S.E., n = 8). * indicates statistical difference with p < 0.01 by analysis of variance.
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Modulating PTRF expression affects membrane repair in skeletal muscle. A, Western blot shows shRNA-mediated down-regulation of PTRF in FDB fibers. Con, control. B, FDB muscle fibers transfected with control plasmid show less FM1-43 dye entry following UV laser wounding (upper panels) as compared with those transfected with shRNA against PTRF (lower panels). Arrowheads indicate the location of laser wounding. Scale bar, 20 μm. C, summary data for panel B. Data represent mean ± S.E., n = 15. D, Western blot analysis shows overexpression of RFP-PTRF in dysferlin−/− and mg53−/− FDB muscle fibers. The endogenous levels of MG53, dysferlin, and PTRF in the different preparations are also shown. E, FDB muscle fibers from dysferlin−/− mice transfected with control plasmid (left) show excessive FM1-43 dye entry following UV laser wounding as compared with those transfected with RFP-PTRF (right; n = 15). G, FDB muscle fibers from mg53−/− mice transfected with control plasmid (left) show similar FM1-43 dye entry following UV laser wounding as compared with those transfected with RFP-PTRF (right, n = 15). Arrowheads indicate the location of laser wounding. F and H, summary data for panels E and G, respectively. Data represent mean ± S.E., n = 15.

membrane was similar to those observed in HEK293 cells, C2C12 myoblasts, and cardiomyocytes (8–11), indicating the requirement of PTRF for MG53 translocation to injury sites.

We next measured the release of an intracellular enzyme, lactate dehydrogenase (LDH), from a population of HepG2 cells following damage with glass microbeads. As shown in Fig. 1E, expression of MG53 or PTRF alone did not improve the membrane repair capacity over the parental HepG2 cells, whereas co-expression of PTRF and MG53 led to significant reduction of LDH release, suggesting that PTRF and MG53 are both required for protection of mechanical damage to cells.

Because skeletal muscle expresses both PTRF and MG53, we used RNAi to knock down the expression of PTRF in skeletal muscle to test whether reduced expression of PTRF produced membrane repair defects. We generated an shRNA probe against PTRF and introduced this plasmid into the FDB muscle of viable wild type mice using electroporation (8, 11, 23). Two weeks after electroporation, Western blotting was performed with transfected FDB muscles that showed effective knockdown of PTRF expression (Fig. 2A). For evaluation of the membrane repair capacity, individual FDB fibers were irradiated with a UV laser to cause localized damage at the sarcolemmal membrane (8, 11, 26). As shown in Fig. 2B, knockdown of PTRF expression led to elevated entry of FM1-43 dye at the UV irradiation site, indicating reduced membrane repair capacity as compared with fibers treated with a control shRNA probe. The extent of membrane repair defects shown in Fig. 2C is similar to those observed in mg53−/− muscle (11), suggesting that reduced expression of PTRF might compromise MG53 function and lead to increased susceptibility to membrane damage.

Bansal et al. (1) showed that dysferlin also contributes to membrane resealing as knock-out mice for dysferlin display membrane repair defects in both skeletal and cardiac muscle (1, 27). To test whether increased expression of PTRF could enhance the membrane repair capacity of skeletal muscle, we used electroporation to overexpress RFP-PTRF in FDB fibers from either dysferlin−/− or mg53−/− mice. As shown in Fig. 2D, the endogenous PTRF protein levels in dysferlin−/− and mg53−/− muscles were comparable with wild type controls, and expression of exogenous RFP-PTRF was similar in mg53−/− and dysferlin−/− muscle. Although overexpression of RFP-PTRF greatly decreased the UV irradiation-induced FM1-43 dye entry in the dysferlin−/− muscle (Fig. 2, E and F), there was no significant change in mg53−/− fibers electroporated with RFP-PTRF (Fig. 2, G and H). In both cases, these experiments used FDB fibers electroporated with RFP expression plasmid as a control. Because overexpression of PTRF can rescue the membrane repair defects in dysferlin−/− muscle, but not in mg53−/− muscle, the functional role of PTRF in membrane repair likely requires the presence of MG53.
Mutations in the human PTRF gene are linked with various diseases (19–22). To test whether such mutations can affect membrane repair capacity, we generated a homologous mouse PTRF mutant (531DelG) for one of the known human muta-
tions (525DelG). Similar to the human mutation (20), the mouse mutation also resulted in mislocation of PTRF to the nucleus (Fig. 3A, see also supplemental Fig. S1 for the location of wild type PTRF in muscle fibers), indicating that the mouse 531DelG mutation can be used as a model to study the human 525DelG function. As shown in Fig. 3, A and B, overexpression of the 531DelG mutant in the dysferlin−/− mice could not improve UV irradiation-induced damage to the FDB fiber, which is in sharp contrast to the significant enhancement of membrane repair function with overexpression of the wild type PTRF gene (Fig. 2, E and F). In vitro studies showed that 531DelG-PTRF expressed in HepG2 cells was mislocalized to the nucleus and insufficient to facilitate GFP-MG53 translocation to the plasma membrane following treatment with saponin (Fig. 3C).

We also performed similar studies with GFP-MG53 expression in H1299 cells, which lack expression of cavin-3 (28), a different member of the cavin gene family. Normal translocation of GFP-MG53 toward the acute membrane injury site was observed in H1299 cells (Fig. 3D), indicating that PTRF specifically interacts with MG53 because cavin-3 is not required for MG53-mediated membrane repair. Thus, PTRF mutations may be linked to compromised membrane repair that could contribute to the muscular dystrophy and cardiac complications observed in affected human patients (19–22).

We recently found that a cholesterol-dependent step for MG53-mediated membrane repair played an important role in protection of ischemia reperfusion-induced damage to cardiomyocytes (10). It is possible that membrane cholesterol, which is normally embedded in the hydrophobic core of an intact membrane and is exposed during injury, could provide a nucleation site for recruitment of MG53-containing vesicles for

![Image](49x496 to 299x676)

**FIGURE 3.** Mutant PTRF cannot rescue membrane repair defects in dysferlin−/− muscle. A, upper panels show mislocalization of RFP-531DelG in the nucleus of the dysferlin−/− muscle fiber (left, RFP fluorescence; right, overlay of bright field and fluorescence image). Lower panels show FM1-43 dye entry in the same FDB fiber following UV laser wounding. B, summary data from multiple experiments show that dysferlin−/− muscle fibers transfected with 531DelG-PTRF (red, n = 12) display similar FM1-43 dye entry as those transfected with RFP as control (black, n = 12), whereas muscle fibers transfected with the wild type PTRF display reduced FM1-43 dye entry (green, n = 12). Data represent mean ± S.E. C, RFP-531DelG expressed in HepG2 cells is localized to nucleus, and GFP-MG53 is present in both intracellular vesicles and plasma membrane (left panel). Fluorescent signals for RFP-531DelG and GFP-MG53 disappear after treatment with 0.005% saponin (right panel, n = 6). D, H1299 cells transfected with GFP-MG53 show trafficking of MG53 to the plasma membrane following treatment with 0.005% saponin. Scale bar, 20 μm.

![Image](127x127 to 485x353)

**FIGURE 4.** PTRF anchors MG53 to membrane cholesterol for initiation of cell membrane repair. A, co-immunoprecipitation (IP) shows physical interaction between PTRF and MG53 in HeLa cells. B, lipid dot-blot analyses reveal PTRF can bind PS and cholesterol, but not PC. MBP-MG53 can bind PS, but not PC and cholesterol. As control, MBP-MBP does not show binding to PS, PC, or cholesterol. Co-incubation with PTRF leads to tethering of MG53 to cholesterol. WB, Western blot. C, treatment of FDB fibers with 5 mM MβCD (15 min at 37 °C) leads to fragility of the sarcotlemmal membrane and defective resealing upon UV laser wounding. MβCD-treated FDB fibers always show contracture with 531DelG-PTRF (red, n = 12). Data represent mean ± S.E.
membrane patch formation (10). A challenge with this model is that MG53 itself cannot bind cholesterol (11); thus, an intermediate molecule would be required to anchor MG53 to the exposed cholesterol at the injury site. To test whether PTRF can participate in this role, we first performed co-immunoprecipitation and found that MG53 and PTRF could physically interact (Fig. 4A) and that the 531DelG-PTRF mutant could not interact with MG53 (supplemental Fig. S2). Next we asked whether PTRF could mediate the MG53 binding to cholesterol. Using a lipid-protein overlay assay (8, 11, 29), we showed that both MG53 and PTRF could interact with phosphatidylserine (PS) and that neither of them could bind phosphatidylcholine (PC) (Fig. 4B); these results were consistent with previous studies (8, 11, 16). Clearly, PTRF could bind cholesterol, whereas MG53 could not. With co-incubation of MG53 with PTRF, we could detect MG53 signal at cholesterol-containing dots, indicating that PTRF could anchor MG53 to cholesterol (Fig. 4D).

To test the effect of cholesterol in membrane repair, we treated FDB fibers with methyl-β-cyclodextrin (MβCD) to deplete cholesterol from the sarcolemmal membrane. Similar to our study with cardiomyocytes (10), this MβCD treatment had a severe impact on the integrity and resealing capacity of skeletal muscle because even prior to UV irradiation, the majority of the treated fibers already showed positive staining with FM1-43 dye due to endogenous expression of PTRF or restoration of any disrupted MG53-PTRF interaction during disease may present an attractive avenue for treatment or prevention of tissue injury in human diseases.

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