Nonmuscle myosin IIA facilitates vesicle trafficking for MG53-mediated cell membrane repair

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ABSTRACT Repair of injury to the plasma membrane is an essential mechanism for maintenance of cellular homeostasis and integrity that involves coordinated movement of intracellular vesicles to membrane injury sites to facilitate patch formation. We have previously identified MG53 as an essential component of the cell membrane repair machinery. In order for MG53 and intracellular vesicles to translocate to membrane injury sites, motor proteins must be involved. Here, we show that nonmuscle myosin type IIA (NM-IIA) interacts with MG53 to regulate vesicle trafficking during cell membrane repair. In cells that are deficient for NM-IIA expression, MG53 cannot translocate to acute injury sites, whereas rescue of NM-IIA expression in these cells can restore MG53-mediated membrane repair. Compromised cell membrane repair is observed in cells with RNAi-mediated knockdown of NM-IIA expression, or following pharmacological alteration of NM-IIA motor function. Together, our data reveal NM-IIA as a key cytoskeleton motor protein that facilitates movement of intracellular vesicles to membrane injury sites.

Key Words: confocal imaging • nucleation • COS-7 cells

Plasma membrane repair is an essential physiological process for maintenance of cellular homeostasis and integrity (1–4). Defects in membrane repair have been linked to the progression of a number of human diseases, including muscular dystrophy, respiratory dysfunction, cardiovascular disease, and neuronal injury (5–9). For membrane resealing to take place, 3 principal steps must occur. First, the cell must be capable of sensing disruption of the plasma membrane and the location of the disruption. Second, the intracellular vesicles necessary to facilitate membrane patch formation must translocate to the injury site. Third, these vesicles must fuse with the plasma membrane and/or each other to form a repair patch at the injury site. Thus, motor-driven vesicle translocation plays an essential role for cell membrane resealing. Steinhardt and colleagues (10) used antibody inhibition and pharmacological approaches to determine that both kinesin and myosin motor proteins are involved in vesicle trafficking during membrane repair and that nonmuscle myosin IIA and IIB (NM-IIA and NM-IIB) have distinct roles in the exocytosis-dependent process of cell membrane repair (11). Bement and colleagues (12–14) demonstrated that local assembly of myosin-2 at the acute wound site created a purse string that facilitates formation of a membrane repair patch.

Several studies identified some of the molecular components involved in cell membrane repair, particularly those specific to striated muscles (15–19). Bansal et al. (17) showed that dysferlin plays an important role in maintenance of sarcolemmal membrane integrity. Various researchers proposed that dysferlin can function as a fusogen to allow vesicles to form a membrane repair patch (4). However, since the initial study by Bansal and colleagues (17), there has been no indication that dysferlin itself can facilitate the rapid translocation of vesicles associated with acute membrane damage. Indeed, dysferlin<sup>−/−</sup> maintains the capacity for vesicle translocation to damage sites on the sarcolemma (17). This suggests that although dysferlin may participate in the final membrane resealing process, proteins other than dysferlin are likely required for nucleation of intracellular vesicles at membrane injury sites.

Abbreviations: co-IP, coimmunoprecipitation; bleb (−), blebbistatin (+); BTS, N-benzyl-p-toluene sulfonamide; FDB, flexor digitorum brevis; LDH, lactate dehydrogenase; NM-IIA, nonmuscle myosin IIA; NM-IIB, nonmuscle myosin IIB; MYHC, myosin heavy chain.

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Our recent studies showed that MG53, a muscle-specific TRIM family protein (TRIM72), is an essential component of the membrane repair machinery in muscle cells, as MG53 ablation results in defective membrane repair, progressive skeletal myopathy (15), and increased vulnerability of cardiomyocytes to ischemia-reperfusion injury (20, 21). In addition, we found that MG53 can interact with dysferlin and is required for dysferlin movement to the acute membrane injury sites (22, 23). Although these studies indicate that MG53 mediates the sensing and vesicle translocation steps of plasma membrane repair, a motor protein must be involved to allow MG53 to move toward the membrane injury site. In an effort to determine molecular partners that contribute to MG53 function during cell membrane repair, we expressed a tagged MG53 construct in differentiated C2C12 myotubes. Following coimmunoprecipitation (co-IP), we found that NM-IIA can physically interact with MG53. We used a combination of molecular and pharmacological interventions together with live cell imaging to find evidence that the NM-IIA motor protein is essential for vesicle trafficking during MG53-mediated cell membrane repair.

MATERIALS AND METHODS

Plasmids and reagents

The myc-MG53 plasmid was generated by inserting a myc tag into the 5’ end of the mouse MG53 cDNA with the following oligo linkers: ATGGAGCAAAAGCTGATTTCTGAGGAGGA TCTGTTCCGG. The sequence encoding the myc-tag amino acid sequence (EQKLISEEDL) is underscored. Expression plasmids for GFP-MG53, RFP-MG53, and HA-MG53 were described previously (22). For knockdown studies, two sets of shRNA oligos were used to target NM-IIA by cloning these sequences into the pU6-mRFP vector to generate the shRNA-NM-IIA1 and shRNA-NM-IIB2 plasmids. The pU6-mRFP was described previously (24). The oligos used for shRNA constructs are as follows: shRNA-IIA1, sense sequence 5’-GAGTACAGAGTACAGATGCTCA-3’; and shRNA-IIB2, sense sequence 5’-GAAGGCTGAGATGCTCA-3’. All plasmids were confirmed by direct sequencing. GFP-NM-IIB and GFP-NM-IIB plasmids were originally developed by Wei and Adelstein (25) and were purchased from Addgene (Cambridge, MA, USA).

Cell culture and imaging

C2C12, COS-7, and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in a humidified environment at 37°C and 5% CO2 in DMEM, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. C2C12 myoblasts were transfected with myc-MG53 and differentiated into myotubes for 6 d before myotubes were resuspended in 0.5 ml modified RIPA buffer plus protease inhibitor cocktail (Sigma) and precleared with a mixture of protein A and protein G beads (KPL, Gaithersburg, MD, USA). Precleared cell lysate was incubated overnight with 10 μg of anti-myc mAb (BD Biosciences, San Jose, CA, USA), or normal mouse IgG (as negative IP control). The resulting immunocomplexes were collected on protein A/G-Sepharose beads and separated by SDS-PAGE. Gel was then stained with Colloidal Blue (Invitrogen, Carlsbad, CA, USA). The protein band of interest was identified using mass spectrometry. Co-IP of MG53 and NM-IIB and NM-IIB was performed in HeLa cells following coexpression of HA-MG53 and GFP-NM-IIB or GFP-NM-IIB, using a similar protocol. Rabbit polyclonal anti-MG53 antibody has been described previously (15). Antibodies against α-tubulin, β-actin, and skeletal muscle myosin heavy chain (fast form; MY32) were from Sigma. Two commercial resources of NM-IIB antibodies were used: epitope-specific NM-IIB antibody (Covance, Princeton, NJ, USA) or antibody raised against the purified NM-IIB (Biomedical Technologies, Berlin, Germany). NM-IIB isoform-specific polyclonal antibodies were from Covance. Anti-HA (clone 12CA5) was from Roche. Anti-RFP was from Abcam (Cambridge, MA, USA).

Membrane repair assay with lactate dehydrogenase (LDH) release after glass microbead-induced injury

C2C12 myoblasts were transfected with pU6-mRFP vector (as control) or pU6-mRFP-shRNA-NM-IIB1 and allowed to differentiate into myotubes for 2 d. Cells were resuspended in BSS buffer (140 mM NaCl, 2.8 mM KCl, 2 mM MgCl2, 10 mM HEPES, and 2 mM Ca2+, pH 7.2) and subjected to mechano-
ical damage induced by glass microbeads (<106 μm diameter; Sigma), as described previously (18). The release of an intracellular enzyme, LDH, from a population of cells following damage with glass microbeads was used as an index for the degree of membrane injury. Total LDH activity in the supernatant was measured (LDH assay kit; Takara, Osaka, Japan) at basal conditions (prior to addition of glass beads), at 3 and 7 min after glass bead-induced injury, or after addition of 1% Triton X-100 to induce complete lysis of the cell and determine the total LDH present.

Statistical analysis

Statistical analyses were performed using the Student’s t test, and values of \( P < 0.05 \) were considered to be statistically significant.

RESULTS

NM-IIA interacts with MG53 and regulates vesicle trafficking in C2C12 cells

Co-IP analysis of myc-MG53 expressed in C2C12 myotubes identified an ~150-kDa protein that associates with MG53 (Fig. 1A). The corresponding gel band was excised, and protein mass fingerprinting identified this protein as NM-IIA (see Table 1). NM-IIA is encoded by the MYH9 gene (26), which contains an open reading frame of 1960 aa with a predicted molecular mass of 220 kDa. The presence of a 150-kDa fragment rather than the full-length protein of 220 kDa is likely due to proteolysis during the co-IP procedure, since specific interaction of MG53 with NM-IIA was observed in HeLa cells that were cotransfected with HA-MG53 and GFP-NM-IIA, where co-IP revealed physical interaction between these two proteins (Fig. 1B). The identity of the 150-kDa fragment could not be verified using two commercial available antibodies against NM-IIA, and these antibodies were inefficient in co-IP assays with MG53 and NM-IIA coexpressed in HeLa cells (not shown). One possible explanation for these results is that the 150-kDa fragment lacks the epitope recognized by these anti-NM-IIA antibodies. Since the protein pulldown or co-IP studies were performed with whole lysates from cells overexpressing the epitope-tagged proteins, the present data do not address whether a direct interaction exists between

### TABLE 1. Identified nonmuscle II A peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>290 TDLLLEPYNK 299</td>
</tr>
<tr>
<td>2</td>
<td>408 EQADEFIALAK 419</td>
</tr>
<tr>
<td>3</td>
<td>712 VVFOEF 718</td>
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<tr>
<td>4</td>
<td>1001 VAEFTNLMEEEEK 1014</td>
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<tr>
<td>5</td>
<td>1816 IQLEEQDNETK 1828</td>
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<tr>
<td>6</td>
<td>1878 QLE EAEEAQ 1888</td>
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MG53 and NM-IIA, or whether an intermediate protein is involved to facilitate the functional interaction between MG53 and NM-IIA.

Western blot showed that NM-IIA is present during C2C12 cell differentiation from myoblasts to myotubes and is also expressed in adult skeletal muscle (Fig. 1C). In contrast, the skeletal muscle myosin heavy chain (MYHC) and MG53 are present only in differentiated C2C12 myotubes. As expected, MYHC is the dominant myosin present in adult skeletal muscle.

Our previous studies show that MG53 can affect intracellular vesicle trafficking in muscle cells (23). To test whether this function of MG53 is dependent on the action of myosin motors, we applied 40 μM bleb (-), a known inhibitor of myosin motors (27), to C2C12 cells transiently transfected with RFP-MG53 and examined whether the movement of MG53-containing vesicles was altered. To prevent photoinactivation of bleb (-), all experiments were performed in the dark and completed within 1 h following the preincubation with bleb (-). As shown in Fig. 1D, in C2C12 myotubes treated with DMSO (as a control), RFP-MG53-labeled intracellular vesicles were abundant and highly mobile. The addition of bleb (-) led to a near-complete immobilization of RFP-MG53-labeled intracellular vesicles. The striking difference in mobility of RFP-MG53-labeled intracellular vesicles in cells treated with bleb (-) can be visualized in the Supplemental Movie S1. On average, the velocity of MG53-containing vesicles was reduced from 25.4 ± 1.0 nm/s (control) to 7.8 ± 0.5 nm/s (+bleb (-)) (P < 0.001; n = 60 from 3 independent experiments; Fig. 1D).

Interference of myosin motor activity compromises MG53-mediated cell membrane repair

Treatment of C2C12 cells with bleb (-) has significant effect on membrane repair. As we previously observed (15), acute damage of C2C12 cells by penetration of a microelectrode resulted in rapid translocation of GFP-MG53 toward the injury sites to form a membrane repair patch. In cells treated with 20 μM bleb (-), there was no accumulation of GFP-MG53 at the acute membrane injury sites (Fig. 2A). The summary data shown in Fig. 2C indicate that MG53-mediated cell membrane repair is compromised in cells treated with bleb (-).

Using isolated mouse FDB muscle fibers, we observed significant contribution of myosin motor activity to the membrane repair function in native skeletal muscle. Since MYHC is the dominant myosin present in skeletal muscle, we first tested the effect of BTS, a known inhibitor of...
MYHC, in muscle cells (28). For this assay, individual FDB fibers were irradiated with a UV laser to induce local damage to the sarcolemmal membrane. As shown in Fig. 2B, the presence of 100 μM BTS led to elevated entry of FM1–43 fluorescent dye at the UV-irradiation site, indicative of reduced membrane repair capacity, as compared with FDB fibers treated with DMSO as a control. Elevated release of FM1–43 dye entry was observed in FDB fibers treated with 100 μM bleb (−), following identical UV-induced damage to the sarcolemmal membrane, indicating inhibition of NM-IIA produced more defects in membrane repair capacity (Fig. 2B). Furthermore, there was no additive effect of combined treatment of bleb (−) and BTS, as the degree of membrane damage in FDB fibers treated with bleb (−)/BTS was similar to those treated with bleb (−) alone. All results are summarized in Fig. 2D. These data suggest that the bleb (−)-sensitive motor activity contributes to the acute membrane repair function in skeletal muscle.

**Knockdown of NM-IIA leads to impairment of cell membrane repair**

Considering the potential nonspecific action of such pharmacological inhibitors, we next focused our studies on the functional impact of altering NM-IIA expression. For this assay, individual FDB fibers were transfected with the control shRNA plasmid. This inhibited expression of NM-IIA in C2C12 cells led to compromised cell membrane repair capacity in response to glass bead-induced injury. Bleb (−) likely acts on NM-IIA to exert its inhibitory role on MG53-mediated vesicle trafficking during cell membrane repair.

**Restoration of NM-IIA in COS-7 cells rescues MG53 translocation during cell membrane repair**

Togo and Steinhardt (11) found that COS-7 cells lack endogenous expression of NM-IIA, and these cells display certain defects in membrane resealing. The COS-7 cells lack endogenous expression of MG53 and thus can be used as a convenient expression system to test the functional interaction between MG53 and NM-IIA in cell membrane repair. If NM-IIA is a motor protein essential for the function of MG53, one would predict that MG53 would not be able to function in membrane repair in COS-7 cells.

Ectopic expression of NM-IIA in COS-7 cells could be achieved by liposome-mediated gene transfection. For subcellular localization and live cell imaging, we introduced GFP-NM-IIA into the COS-7 cells (Fig. 4A). Using confocal microscopic imaging of COS-7 cells cotransfected with GFP-NM-IIA and RFP-MG53, we monitored the coordinated movement of NM-IIA and MG53 associated with repair of acute injury to the plasma membrane. As shown in Fig. 4B, in response to microelectrode-induced mechanical injury to the plasma membrane, cotranslocation of GFP-NM-IIA and RFP-MG53 could be observed during the initial stage of cell membrane repair (5 to 10 s), with the final repair patch densely labeled with MG53 that is surrounded by NM-IIA (25 to 30 s and enlarged image, right panel). This repair patch is similar to the earlier report of a “purse-string like structure” by Bennett et al. (14).

Interestingly, GFP-NM-IIA expressed in COS-7 cells appeared in two distinct localization patterns. Many cells displayed a cytosolic pattern for GFP-NM-IIA (Fig. 4C, middle panel), while other cells displayed GFP-NM-IIA protein expression mainly bound to filamentous structures (Fig. 4C, bottom panel). Using our microelectrode cell-wounding assay, we found that RFP-MG53 could not accumulate to the membrane damage site in COS-7 cells in the absence of NM-IIA (Fig. 4C, top panel), whereas COS-7 cells expressing the cytosolic, soluble form of GFP-NM-IIA showed rapid RFP-MG53 accumulation to sites of membrane injury. Interestingly, RFP-MG53 cannot form a membrane repair patch in COS-7 cells displaying the filamentous form of GFP-NM-IIA (Fig. 4C, D).

The specificity of NM-IIA in facilitating MG53-mediated vesicle translocation was further tested using coexpression of GFP-NM-IIA in COS-7 cells. On the basis of co-IP, we found that NM-IIA could not interact with MG53 (Fig. 5A). Interestingly, overexpression of GFP-NM-IIA failed to rescue RFP-MG53 translocation to the membrane injury site (Fig. 5B). The striking difference in the role of NM-IIA and NM-IIB in facilitating translocation of MG53 to acute injury sites in COS-7 cells can...
be seen in Supplemental Movie S3. These results show that NM-IIA appears to be an obligatory factor for MG53-mediated membrane repair in COS-7 cells.

**DISCUSSION**

In this study, we identify NM-IIA as a key molecular motor that moves MG53-containing vesicles to membrane injury sites to reseal membrane damage in both native muscle cells and in reconstitution studies in nonmuscle cells. Pharmacological intervention to inhibit NM-IIA motor function and shRNA knockdown of NM-IIA expression leads to compromised MG53-mediated cell membrane repair. While NM-IIA represents a minor component of the myosin expressed in adult skeletal muscle, our functional studies indicate that NM-IIA plays an important role in repair of membrane damage in skeletal muscle.

In COS-7 cells that lack endogenous NM-IIA expression (11), MG53 cannot move to the acute membrane injury sites, while rescuing NM-IIA expression in COS-7 can restore MG53-vesicle accumulation at such sites. This effect appears to be specific for NM-IIA, since overexpression of NM-IIB was ineffective in rescuing the movement of MG53-containing vesicles toward the acute injury sites in COS-7 cells. Our study further revealed that when GFP-NM-IIA was confined to the filamentous structures, RFP-MG53 cannot move to the injury sites, suggesting that a mobile pool of NM-IIA is likely required for the rapid accumulation of MG53-containing vesicles at the injury sites.
Nonmuscle myosin has been shown to participate in many cellular functions (29), including myoblast fusion (30), membrane docking (31), regulation of actin network rearrangement for cell-scale actin treadmilling (32), regulation of vesicle trafficking from Golgi to plasma membrane (33), and wound closure (12). Previous work by Togo and Steinhardt (11) showed that following membrane disruption there was an acute response driven by vesicle fusion close to the site of cell injury and a recruitment response that involves mobilization from a vesicle pool deeper inside the cell. These studies used an antisense approach to demonstrate that NM-IIB mediates the initial response while NM-IIA is responsible for the later vesicle mobilization (11). In our study, we find that MG53 translocation is dependent on the presence of NM-IIA, while NM-IIB is not required for MG53 movement to injury sites. This suggests that MG53 is likely present in the pool of vesicles found deeper within the cell that are independent of NM-IIB activity found to be important in the previous studies. It is also possible that differences in the methodology used between our studies and those of Togo and Steinhardt (11) could explain differential findings on the importance of NM-IIB in membrane repair. In this study, we measured MG53-containing vesicle translocation, whereas the previous study measured the efflux or influx of dyes following membrane injury. As shown in our early study (15), while entry of extracellular Ca\(^{2+}\) plays an important role in the fusion process of intracellular vesicles to the injury plasma membrane, the movement of MG53-containing vesicles to the acute injury site can occur in the absence of Ca\(^{2+}\) entry. Thus, it is possible that while NM-IIB is not required for movement of MG53-containing vesicles to

Figure 4. Restoration of NM-IIA rescues MG53-translocation during cell membrane repair in COS-7 cells. A) Cell lysates extracted from C2C12 (lane 1), COS-7 cells (lane 2), and COS-7 cells transfected with GFP-NMIIA (lane 3) were analyzed by Western blot with anti-NM-IIA. B) Comovement of GFP-NM-IIA and RFP-MG53 in COS-7 cells following acute injury with a microelectrode penetration into the cell membrane. Arrows indicate sites of microelectrode penetration. Images were taken at different times postinjury. Enlarged overlay image (at 30 s postinjury, right panel) shows MG53-containing repair patch surrounded by NM-IIA. C) COS-7 cells transfected with RFP-MG53 alone or cotransfected with RFP-MG53 and GFP-NM-IIA were subjected to microelectrode injury and time-dependent confocal analysis at 24 h after transfection. No RFP-MG53 accumulation to the injury site was observed in cells transfected with RFP-MG53 alone (top panels). Rapid recruitment of RFP-MG53-containing vesicles toward the injury site (arrows) was observed in cells expressing the soluble forms of GFP-NM-IIA(S) (middle panels). No accumulation of RFP-MG53 at injury sites was observed in cells expressing the filamentous forms of GFP-NM-IIA(F) (bottom panels). D) Summary data for time-dependent accumulation of RFP-MG53 at the injury sites in COS-7 cells transfected with RFP-MG53 alone or cotransfected with RFP-MG53 and GFP-NM-IIA. Data are means ± se (n=14 cells/group from 4 independent experiments). Arrow indicates initiation of microelectrode penetration. Scale bars = 10 μm.
the injury site, it may contribute to the overall Ca\(^{2+}\)-dependent process of membrane resealing.

Confocal microscopy imaging revealed a colocalization of MG53 and NM-IIA in the formation of the membrane repair patch. MG53 is enriched inside the repair patch, whereas NM-IIA appeared to form a ring-like structure surrounding the repair patch. This structure is strikingly similar to the earlier work by Bement and colleagues (12, 14), who demonstrated that myosin-2 forms a purse ring for wound closure. Our studies suggest that NM-IIA is the myosin-2 isoform that likely facilitates the rescaling process of the acute membrane damage. Further examination for the interaction of MG53 with other cytoskeletal components may provide insights into the mechanism that underlies the formation and progression of a purse ring associated with membrane repair. Our identification for NM-IIA as a critical molecular motor in transporting MG53-containing vesicles toward the acute membrane injury sites in both myogenic and nonmyogenic cells should not only broaden our understanding of the basic biology of tissue repair, but also provide alternative targets for treatment of human diseases associated with defective membrane repair.

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Figure 5. NM-IIA cannot facilitate MG53-translocation in COS-7 cells during acute injury. A) CoIP showed that NM-IIA and MG53 do not interact with each other. HeLa cells were cotransfected with HA-MG53 and GFP-NM-IIA. Cell lysates were immunoprecipitated with anti-HA or anti-NM-IIA antibody and Western blotted with anti-MG53 or anti-NM-IIA antibodies, respectively. B) RFP-MG53 shows no translocation to membrane injury site when expressed alone in COS-7 cells (top panels) or coexpressed with GFP-NM-IIA (bottom panels). Scale bars = 10 μm.

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