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Nonmuscle Myosin IIA and IIB Have Distinct Functions in the Exocytosis-dependent Process of Cell Membrane Repair

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Vesicle generation, recruitment, and exocytosis are essential for repairing disruptions of cell membranes. The functions of nonmuscle myosin IIA and IIB in this exocytotic process of membrane repair were studied by the antisense technique. Knockdown of myosin IIB suppressed wound-induced exocytosis and the membrane resealing process. Knockdown of myosin IIA did not suppress exocytosis at an initial wound and had no inhibitory effect on the resealing at initial wounds but did inhibit the facilitated rate of resealing normally found at repeated wounds made at the same site. COS-7 cells, which lack myosin IIA, did not show the facilitated response of membrane resealing to a repeated wound. S91 melanoma cells, a mutant cell line lacking myosin Va, showed normal membrane resealing and normal facilitated responses. We concluded that myosin IIB was required for exocytosis and therefore cell membrane repair itself and that myosin IIA was required in facilitation of cell membrane repair at repeated wounds. Myosin IIB was primarily at the subplasmalemma cortex and myosin IIA was concentrated at the trans-Golgi network consistent with their distinct roles in vesicle trafficking in cell membrane repair.

INTRODUCTION

Myosins are a large family of structurally diverse molecular motors. To date, at least 15 structurally distinct classes of myosin heavy chains have been identified (Sellers, 2000; Berg *et al.*, 2001). Conventional nonmuscle myosin II is comprised of two genetically distinct isoforms referred to as myosin IIA and IIB (Simons *et al.*, 1991). Different isoforms of myosin II localize differently within individual cells, and these different distributions in the cell suggest that the two proteins have some important functional differences (Maupin *et al.*, 1994; Rochlin *et al.*, 1995; Kelley *et al.*, 1997). In nonmuscle cells, myosin II has diverse functions including cytoplasmic contractility (Condeelis and Taylor, 1977), cytokinesis (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987), capping of cell-surface components (Pasternak *et al.*, 1989), polarization of cell locomotion (Wessels *et al.*, 1988), and neurite outgrowth (Wylie *et al.*, 1998; Wylie and Chantler, 2001). Myosin II is also suggested to be involved in membrane trafficking within the cell. It has been proposed that myosin IIB is involved in exocytosis, because microinjection of polyclonal antibody against myosin IIB suppressed neurotransmitter release (Mochida *et al.*, 1994; Mochida, 1995). Although it has not been clear how many populations of vesicles bud off the trans-Golgi network (TGN), the p200/myosin II protein, analogous to nonmuscle myosin IIA heavy chain, has been reported to be on a specific subset of TGN-derived vesicles (Narula *et al.*, 1992; Narula and Stow, 1995; Ikonen *et al.*, 1997; Musch *et al.*, 1997; Heimann *et al.*, 1999). However, there are conflicting reports about whether p200/myosin II is an essential participant in the vesicle budding reaction (Musch *et al.*, 1997; Simon *et al.*, 1998). It has also been proposed that unconventional myosins type I, V, VI, and VII, are involved in membrane trafficking (Tuxworth and Titus, 2000).

The disruption of cell plasma membranes frequently occurs in many animal tissues, and cells survive these disruptions by restoring the integrity of the plasma membrane (McNeil and Steinhardt, 1997; McNeil and Terasaki, 2001). Small disruptions on the order of 1 μm evoke the calcium-dependent exocytosis of vesicles near the wound site. This response is essential for successful membrane resealing (Steinhardt *et al.*, 1994; Bi *et al.*, 1995, 1997; Miyake and McNeil, 1995; Togo *et al.*, 1999; Reddy *et al.*, 2001). Exocytosis promotes resealing by decreasing membrane tension (Togo *et al.*, 2000). Vesicles forming exocytotic figures required for membrane resealing were selectively blocked by inhibitors of kinesin and myosin motors in a two-step process in sea urchin embryos (Bi *et al.*, 1997). In Swiss 3T3 fibroblasts, it had been previously shown that inhibition of kinesin motor activity inhibited membrane resealing (Steinhardt *et al.*, 1994). Furthermore, disruption of cortical actin filaments by cytochalasin D inhibited wound-induced exocytosis in 3T3 cells (Togo *et al.*, 1999), suggesting that myosin motor activity is involved in wound-induced exocytosis. Recently, we found that a second membrane disruption at the same surface site as the initial wound resealed more quickly than the initial wound, and the BFA sensitivity of the increased rate of resealing at the second wound suggested that this facilitated response required new TGN-derived vesicles (Togo *et al.*, 1999, 2003).

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Abbreviations used: BFA, brefeldin A; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PKC, protein kinase C; TGN, trans-Golgi network.

The aim of the present study was to further define which myosins are involved in membrane resealing and facilitation of membrane resealing in Swiss 3T3 fibroblasts. We applied the antisense technique to knockdown nonmuscle myosin IIA and IIB in 3T3 cells and also investigated characteristics of membrane resealing of COS-7 cells, which are missing nonmuscle myosin IIA (Tullio *et al.*, 1997), and S91 cells, a mutant cell line lacking myosin Va (Wu *et al.*, 1997).

MATERIALS AND METHODS

Cell Preparation

Swiss 3T3 fibroblasts were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 8% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) and 50 μg/ml gentamicin (Invitrogen). African green monkey kidney cell lines, COS-7 and CV-1, were cultured in DMEM containing 10% FBS and 50 μg/ml gentamicin. S91 mouse melanoma cells were cultured in Ham's F10 medium (Invitrogen) containing 15% horse serum, 2.5% FBS, and 50 μg/ml gentamicin.

Cells for experiments were plated on cover glass-inserted plastic dishes (35 × 10 mm) and were grown for 1–2 d before use. During wound experiments, the cells were maintained in 1.8 mM Ca^{2+} Ringer's solution. Ca^{2+}-free Ringer's solution contained 138 mM NaCl, 2.7 mM KCl, 1.06 mM $MgCl_2$, 5.6 mM D-glucose, and 12.4 mM HEPES (pH 7.25). A stock solution of 100 mM $CaCl_2$ was used to adjust the concentration of Ca^{2+}.

Treatment of Cells with Antisense Oligonucleotides

Phosphorothioate-modified oligonucleotides and fluorescein-labeled phosphorothioate-modified oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and Proligo Japan (Kyoto, Japan). The antisense sequence was based on the start codon and additional downstream bases of mRNA sequences of rat myosin IIA and mouse myosin IIB (accession numbers, U31463 and U34303, respectively). Scrambled sequences of the antisense were used for control experiments. The antisense and scrambled sequences of myosin IIA were 5′-tgcagcctgctgggccatg-3′ and 5′-ccacgtgctgcgagtgtgc-3′, respectively. The antisense and scrambled sequences of myosin IIB were 5′-tccagttctctgggccatt-3′ and 5′-ttactctcgcagtcggtct-3′, respectively.

3T3 cells (typically 50% confluent in cover glass inserted Petri dishes) were washed once with DMEM and incubated with DMEM containing 1 μM oligonucleotide and 10 μg/ml Lipofectin (Invitrogen) for 6 h at 36°C. Culture medium was then replaced with DMEM containing 2% FBS and 1 μM oligonucleotides and further incubated at 36°C for 42 h.

Western Blotting

For analysis of protein expression of myosin IIA and IIB, cells were washed twice with PBS and lysed in lysis buffer. Lysis buffer consisted of 20 mM Tris (pH 7.5), 1.2% Triton X-100, 0.1% 2-mercaptoethanol, 500 μM AEBSF, 150 nM aprotinin, 1 μM E-64, 0.5 mM EDTA, and 1 μM leupeptin. The cell lysates were boiled for 2 min in SDS-PAGE sample buffer, and equivalent amounts of protein were separated on an 8% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane. Myosin antibodies were obtained from Covance Research Products (Denver, PA): polyclonal antibody against nonmuscle myosin II heavy chain isoform had species reactivity to human, rodent, and bovine myosin IIA. This antibody was raised against the peptide GKADGAEAKPAE corresponding to the C-terminus of human nonmuscle myosin heavy chain isoform A (MHC-A). Polyclonal antibody against nonmuscle myosin II heavy chain isoform B had species reactivity to human and rodent myosin IIB. This antibody was raised against the peptide SDVNETQPPQSE corresponding to the C-terminus of human nonmuscle myosin heavy chain isoform B (MHC-B). Each antibody recognized a single band (200 kDa) in 3T3 cells by Western blot. The membrane was incubated for 1 h with blocking solution (TBS, pH 7.6, 5% nonfat dried milk, 0.1% Tween-20) and further incubated for 1 h in blocking solution containing primary antibodies with following dilutions: myosin IIA (lot 130944001 and 135025001), 1/500; myosin IIB (lot 139019002), 1/1000; and actin, 1/5000. The mAb used against actin was obtained from Calbiochem (San Diego, CA). After washing with TBS (pH 7.6) containing 0.1% Tween-20, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ) for 1 h. Immunoreactive bands were detected by ECL Western blotting analysis reagents (Amersham Biosciences) and exposed to chemiluminescence film (Hyperfilm ECL; Amersham Biosciences).

Immunofluorescence Staining

3T3 cells were treated with antisense and scrambled oligos for myosin IIA and IIB as described above. They were fixed with 4% formaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 5 min, and stained with antibodies against nonmuscle myosin IIA, IIB, (Covance Research Products, described above) and a monoclonal anti-Golgi 58K protein from mouse that detects a peripheral Golgi membrane protein (Sigma, St. Louis, MO); rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) were used for detection. Staining patterns were analyzed by fluorescence microscope (DMRBE, Leica, Exton, PA) and SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI). Exposure time and gain level of the camera remained constant between control (scrambled oligo) and antisense-treated cells.

Analysis of Exocytosis by FM 1–43 Destaining

3T3 cells were incubated with fresh culture medium containing 4 μM FM 1–43 (Molecular Probes) overnight. Each dish was washed with 1.8 mM Ca^{2+} Ringer's solution just before the experiment. Acquisition of fluorescent images was performed using a SIT68 video camera (MTI, Michigan City, IN) linked to a Zeiss IM-35 inverted microscope (Thornwood, NY). FM 1–43 fluorescence was excited at 495 nm. Images were digitized by a Digidata 2000 AD board (Axon Instruments, Union City, CA), and were acquired at 4-s intervals by averaging four frames for each image. All processes were controlled by Axon Imaging Workbench (Axon Instruments). Cells were wounded by the same system as for the resealing assay. Changes of local fluorescent intensity were localized to the area around the wound, and the change in FM 1–43 fluorescence in a circular region around the wound site (5-μm diameter) was analyzed.

Membrane Resealing Assay

Membrane resealing was monitored by measuring emission fluorescence of the calcium sensitive dye fura-2. Fura-2 was introduced into the cells by AM-ester loading. Fura-2 AM (Molecular Probes), 10 mM in DMSO as a stock, was mixed with same volume of Pluronic F-127 (25% wt/wt in DMSO) before dilution to a final loading concentration of 5 μM in 1.8 mM Ca^{2+} Ringer's solution containing 8% FBS for 3T3 cells or in DMEM containing 10% FBS for COS-7 and CV-1 cells. Fura-2 AM was loaded at room temperature (25°C, 3T3) or 37°C (COS-7, CV-1) for 1 h and then washed with Ringer's solution containing 1.8 mM Ca^{2+}.

For tests of membrane resealing, fura-2–loaded cells were wounded with a glass needle using an Eppendorf 5242 microinjector and 5170 micromanipulator (Brinkmann Instruments, Westbury, NY) mounted on a Zeiss IM-35 inverted microscope, and fura-2 fluorescence was monitored. The time setting for wound was 0.3 s. All wound experiments were performed at 25°C. A persistent decrease of 357-nm excited fluorescent intensity (as an indicator of dye loss) together with a persistent increase of the ratio of fluorescent intensity excited by 385/357-nm light (as an indicator of increasing intracellular Ca^{2+} concentration) indicated resealing failure. A transient decrease of 357-nm excited fluorescent intensity indicated successful resealing. The interval between wound and when the fluorescent intensity reached a constant value was defined as resealing time. Because the dye leaked out completely when disruption of the plasma membrane did not reseal, loading of fura-2 at 37°C into COS-7 and CV-1 cells did not result in compartmentalization of the dye.

RESULTS

Knockdown of Nonmuscle Myosin IIA and IIB by Antisense Oligonucleotide in Swiss 3T3 Fibroblasts

To investigate if treatments with antisense oligonucleotides suppress the expression of nonmuscle myosin IIA and IIB, Swiss 3T3 fibroblasts were treated with antisense or scrambled (control) oligonucleotide, and cellular proteins were extracted. Sequence information for mRNA of rat nonmuscle myosin IIA and mouse nonmuscle myosin IIB, available from gene databanks, were used to design antisense and control oligonucleotides. Fluorescein-labeled phosphorothioate-modified oligonucleotides labeled 90% of the cells used (Figure 1A). Immunoblot analysis using antibodies against nonmuscle myosin IIA, IIB and actin showed that each antisense treatment specifically decreased the amount of each myosin protein (Figure 1B). Average optical density of immunoreactive bands decreased to 43.3% ± 6.6 (n = 3) and 45% ± 2.8 (n = 3) when cells were treated with antisense oligonucleotides for myosin IIA and IIB, respectively (Figure 1C). The amount of actin protein remained unaffected (Figure 1B). Control scrambled oligonucleotides did not suppress the expression of either myosin. These data demonstrate that the antisense oligonucleotides were acting in the expected manner, diminishing expression of nonmuscle my-

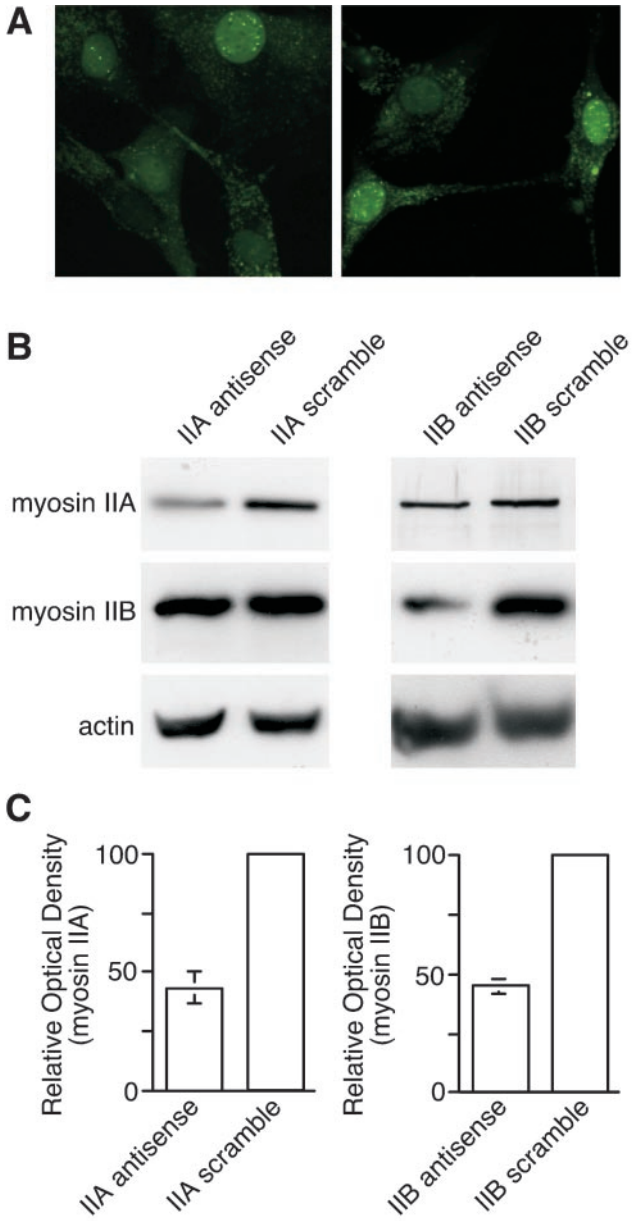

Figure 1. (A) Cellular distribution of fluorescein-conjugated antisense oligonucleotide in 3T3 cells. Cells were transfected with antisense of nonmuscle myosin IIA (left) or IIB (right) for 6 h. Cells were then rinsed and examined live under a fluorescence microscope. (B) Immunoblot analysis. 3T3 cells were transfected with antisense or scrambled oligonucleotides of nonmuscle myosin IIA and IIB. Total cellular proteins were separated by SDS-PAGE and subjected to immunoblot analysis using antibodies against nonmuscle myosin IIA, IIB, and actin. (C) Quantitative analysis of the Western blotting results. The obtained data represent mean values ± SEM (n = 3).

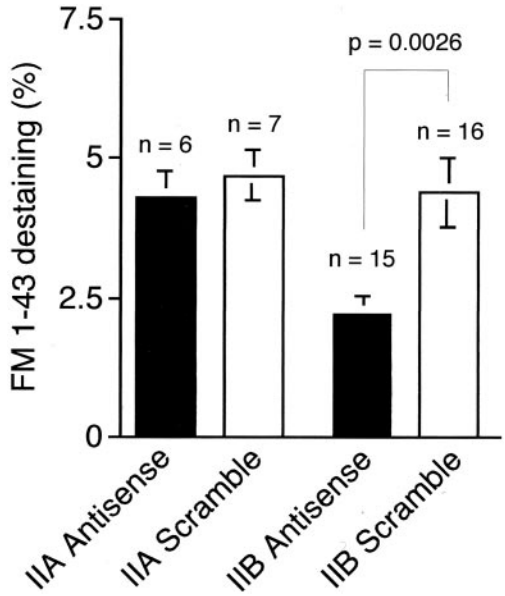

Figure 2. FM 1–43 destaining at an initial wound in cells treated with oligonucleotides. Cells were transfected with antisense or scrambled oligonucleotide of nonmuscle myosin IIA or IIB. At 36 h after the transfection, cells were loaded with FM 1–43 overnight and washed with 1.8 mM Ca^{2+} Ringer's solution. Forty-eight hours after the transfection, the local fluorescent intensity around the disruption site (5-μm diameter) was analyzed at 4-s intervals to determine the degree of destaining.

osin IIA or IIB encoded by sequence complementary to the antisense oligonucleotide.

Wound-induced Exocytosis Is Significantly Decreased by IIB Antisense Treatment But Not by IIA Antisense Treatment

We first investigated whether the treatment with antisense oligonucleotide of nonmuscle myosin IIs inhibited exocytosis after wounding. To observe exocytosis accompanying microdisruptions, the fluorescent dye FM 1–43 was preloaded by endocytosis during an overnight incubation. FM 1–43 intercalates into the outer leaflet of lipid bilayers and is much more fluorescent in hydrophobic than in hydrophilic environments (Cochilla *et al.*, 1999). When cells are incubated with the dye and later washed, dye remaining in the plasma membrane rapidly diffuses away, leaving only dye that is trapped in the endocytosed vesicles. Subsequent delivery of the labeled vesicles into the plasma membrane by exocytosis allows diffusion of the dye into the external media and results in a loss of cellular fluorescence near the wound site. Treatment with a IIB scramble antisense nucleotide gave normal levels of wound-induced FM1–3 destaining of 4.4 ± 0.61% (n = 16), whereas treatment with antisense IIB reduced destaining to 2.2 ± 0.34% (n = 15; Figure 2). Treatment with scramble and antisense to IIA showed no effect on exocytosis, scramble destaining, 4.7 ± 0.46% (n = 7); and antisense destaining 4.3 ± 0.47% (n = 6; Figure 2).

Antisense Oligonucleotide of Nonmuscle Myosin IIB Inhibits Membrane Resealing at an Initial Wound

To investigate the role of nonmuscle myosins IIA and IIB in membrane resealing, 3T3 cells were treated either with antisense oligonucleotide of nonmuscle myosin IIA or IIB. These cells were loaded with fura-2 AM, and changes of fura-2 fluorescence were monitored during wounding experiments. As described previously (Steinhardt *et al.*, 1994), a membrane disruption (arrows in Figure 3) was indicated by sharp rise in the emission ratio of fura-2 excited at 385 and 357 nm, and the loss of the dye resulted in a decrease in the fluorescent intensity excited at 357 nm. When the cell resealed, the decrease in fluorescent intensity stopped (bar in Figure 3). To compare timing of membrane resealing in

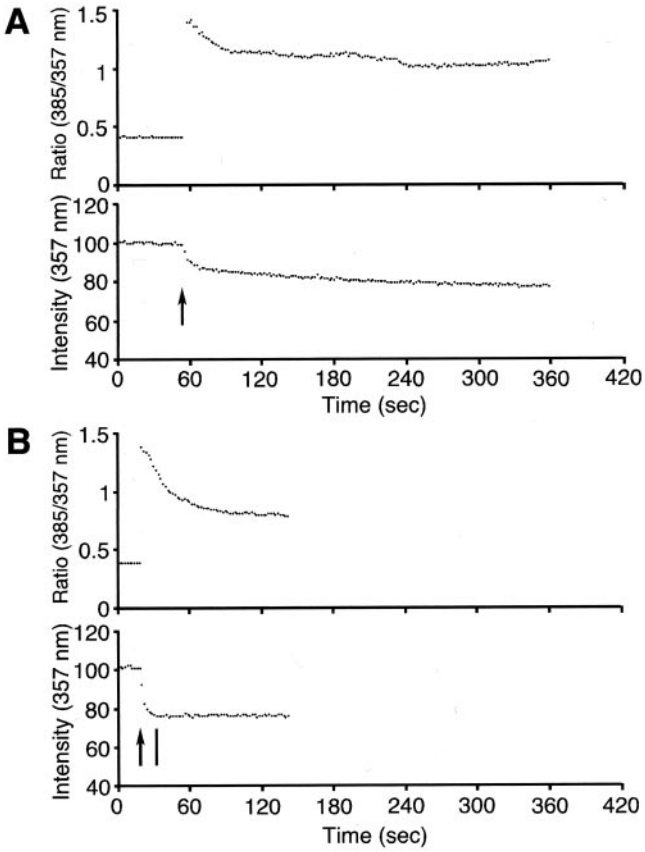

Figure 3. Effect of IIB antisense on membrane resealing. Cells were transfected with antisense (A) or scrambled (B) oligonucleotide of nonmuscle myosin IIB. Then cells were loaded with fura-2 AM 48 h after the transfection and wounded at indicated times by arrows. Bars mark the completion times of membrane resealing. Excitation wavelength, 357 and 385 nm; emission, 510 nm.

each condition, the resealing rate was defined as the inverse of the resealing time in seconds. For cells that failed to reseal, the rate was defined as zero. When the cells were treated with antisense or scrambled oligonucleotide of myosin IIA, the average resealing rate at an initial wound was 0.066 ± 0.013 (n = 30) or 0.076 ± 0.014 (n = 28), respectively (Table 1), and there was no significant difference between these values (p = 0.5983, Student's *t* test). These resealing rates were statistically identical to the value of untreated cells reported previously (Togo *et al.*, 1999, 2000). Therefore, these results indicate that membrane resealing at the initial wound was not affected by the treatments with IIA antisense.

When the cells were treated with antisense oligonucleotide of myosin IIB (Figure 3A), the percentage of successful membrane resealing was 46.7%, and the average resealing rate at these initial wounds was 0.029 ± 0.011 (n = 30; Table 1). On the other hand, the percentage of resealing was 84.2%, and the resealing rate was 0.069 ± 0.011 (n = 19) when the cells were treated with scrambled oligonucleotide (Table 1; Figure 3B). This resealing rate for scrambled oligonucleotide was statistically identical to the value of untreated cells reported previously (Togo *et al.*, 1999, 2000). These results indicate that nonmuscle myosin IIB is needed for membrane resealing at initial wounds in 3T3 fibroblasts.

Table 1. Effect of antisense oligonucleotide of nonmuscle myosin IIA and IIB on membrane resealing in Swiss 3T3 fibroblasts

	Initial wound		Second wound	
Treatment	Resealing rate	n	Resealing rate	n
IIA antisense	0.066 ± 0.013	30	0.051 ± 0.016	25
IIA scrambled	0.076 ± 0.014	28	0.13 ± 0.022*	21
IIB antisense	0.029 ± 0.011	30	—	—
IIB scrambled	0.069 ± 0.011	19	—	—

Membrane resealing was monitored by photometric measurement of fura-2 fluorescence and subsequent visual inspection. The resealing rate was defined as the inverse of the resealing time in seconds. For cells that failed to reseal, the rate was defined as zero. Values are mean ± SE. Student *t* test: IIA antisense initial vs IIA scramble initial, p = 0.5983; IIB antisense initial vs IIB scramble initial, p = 0.0176; IIA antisense initial vs IIA antisense second, p = 0.4653; and IIA scramble initial vs IIA scramble second, p = 0.0207.

Antisense Oligonucleotide of Nonmuscle Myosin IIA Inhibits Facilitation of Membrane Resealing But Not the Resealing of an Initial Wound

To test the role of nonmuscle myosin IIA in facilitated response, 3T3 cells were treated with antisense or scrambled oligonucleotides of nonmuscle myosin IIA and wounded twice. Contrary to the initial wound, the facilitated resealings at second wounds were suppressed in the majority of the antisense-treated cells; resealings at the second wounds were same or slower than at the initial wounds (Figure 4, Table 1). A typical fura-2 recording example is shown in Figure 4A. However, scrambled oligonucleotide did not have any inhibitory effect on the facilitated response of membrane resealing (Figure 4B). Figure 4C summarizes the results of the double-wound experiments. The majority of data points from antisense-treated cells fell below the diagonal, indicating that the second resealing rate was lower than the first rate. Average ratio of first and second resealing rate (2nd/1st) of control (scrambled oligonucleotides-treated) cells was 1.97 ± 0.47 (n = 21), whereas the ratio of antisense-treated cells was 0.80 ± 0.25 (n = 25; Figure 4D). These results indicate that nonmuscle myosin IIA is required for facilitation of membrane resealing at repeated wounds.

COS-7 Cells Do Not Show a Facilitated Response during Repeated Wounds

COS-7, a monkey kidney cell line, was established from the CV-1 cell line (Gluzman, 1981) and lacks nonmuscle myosin IIA (Tullio *et al.*, 1997). Western blotting analysis confirmed this, whereas CV-1 cells do have nonmuscle myosin IIA (Figure 5A). To further check whether nonmuscle myosin IIA is required for facilitated membrane resealing at repeated wounds, fura-2–loaded COS-7 and CV-1 cells were wounded twice, and initial and second resealing rates were compared. The results of double-wound experiments are summarized in Figure 5, B and C, where initial rates are plotted on the X axis and second rates on the *Y*. Points above the diagonal graphically show faciliatation. The majority of data points from COS-7 cells were scattered around the diagonal (Figure 5B), and there was no significant difference in the rates of resealing to first and second wounds, 0.08 ± 0.01 (n = 17) and 0.08 ± 0.01 (n = 17), respectively. On the other hand, comparisons of the resealing rates to first and second wounds in CV-1 cells, initial 0.08 ± 0.02 (n = 13); second

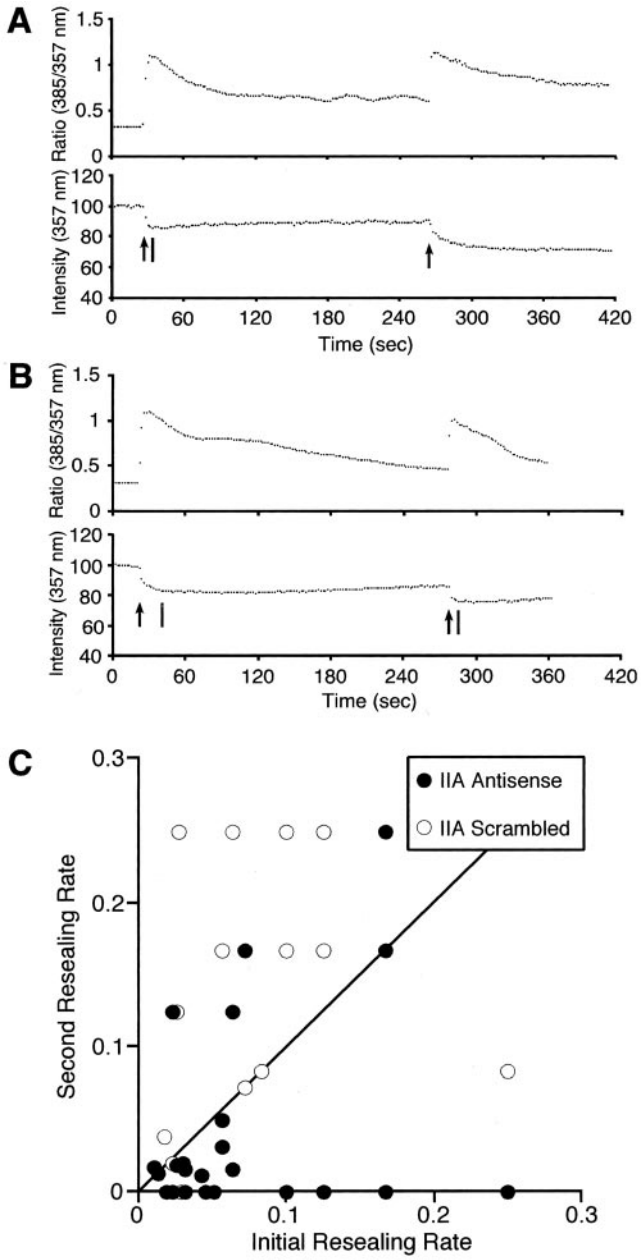

Figure 4. (A, B) Fura-2 recordings from the cells treated with antisense and scrambled oligonucleotide of nonmuscle myosin IIA during double-wound experiments. Cells were transfected either with antisense (A) or scrambled (B) oligonucleotide of nonmuscle myosin IIA. Then cells were loaded with fura-2 AM 48 h after the transfection and wounded twice at indicated times by arrows. Bars indicate the completion time of membrane resealing. Excitation wavelength, 357 and 385 nm; emission, 510 nm. (C) Resealing rates to first and second wound. Each point represents one experiment. The resealing rate was defined as the inverse of the resealing time in seconds. For cells that failed to reseal, the rate was defined as zero. Points above diagonal show facilitation at second wounds.

0.15 ± 0.02 (n = 13), clearly showed facilitation (Figure 5C). These results indicate that COS-7 cells do not have the facilitated response of membrane resealing and suggest that the facilitated resealing at repeated wounds requires nonmuscle myosin IIA.

S91 Cells Show Normal Membrane Resealing and Facilitated Response

To determine whether myosin Va is involved in membrane resealing, we investigated the characteristics of membrane resealing in S91 melanoma cells. This cell line was originally derived from a tumor carried by a mouse from the inbred strain DBA/2J, which is homozygous d^v/d^v (Jenkins *et al.*, 1981) and is lacking myosin Va (Wu *et al.*, 1997).

When the cells were wounded, the average resealing rate at an initial wound was 0.069 ± 0.011 (n = 22), and the percentage of membrane resealing was 81.8%. This indicated that membrane resealing at the initial wound was normal in S91 cells.

We next investigated the resealing rates to repeated wounds in S91 cells. S91 cells showed clear evidence of facilitated responses: initial rate 0.08 ± 0.01 (n = 18), second rate 0.13 ± 0.02 (n = 18). The majority of data points plotted above the diagonal, indicating that the second resealing rate was faster than the first rate (Figure 5D). These results suggest that myosin Va is not involved in vesicle generation, transport, and exocytosis during membrane resealing in S91 cells.

Finally, Figure 5E summarizes the significant of differences between first and second rates of resealing in all three cell lines, confirming a role for myosin IIA in facilitated cell membrane responses and ruling out a role for myosin Va, at least in S91 cells.

Distribution of Nonmuscle Myosins IIA and IIB

To investigate the distributions of nonmuscle myosin IIA and IIB in 3T3 cells, cells were treated with antisense and scrambled oligo nucleotides for myosin IIA and IIB, and immunostaining was carried out with the antibodies to myosin IIA and IIB together with anti-Golgi 58K protein antibody that recognizes the Golgi membrane 58-kDa protein (Bloom and Brashear, 1989). As shown in Figure 6, A and A′, myosin IIA colocalizes with the 58-kDa Golgi peripheral membrane protein in an asymmetric perinuclear manner when cells were treated scrambled oligonucleotide of myosin IIA. When cells were treated with antisense oligonucleotide of myosin IIA, the amount of myosin IIA at Golgi region was decreased (Figure 6, B and B′). These results suggest that myosin IIA is enriched in the Golgi apparatus, which is consistent with the requirement for IIA in the facilitated rates of membrane repair and the need for new vesicles for repair at second wounds. Myosin IIB was abundant in the cell periphery, and stress fibers when cells were treated with scrambled oligonucleotide of myosin IIB (Figure 6C). When cells were treated with antisense oligonucleotide of myosin IIB, the amount of myosin IIB was decreased (Figure 6D), consistent with a role in trafficking just beneath the plasma membrane during exocytosis. There were no signs of "purse string" distributions of myosin IIB (Bement *et al.*, 1999) when cells were rapidly fixed after wounding (unpublished data).

DISCUSSION

Role of Nonmuscle Myosin IIB in Membrane Resealing and Exocytosis

We have previously shown that Ca^{2+}-dependent exocytosis is essential for successful repair of disrupted cell membrane (Steinhardt *et al.*, 1994; Bi *et al.*, 1995, 1997; Togo *et al.*, 1999). In the present study, we showed that both membrane resealing and exocytosis triggered by calcium influx at the wound was suppressed by the treatment with antisense

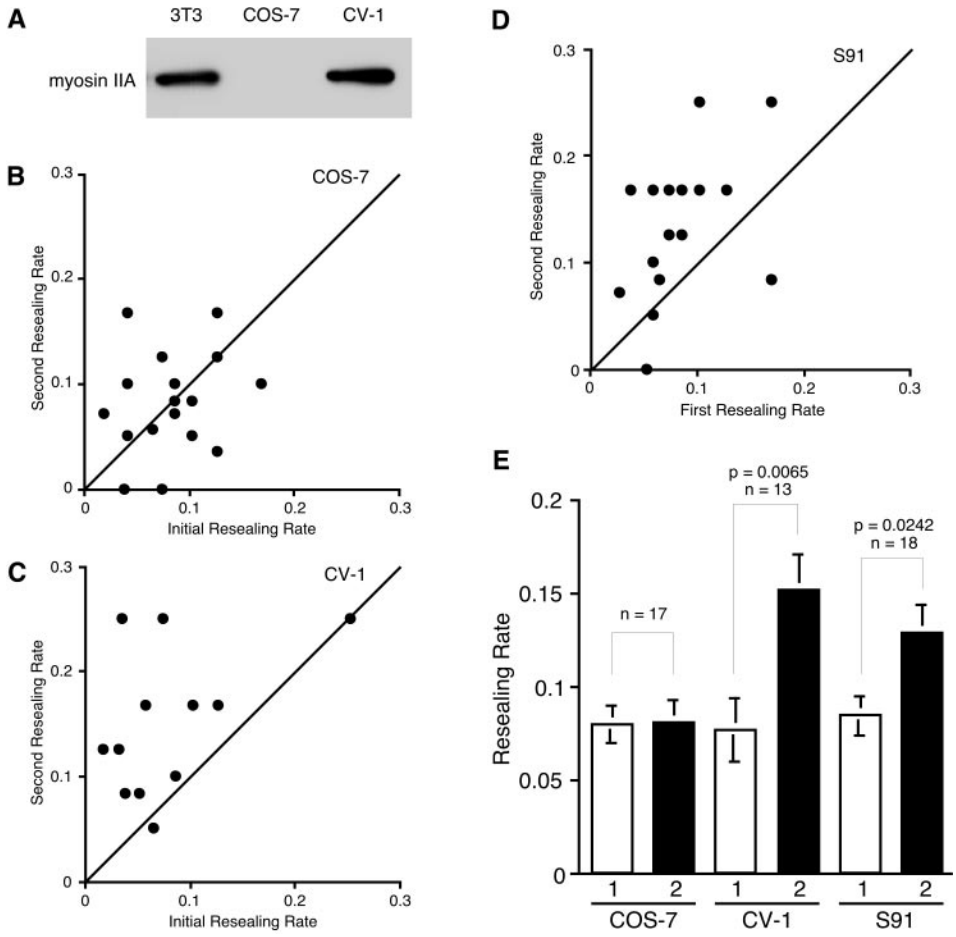

Figure 5. Resealing in cell lines lacking myosins. (A) Immunoblot analysis. Total cellular proteins of 3T3, COS-7, and CV-1 cells were separated by SDS-PAGE and subjected to immunoblot analysis using an antibody against nonmuscle myosin IIA. (B–D) Resealing rates to first and second wound. Fura-2–loaded COS-7, CV-1, and S91 cells were wounded twice and resealing rates were compared. Each point represents one experiment. The resealing rate was defined as the inverse of the resealing time in seconds. For cells that failed to reseal, the rate was defined as zero. Points above diagonal show facilitation at second wound. (E) Average resealing rates after a initial and second wound. Values are mean ± SE.

oligonucleotide of nonmuscle myosin IIB (Figures 1–3; Table 1). Myosin IIB was abundant in the cell periphery, which is consistent with a role in the transport of vesicles through the actin cortex to the plasma membrane near the disrupted site. Alternately, myosin IIB might be essential to an actin rearrangement permissive for exocytosis (Trifaro *et al.,* 2002; Rose *et al.*, 2003). We saw no evidence of myosin IIB "purse-string" formation. Myosin purse-strings have been shown to play a role in healing large wounds in *Xenopus* oocytes (Bement *et al.*, 1999). There are previous suggestions of myosin II mediated vesicle transport. 1) Vesicles move beneath the plasma membrane using myosin motor activity in PC-12 cells (Lang *et al.,* 2000). 2) Myosin inhibitor, butanedione monoxime (BDM), blocks the step of vesicle recruitment just before docking for Ca^{2+}-regulated exocytosis in membrane resealing (Bi *et al.,* 1997). 3) Myosin II is purified with vesicles (Costa *et al.,* 1999; Miller and Sheetz, 2000). 4) It has been shown that myosin II can carry vesicles along actin filament in vitro (Sandberg *et al.,* 2000). 5) Injection of polyclonal antibody against nonmuscle myosin IIB inhibited neurotransmitter release (Mochida *et al.,* 1994; Mochida, 1995).

However, most evidence implicates myosin class V for a role in vesicle transport (Reck-Peterson *et al.,* 2000; Tuxworth and Titus, 2000; Ohyama *et al.,* 2001; Stachelek *et al.,* 2001; Brown *et al.,* 2002; Langford, 2002; Schott *et al.,* 2002; Rose *et al.,* 2003). The evidence for a role for myosin V in particular instances does not exclude a possible role for myosin type II. In fact myosin V and II can colocalize to the same vesicle (Costa *et al.,* 1999; Miller and Sheetz, 2000). More recently myosin II has been found associated with a substantial fraction of axoplasmic organelles and is postulated to play a transport role for those organelles in axons (DeGiorgis *et al.,* 2002), a finding that supports earlier results from antibodies (Sandberg *et al.,* 2000). Likewise, our finding that myosin Va is not required to reseal membrane disruptions in S91 melanoma cells does not rule out that another uncharacterized myosin type V could be needed for resealing in these cells. We have no evidence on myosin Vb and the new myosin class Vc that has been recently characterized in epithelial cells (Rodriguez and Cheney, 2002).

The Role of Nonmuscle Myosin IIA in the Facilitated Response of Membrane Resealing and Vesicle Generation at Golgi

Our present study demonstrated clearly that nonmuscle myosin IIA was needed for the facilitated rates of membrane resealing of repeated wounds at the same site. Knockdown of nonmuscle myosin IIA by treatment with antisense oligonucleotide inhibited facilitation in 3T3 fibroblasts (Figures 1 and 4). COS-7 cells, which do not have nonmuscle myosin IIA, did not show facilitated response at repeated membrane disruptions, whereas facilitation of CV-1 cells, from which they were derived, was normal (Figure 5).

We have previously shown that Gö-6976, a specific inhibitor for Ca^{2+}-dependent PKC isozymes (Martiny-Baron *et al.,* 1993), and brefeldin A (BFA), a fungal metabolite that inhibits binding of ADP-ribosylation factor to the Golgi (Klausner *et al.,* 1992), inhibits facilitated membrane resealing at repeated wounds in 3T3 cells (Togo *et al.,* 1999). This

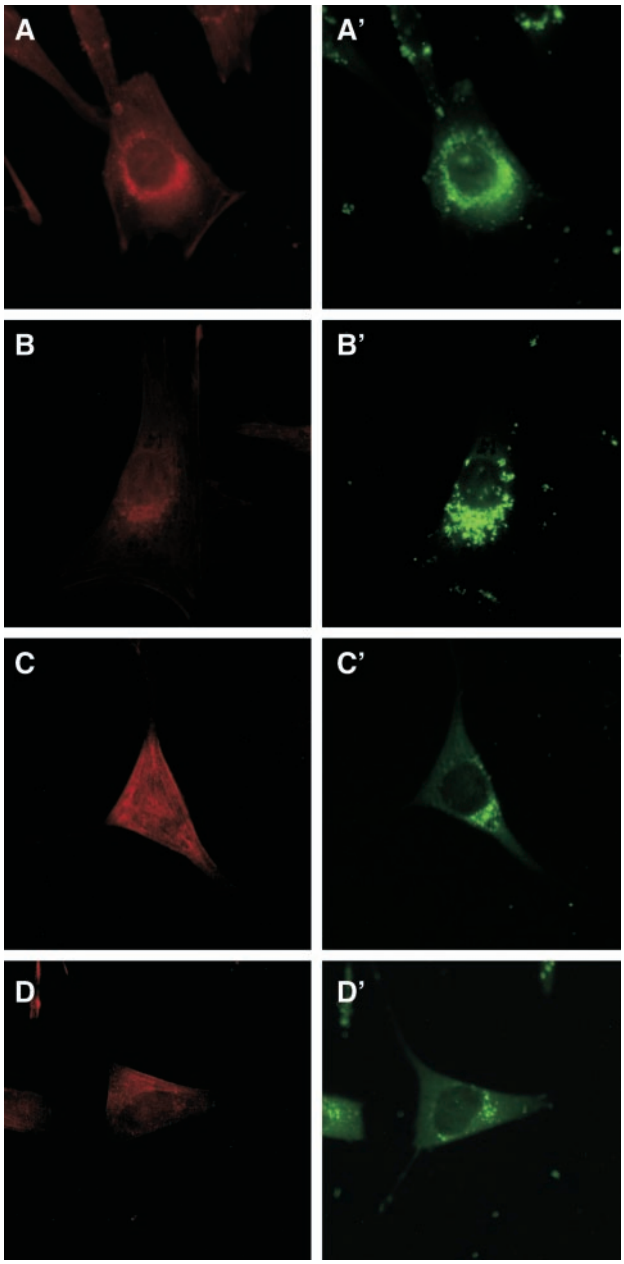

Figure 6. Localization of nonmuscle myosin IIA and IIB in 3T3 cells. 3T3 fibroblasts were treated either with scrambled (A and A′) or antisense oligonucleotide (B and B′) of myosin IIA, or with scrambled (C and C′) or antisense oligonucleotide (D and D′) of myosin IIB, and double-labeled by immunofluorescence with antibodies to myosin IIA (A and B), myosin IIB (C and D), and Golgi 58K protein (A′–D′).

inhibition of facilitated resealing and our previous studies of FM 1–43 destaining assay suggested that facilitation of membrane resealing requires exocytosis of a new vesicle pool generated via a PKC-dependent and BFA-sensitive process (Togo *et al.*, 1999).

Therefore, both a myosin IIA-dependent and a BFA-sensitive process are required for facilitation of the membrane resealing at repeated wounds. We propose that nonmuscle myosin IIA is involved in vesicle generation at TGN at facilitated response of the membrane resealing. Several lines of evidence suggest this possibility. First, it has been reported that the p200/myosin II, analogous to nonmuscle myosin IIA heavy chain, is involved in vesicle generation at TGN in many cells (Narula *et al.*, 1992; Narula and Stow, 1995; Ikonen *et al.*, 1997; Musch *et al.*, 1997; Heimann *et al.*, 1999). Second, binding of myosin IIA to the Golgi is known to be BFA sensitive (Ikonen *et al.*, 1997). Third, myosin II was localized by immunofluorescence in a variety of cultured cells using an antiserum that recognizes nonmuscle myosin II. In NRK cells, myosin II, detected by an antibody against chick intestinal brush border nonmuscle myosin II, localizes at the Golgi (Ikonen *et al.*, 1997). In 3T3 cells, myosin IIA localizes at the Golgi (Heimann *et al.*, 1999; this study).

In vitro analysis suggests that p200/myosin II is involved in a part of constitutive basolateral transport (Narula *et al.*, 1992; Narula and Stow, 1995; Ikonen *et al.*, 1997; Musch *et al.*, 1997; Heimann *et al.*, 1999). Therefore, cells may use this constitutive pathway or regulated secretion pathway to refill the vesicle pool that is depleted at initial wounds. TGN-derived vesicles can undergo Ca^{2+}-regulated exocytosis (Maletic-Savatic and Malinow, 1998). There is a conflicting report proposing that p200/myosin II is not an essential participant in the vesicle budding reaction of constitutive secretory pathways and that the p200 antibody used in functional studies cross reacted with coatomer (Simon *et al.*, 1998). This finding calls into question the functional evidence for myosin IIA at the TGN. However, using a different antibody we could confirm its concentration at the TGN. This confirmation of localization, together with our finding of an essential role for myosin IIA in BFA-sensitive facilitated membrane repair, suggests that myosin IIA does have a functional role in membrane trafficking at the Golgi.

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