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S100A10 is required for the organization of actin stress fibers and promotion of cell spreading

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Abstract Dynamic remodeling of the actin cytoskeleton is crucial for biological processes such as cell migration and cell spreading. S100A10 is a member of the S100 protein family and is involved in intracellular trafficking and cell migration. In this study, we examined the role of S100A10 in actin cytoskeletal organization and cell spreading. Depletion of S100A10 induced disruption of stress fiber formation and delay in cell spreading. Rac1 activation during spreading was suppressed by S100A10 knockdown, and exogenous expression of active Rac1 restored the ability of cells to spread in the absence of S100A10. Our results demonstrate the crucial role of S100A10 in actin dynamics promoting cell spreading via Rac1 activation.

Keywords Cell spreading · Rac1 · S100A10 · Actin cytoskeleton

Introduction

Remodeling of the actin cytoskeleton is critical for fundamental biological functions such as cell migration and cell spreading. During cell migration, membrane protrusions called lamellipodia originate in the direction of movement [1]. Lamellipodia consist of dynamically reorganized branched or unbranched actin filaments and generate the traction forces that are necessary for cell migration [2, 3]. Robust formation of membrane protrusions along the periphery of cells is also observed during cell spreading. Upon their adhesion to the extracellular matrix, cells form focal complexes to attach to the matrix and then initiate the formation of lamellipodia to facilitate spread. Accumulating evidence has shown that a small GTPase, Rac1, plays a crucial role in the dynamic reorganization of the actin cytoskeleton to promote membrane extension [4]. A number of pathways that activate Rac1 have been identified. We previously reported that the ILK-PINCH1-Rsu1 pathway was required for the activation of Rac1 during spreading [5]. However, it is still unknown whether additional factors are also involved in Rac1 activation and subsequent actin cytoskeleton remodeling for cell migration and spreading.

S100A10, also known as p11, is a member of the S100 protein family, which generally modulates the functions of target proteins in response to intracellular Ca^{2+} signals [6]. S100 proteins are composed of an N-terminal and a C-terminal EF-hand Ca^{2+}-binding motif separated by a linker region; and through their interactions with numerous proteins are involved in a variety of cellular functions such as proliferation, differentiation, apoptosis, energy metabolism, and migration [7–10]. In contrast to other S100 proteins, S100A10 is Ca^{2+}-insensitive because of the amino acid replacement in the Ca^{2+}-binding loops and thus remains in a permanently active state [11, 12]. S100A10 is found to be higher in some cancer tissues and is thought to be involved in the promotion of cell invasion and migration [13–17]. The majority of S100A10 remains tightly associated with the membrane-binding protein annexin A2 (ANX2) [18, 19]. The ANX2-S100A10 complex is a heterotetrameric complex in which a central S100A10 dimer interacts with two ANX2

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chains [20]. ANX2 has been shown to be a critical protein involved in membrane transport events such as endocytosis and exocytosis [21, 22]. ANX2 is also an actin-binding protein and is capable of bundling actin filaments [23, 24]. Previous studies have shown that ANX2 is required for actin dynamics to promote cell migration and spreading [25], but whether S100A10 is necessary for dynamic remodeling of the actin cytoskeleton still remains elusive.

In order to gain more insight into the role of S100A10 in actin dynamics, we used RNA interference to deplete S100A10 in cells. In this study, we show that S100A10 is required for actin stress fiber formation and Rac1 activation to promote cell spreading.

Materials and methods

Cells, antibodies, and DNA constructs

HeLa and MDA-MB-231 cells were maintained in DMEM supplemented with 10 % FBS. Antibodies for S100A10 and β-actin were obtained from Sigma (St. Louis, MO), and anti-Rac1 and anti-Rho antibodies were purchased from BD Biosciences (San Jose, CA). Plasmids encoding wild type and active Rac1 were generously provided by Dr. Kaibuchi (Department of Cell Pharmacology, Nagoya University Graduate School of Medicine).

Generation of cell lines

293T cells were transfected with pQCXIH (Clontech; Mountain View, CA) vector encoding either wild type or active Rac1 together with pVPack-GP and pVPack-Ampho vector (Stratagene, Tokyo, Japan). After 48 h, the cell supernatant was added to HeLa cells in the presence of 2 ug/ml of polybrene (Sigma). Infected cells were selected for hygromycin resistance, and polyclonal cell lines that constitutively expressed each gene were generated.

Immunoblot analysis

Cells were lysed with Laemmli sample buffer (20 % glycerol, 135 mM Tris–HCl pH 6.8, 4 % SDS, 10 % 2-mercaptoethanol, 0.003 % BPB) and boiled for 5 min. The protein concentration of each lysate was measured using the RC-DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein from each cell lysate were separated on SDS–polyacrylamide electrophoresis (SDS–PAGE) gels and transferred onto PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 1 % nonfat milk, incubated with each primary antibody for 1 h, washed with TBS-T buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05 % Tween 20), and incubated with the secondary antibodies. The proteins were visualized using enhanced chemiluminescence (GE Healthcare BioSciences, Uppsala, Sweden).

siRNA transfection

The sequences of the siRNAs used to knock down S100 A10 were 5′-CCUCACCAUUGCAUGCAAUTT-3′ (siRNA#1) and 5′-GAGAUGGCAAAGUGGGCUUTT-3′ (siRNA#2). The control siRNA sequence targeting the luciferase gene was 5′-CU UACGCUGAGUACUUCGATT-3′. siRNAs were obtained from Sigma. Cells were transfected with 20 nM of siRNA using Lipofectamine RNAiMAX (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions.

Rho and Rac activity assay

Cells were lysed with pull-down lysis buffer [25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 % glycerol, 1 % NP40, 5 mM $MgCl_2$, protease inhibitor cocktail (Roche, Basel, Switzerland), and 1 mM PMSF] and incubated with GST-PAK-PBD or GST-Rhotekin-RBD protein bound to glutathione-agarose beads (Sigma) for 1 h at 4 °C. The beads were washed four times with pull-down buffer and then subjected to western blot analysis. Total protein was detected by immunoblotting whole cell lysates.

Cell spreading assay

Cells were seeded at a density of 2.0×10^4 cells per well in a 48-well plate coated with fibronectin and fixed 30 or 60 min later. Spread and non-spread cells were counted in five randomly selected fields. Non-spread cells were defined as small, round cells with few or no membrane protrusions, whereas spread cells were defined as large cells with extensive visible lamellipodia. The data are presented as the average of results from three independent experiments.

Results

In order to gain further insight into the role of S100A10 in actin cytoskeleton organization, we used two different siRNAs to deplete S100A10 in cells. HeLa cells were transfected with control or S100A10 siRNAs, and 72 h later the cells were lysed and expression of S100A10 was determined. Both siRNAs targeting S100A10 efficiently suppressed its expression (Fig. 1a). Interestingly, S100A10 knockdown cells became more round compared to control siRNA-transfected cells (Fig. 1b). Using these siRNAs, we examined the effects of S100A10 depletion on actin

cytoskeleton organization. Cells cultured on fibronectin-coated glass coverslips were transfected with either control or S100A10 siRNAs, and 72 h later were fixed and immunostained for F-actin using rhodamine-conjugated phalloidin. Actin stress fiber organization was found to be disrupted in the S100A10-knockdown cells (Fig. 1c). Since the formation of focal adhesion is dependent on actin stress fiber formation, we also immunostained cells for vinculin, which is a major component of focal adhesions. Consistent with the disruption of stress fiber formation, disorganization of focal adhesions was evident in the absence of S100A10 expression (Fig. 1c). These results suggest that S100A10 is required for the organization of stress fibers and the formation of focal adhesions. Next, we determined whether S100A10 knockdown diminished the activity of RhoA, a small GTPase essential for stress fiber formation [26]. HeLa cells transfected with control or S100A10 siRNA were lysed and affinity precipitated with GST-Rhotekin-RBD to determine the amount of active RhoA in cells. As shown in Fig. 1d, the activity of RhoA was not suppressed by the depletion of S100A10.

Dynamic remodeling of actin organization is crucial for the promotion of cell spreading. We tested whether cell spreading was delayed in the absence of S100A10. HeLa cells transfected with siRNAs were resuspended in serum-free medium and seeded onto a fibronectin-coated surface. At 30 or 60 min post-seeding, the cells were fixed, and the ratio of spread cells was evaluated. Approximately 50 % of control siRNA-transfected cells were spread 30 min after seeding, whereas only 15 % of the S100A10-depleted cells showed spread morphology (Fig. 2). A significant difference in the ratio of spread cells between control and S100A10 siRNA-transfected cells was also observed 60 min after seeding (Fig. 2). These results suggest that S100A10 is required for the promotion of cell spreading.

Previous studies have shown that activation of the small GTPase, Rac1, is essential for the promotion of cell spreading [27, 28]. We determined the activity of Rac1 in control and S100A10 siRNA-transfected cells during spreading. siRNA-transfected cells were resuspended and seeded onto a fibronectin-coated surface, and 30 min later the cells were lysed and affinity precipitated with GST-PAK-PBD bound to glutathione beads. In this assay, the active form of Rac1 is preferentially affinity precipitated. During cell spreading, the amount of active Rac1 was significantly lower in S100A10-knockdown cells compared to control cells (Fig. 3a). We next tested whether exogenous expression of active Rac1 could overcome the spreading defect induced by S100A10 depletion. HeLa cells constitutively expressing either wild type (WT-Rac)

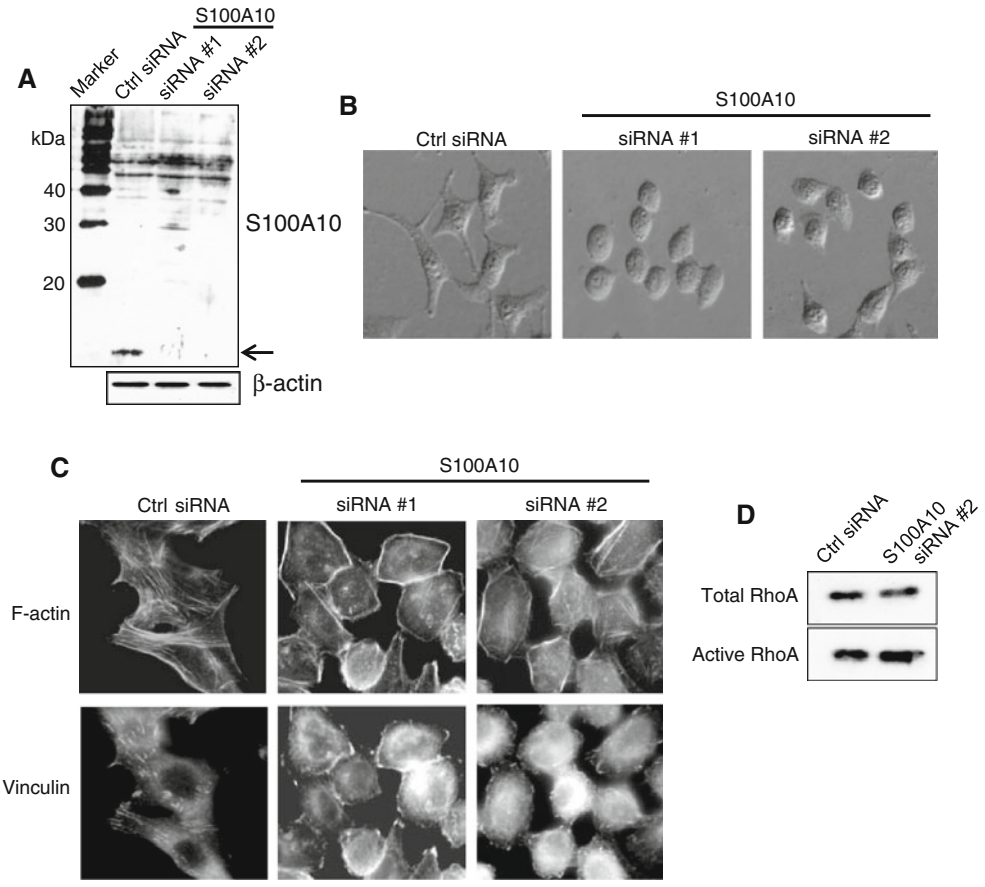

Fig. 1 Depletion of S100A10 disrupts actin stress fiber formation. **a** HeLa cells were transfected with the indicated siRNAs, and cells were lysed and immunoblotted with anti-S100A10 antibody 72 h later. The *arrow* indicates S100A10. **b** HeLa cells were transfected with the indicated siRNAs, and pictures were taken to visualize cellular morphology 72 h later. **c** HeLa cells cultured on fibronectin-coated glass coverslips were transfected with the indicated siRNAs. At 72 h post-transfection, cells were fixed and immunostained with rhodamine-conjugated phalloidin and anti-vinculin antibody. **d** HeLa cells were transfected with the indicated siRNAs, and cells were lysed and affinity precipitated by GST-Rhotekin-RBD bound to glutathione-agarose beads 72 h later. The immunoprecipitates were blotted with anti-RhoA antibody

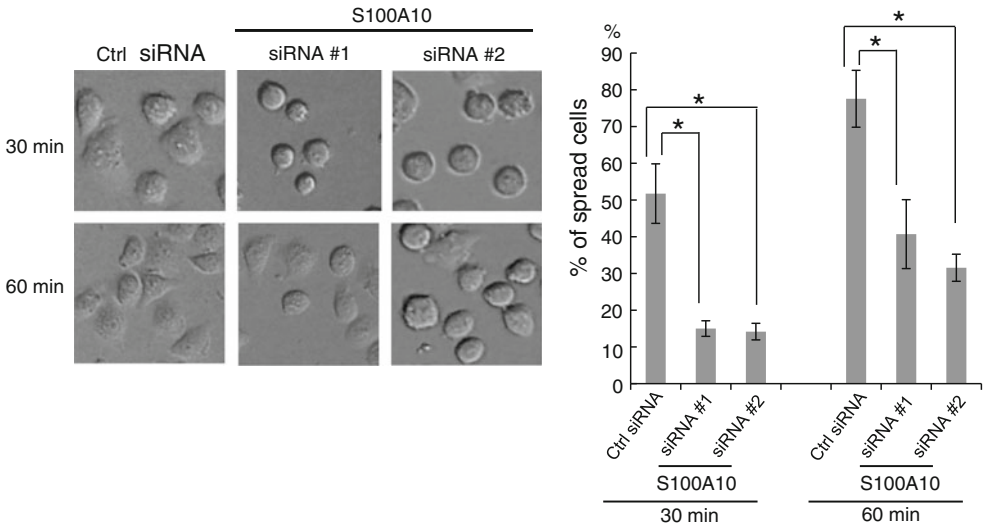

Fig. 2 S100A10 knockdown suppresses cell spreading. siRNA-transfected HeLa cells were resuspended and seeded onto the fibronectin-coated surface. At 30 and 60 min post-seeding, the cells were fixed and pictures were taken. Representative pictures are shown. Spread and non-spread cells were counted in five randomly selected fields and three independent experiments were performed. The *graph* shows the ratio of spread cells (mean ± SD; $*P < 0.05$)

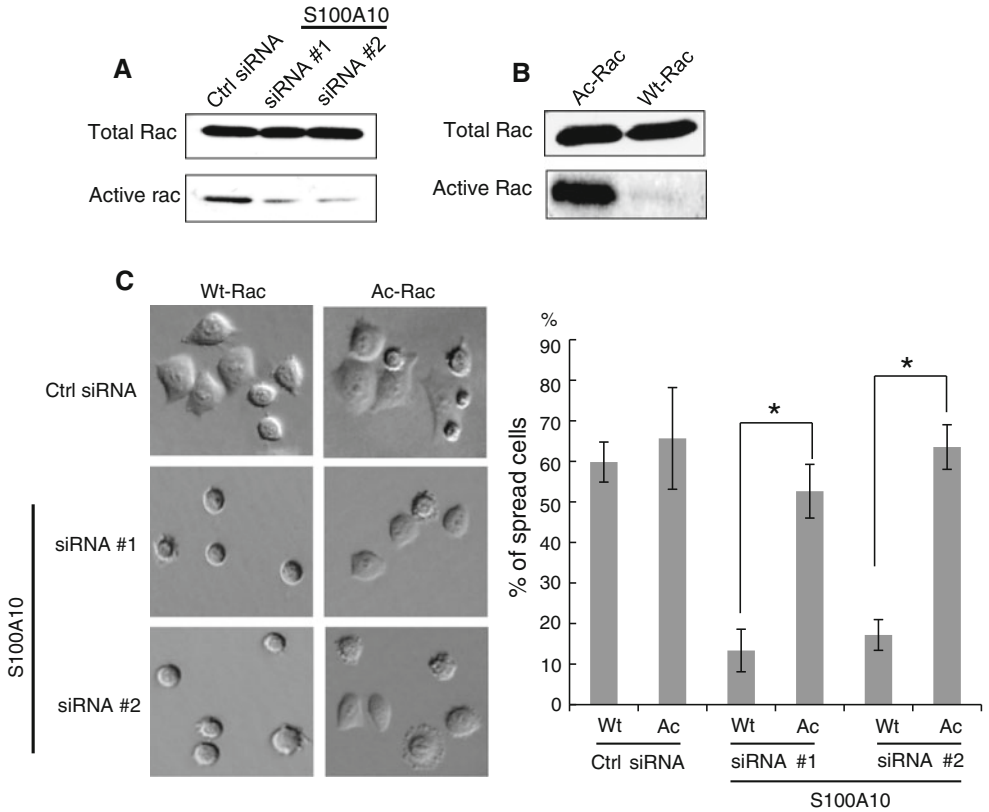

Fig. 3 S100A10 regulates Rac1 activity during cell spreading. **a** siRNA-transfected HeLa cells were resuspended and seeded onto the fibronectin-coated surface. After 30 min, the cells were lysed and affinity precipitated by GST-PAK-PBD bound to glutathione-agarose beads. The immunoprecipitates were blotted with anti-Rac1 antibody. **b** HeLa cells constitutively expressing wild type (Wt-Rac) or active Rac1 (Ac-Rac) were generated by retrovirus infection. Levels of active Rac1 were measured by affinity precipitation using GST-PAK-PBD. **c** Wt-Rac and Ac-Rac cells were transfected with the indicated siRNAs, and cells were resuspended and seeded onto the fibronectin-coated surface 72 h later. After 30 and 60 min, the cells were fixed and subjected to imaging. Representative images are shown. Spread and non-spread cells were counted in five randomly selected fields and three independent experiments were performed. The *graph* shows the ratio of spread cells (mean ± SD; $*P < 0.05$)

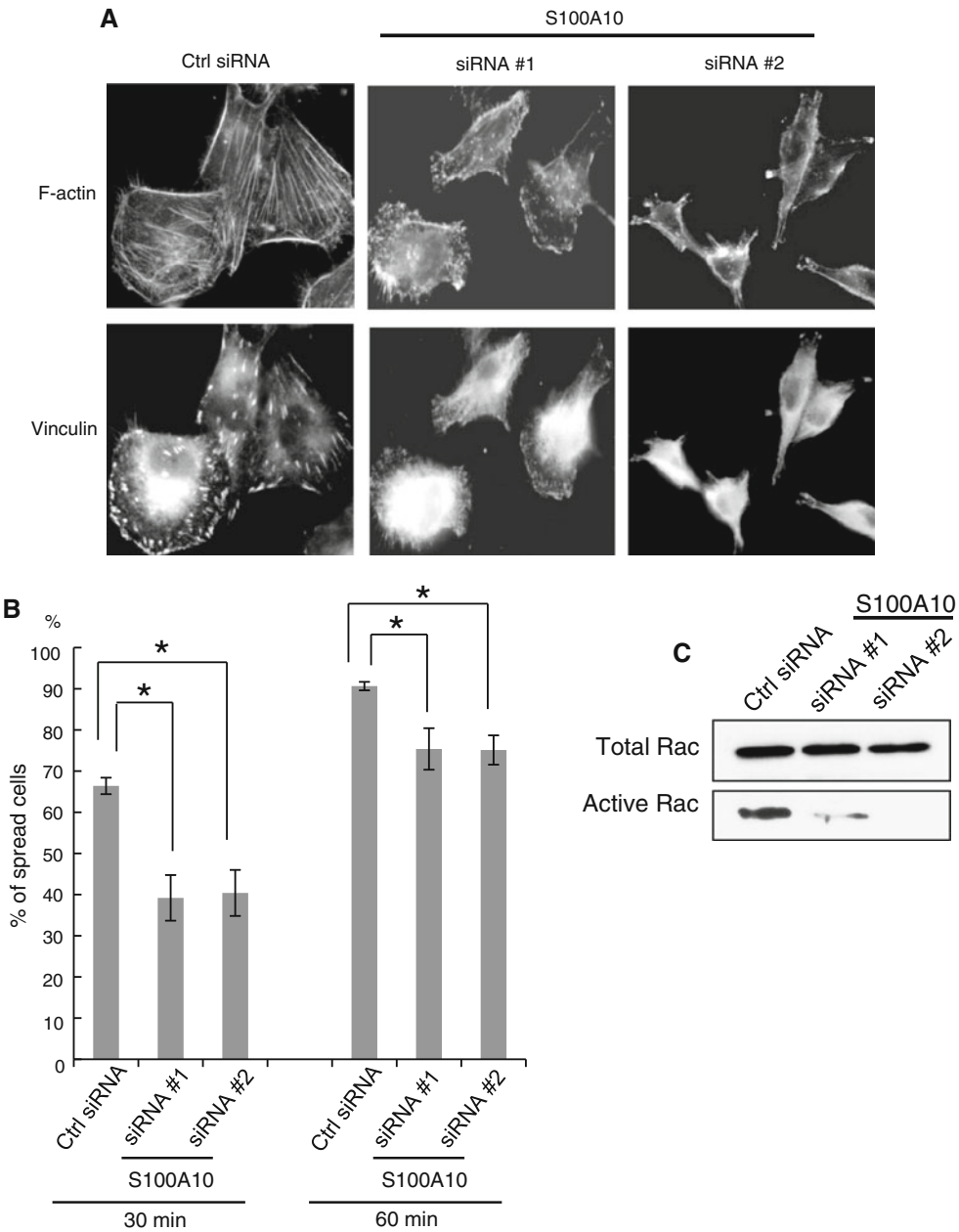

Fig. 4 S100A10 regulates stress fiber formation and spreading in MDA-MB-231 cells. **a** MDA-MB-231 cells cultured on fibronectin-coated surface were transfected with the indicated siRNAs. After 72 h, the cells were fixed and immunostained with rhodamine-conjugated phalloidin and anti-vinculin antibody. **b** siRNA-transfected MDA-MB-231 cells were seeded onto the fibronectin-coated surface and fixed 30 min and 60 min later. Spread and non-spread cells were counted in five randomly selected fields and three independent experiments were performed. The *graph* shows the ratio of spread cells (mean ± SD; $*P < 0.05$). **c** siRNA-transfected MDA-MB-231 cells were suspended and seeded onto the fibronectin-coated surface. After 30 min, the cells were lysed and affinity precipitated using GST-PAK-PBD bound to glutathione-agarose beads. The immunoprecipitates were blotted with anti-Rac1 antibody

or active Rac (Ac-Rac) were generated by retrovirus infection. Results from the pull-down assay clearly showed an increase in active Rac1 levels in Ac-Rac cells compared to WT-Rac cells (Fig. 3b). These cells were transfected with either control or S100A10 siRNAs, and cell spreading was evaluated 72 h later. As shown in Fig. 3c, the expression of active Rac1 significantly restored ability of cells to spread. These results indicate that S100A10 controls cell spreading via Rac1 activation.

In order to further confirm the requirement of S100A10 for cell spreading, we used another cell line, MDA-MB-231, in our assays. As observed in HeLa cells, silencing of S100A10 induced the disruption of stress fiber and focal adhesion formation in MDA-MB-231 cells (Fig. 4a). We also examined cell spreading in the absence of S100A10. As shown in Fig. 4b, cell spreading was significantly delayed upon S100A10 knockdown. In addition, Rac1 activation during cell spreading was suppressed by S100A10 depletion (Fig. 4c). These results further confirm the crucial role of S100A10 in actin cytoskeleton organization and cell spreading.

Discussion

In this study, we showed that depletion of S100A10 using two different siRNAs induced the disruption of actin stress fiber and focal adhesion formation in HeLa and MDA

-MB-231 cells. Actin organization is regulated by a diverse set of signaling proteins. One of the major players is a small GTPase, RhoA [26]. Activated RhoA promotes the activation of downstream effectors such as ROCK and induces actin filament formation [29]. Inhibition of RhoA activation is known to induce disorganization of stress fiber formation. We tested whether the disruption of stress fiber formation by S100A10 depletion was mediated by RhoA inactivation, but did not observe any decrease of RhoA activity in the absence of S100A10 expression. For the organization of actin stress fibers, S100A10 may be required for the activation of a signaling pathway downstream of RhoA. Alternatively, S100A10 may regulate stress fiber formation independent of RhoA. Stress fibers are composed of 10-30 actin filaments, and numerous actin-binding proteins are required for actin bundling [30]. Previously, ANX2 has been shown to be an actin-binding protein that is critical for the regulation of actin dynamics [24]. Although further analysis is necessary, it is reasonable to hypothesize that S100A10 is required for ANX2 to maintain actin stress fiber organization.

We found that S100A10 is involved in the process of cell spreading. In the absence of S100A10, cell spreading was delayed in both HeLa and MDA-MB-231 cells. Upon cell adhesion to the extracellular matrix, a number of signaling pathways are triggered to activate Rac1 for the induction of actin reorganization at the periphery of cells to promote membrane extension [28]. We observed that activation of Rac1 during cell spreading was suppressed in the absence of S100A10. In addition, the expression of active Rac1 restored the ability of cells to spread. These results suggest that delayed cell spreading by S100A10 knockdown is mediated by the suppression of Rac1 activation. It has been demonstrated previously that S100A10 depletion suppresses cell migration [16]. Activation of Rac1 is crucial for the membrane extension to promote migration [4]. Since common regulatory mechanisms are utilized in the generation of membrane protrusions during cell spreading and migration, it is possible that S100A10 may also be required for Rac1 activation during cell migration.

In summary, we have shown that S100A10 is required for the organization of actin stress fibers and the formation of focal adhesions. In addition, we have demonstrated that S100A10 is required for the activation of Rac1 to promote membrane extension for cell spreading. Our results suggest that S100A10 contributes to the dynamic remodeling of the actin cytoskeleton by modulating the activity of Rac1.

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References

1. Raftopoulou M, Hall A (2004) Cell migration: Rho GTPases lead the way. Dev Biol 265:23–32
2. Ridley AJ (2006) Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends Cell Biol 16:522–529
3. Urban E, Jacob S, Nemethova M, Resch GP, Small JV (2012) Small, electron tomography reveals unbranched networks of actin filaments in lamellipodia. Nat Cell Biol 12:429–435
4. Parri M, Chiarugi P (2010) Rac and Rho GTPases in cancer cell motility control. Cell Commun Signal 8:23
5. Ito S, Takahara Y, Hyodo T, Hasegawa H, Asano E, Hamaguchi M, Senga T (2010) The roles of two distinct regions of PINCH-1 in the regulation of cell attachment and spreading. Mol Biol Cell 21:4120–4129
6. Rescher U, Gerke V (2008) S100A10/p11: family, friends and functions. Pflugers Arch 455:575–582
7. Donato R (2001) S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int J Biochem Cell Biol 33:638–668
8. Marenholz I, Heizmann CW, Fritz G (2004) S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophy Res Commun 322:1111–1122
9. Salama I, Malone PS, Mihaimeed F, Jones JL (2007) A review of the S100 proteins in cancer. Eur J Surg Oncol 34:357–364
10. Donato R, Cannon BR, Sorci G, Riuzzi F, Hsu K, Weber DJ, Geczy CL(2012) Functions of S100 proteins. Curr Mol Med (in press)
11. Réty S, Sopkova J, Renouard M, Osterloh D, Gerke V, Tabaries S, Russo-Marie F, Lewit-Bentley A (1999) The crystal structure of a complex of p11 with the annexin II N-terminal peptide. Nat Struct Biol 6:89–95
12. Gerke V, Weber K (1985) The regulatory chain in the p36-kd substrate complex of viral tyrosine-specific protein kinases is related in sequence to the S-100 protein of glial cells. EMBO J 4:2917–2920
13. Rust R, Visser L, van der Leij J, Harms G, Blokzijl T, Deloulme JC, van der Vlies P, Kamps W, Kok K, Lim M, Poppema S, van den Berg A (2005) High expression of calcium-binding proteins, S100A10, S100A11 and CALM2 in anaplastic large cell lymphoma. Br J Haematol 131:596–608
14. Ito Y, Arai K, Nozawa R, Yoshida H, Higashiyama T, Takamura Y, Miya A, Kobayashi K, Kuma K, Miyauchi A (2007) S100A10 expression in thyroid neoplasms originating from the follicular epithelium: contribution to the aggressive characteristic of anaplastic carcinoma. Anticancer Res 27:2679–2683
15. Liu J, Li X, Dong GL, Zhang HW, Chen DL, Du JJ, Zheng JY, Li JP, Wang WZ (2008) In silico analysis and verification of S100 gene expression in gastric cancer. BMC Cancer 8:261
16. Jhung MJ, Murzik U, Wehder L, Hemmerich P, Melle C (2010) Regulation of cellular actin architecture by S100A10. Exp Cell Res 316:1234–1240
17. McKeirnan E, McDermott EW, Evoy D, Crown J, Duffy MJ (2011) The role of S100 genes in breast cancer progression. Tumour Biol 32:441–450
18. Thiel C, Osborn M, Gerke V (1992) The tight association of the tyrosine kinase substrate annexin II with the submembranaous cytoskeleton depends on intact p11- and Ca(2+)-binding sites. J Cell Sci 103:733–742

19. Zobiack N, Gerke V, Rescher U (2001) Complex formation and submembranous localization of annexin 2 and S100A10 in live HepG2 cells. FEBS Lett 500:137–140
20. Lewit-Bentley A, Rety S, Sopkova-de Oliveira Santos J, Gerke V (2000) S100-annexin complexes: some insights from structural studies. Cell Biol Int 24:799–802
21. Harder T, Gerke V (1993) The subcellular distribution of early endosomes is affected by the annexin ll2p11(2) complex. J Cell Biol 123:1119–1132
22. Grieve AG, Moss SE, Hayes MJ (2012) Annexin A2 at the interface of actin and membrane dynamics: a focus on its roles in endocytosis and cell polarization. Int J Cell Biol 2012:852430
23. Hayes MJ, Rescher U, Gerke V, Moss SE (2004) Annexin-actin interaction. Traffic 8:571–576
24. Hayes MJ, Shao D, Bailly M, Moss SE (2006) Regulation of actin dynamics by annexin 2. EMBO J 25:1816–1826
25. Babbin BA, Parkos CA, Mandell KJ, Winfree LM, Laur O, Ivaov AL, Nusrat A (2007) Annexin 2 regulates intestinal epithelial cell spreading and wound closure through Rho-related signaling. Am J Pathol 170:951–966
26. Hall A (2005) Rho GTPases and the control of cell behaviour. Biochem Soc Trans 33:891–895
27. Price LS, Leng J, Schwartz MA, Bokoch GM (1998) Activation of Rac and Cdc42 by integrins mediates cell spreading. Mol Biol Cell 9:1863–1871
28. Sepulveda JL, Wu C (2006) The parvins. Cell Mol Life Sci 63:25–35
29. Amano M, Nakayama M, Kaibuchi K (2010) Rho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity. Cytoskeleton 67:545–554
30. Pellegrin S, Mellor H (2007) Actin stress fibres. J Cell Sci 120:3491–3499