The S100A4 Metastasis Factor Regulates Cellular Motility via a Direct Interaction with Myosin-IIA

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Abstract

S100A4, a member of the Ca\(^{2+}\)-dependent S100 family of proteins, is a metastasis factor that is thought to regulate the motility and invasiveness of cancer cells. Previously, we showed that S100A4 specifically binds to nonmuscle myosin-IIA and promotes the unassembled state. S100A4, thus, provides a connection between the actomyosin cytoskeleton and the regulation of cellular motility; however, the steps or steps in the motility cycle that are affected by S100A4 expression have not been investigated. To examine how the biochemical properties of S100A4 affect cell motility, we determined the effect of S100A4 expression on protrusive behavior during chemotactant-stimulated motility. Our studies show that S100A4 modulates cellular motility by affecting cell polarization, with S100A4 expressing cells displaying fewer side protrusions and extensive forward protrusions during chemotaxis compared with control cells. To establish a direct link between S100A4 and the regulation of myosin-IIA function, we prepared an antibody to the S100A4 binding site on the myosin-IIA heavy chain that has comparable effects on myosin-IIA assembly as S100A4. Microinjection experiments show that the antibody elicits the same effect on cell polarization as S100A4. Our studies show for the first time that S100A4 promotes directional motility via a direct interaction with myosin-IIA. These findings establish S100A4 as a critical regulator of myosin-II function and metastasis-associated motility. (Cancer Res 2006; 66(10): 5173-80)

Introduction

S100A4 (mts1) belongs to the S100 family of Ca\(^{2+}\)-binding proteins (1). These proteins typically form homodimers (10-12 kDa per subunit) that are characterized by their solubility in saturated 100% ammonium sulfate (2). There are >20 known members, most of which are expressed in a highly tissue specific manner (1, 3). Importantly, most S100 family members display a high degree of target specificity, suggesting that individual S100 proteins regulate specific cellular processes.

Elevated S100A4 expression levels are associated with metastatic cancers, and S100A4 has emerged as a prognostic marker for poor patient survival in a number of cancers (4, 5). In addition, animal studies have provided evidence supporting the involvement of S100A4 in tumor progression and metastasis. In orthotopic models, overexpression of S100A4 in nonmetastatic mammary tumor cells confers a metastatic phenotype (6, 7), whereas inhibition of S100A4 expression suppresses the metastatic capacity of tumor cells (8, 9). S100A4 itself is not tumorgenic as transgenic mice that overexpress S100A4 do not develop tumors (10); however, in a tumorigenic background, S100A4 expression in the tumor cells facilitates the development of highly aggressive primary tumors and the formation of metastases (10, 11). In addition, the progeny from mice expressing the polyoma virus middle T antigen crossed with mice carrying null alleles for S100A4 display a significant decrease in metastases (12). Altogether, these observations suggest that S100A4 is not simply a marker for metastatic disease but rather has a causal role in mediating this process.

The transition from benign tumor growth to malignancy is described by the three-step hypothesis of metastasis, which involves (a) attachment to the extracellular matrix, (b) local proteolysis, and (c) subsequent migration (13). Of particular relevance to the role of S100A4 in promoting a metastatic phenotype, several studies suggest that S100A4 expression levels correlate strongly with cell motility. For instance, fibroblasts and epithelial tumor cells that overexpress S100A4 display increased migratory properties (14), and S100A4 expression is associated with mesenchymal cell morphology and motility (15). Conversely, ablation or reduction of S100A4 expression in tumor cells correlates with decreased cellular motility (9, 16). A consideration of all these data suggests that S100A4 may regulate the motility of tumor cells.

In vitro studies show that S100A4 specifically binds to nonmuscle myosin-IIA and inhibits the assembly of myosin-IIA monomers into filaments and promotes the disassembly of preexisting myosin-IIA filaments in a calcium-dependent manner (17). The S100A4 binding site maps to residues 1909 to 1924 in the COOH-terminal end of the coiled coil region of the myosin-IIA heavy chain (17, 18). Although this segment contains a protein kinase C (PKC) phosphorylation site at Ser\(^{1917}\), S100A4 binding is not affected by PKC phosphorylation. Rather, phosphorylation on Ser\(^{1944}\) by casein kinase 2 (CK2), which is located 20 residues downstream of the S100A4 binding site, inhibits S100A4 binding and protects against S100A4-induced destabilization of myosin-IIA filaments (19). Thus, heavy-chain phosphorylation on the CK2 site and calcium binding to S100A4 modulate the S100A4/myosin-IIA interaction.

To examine how the biochemical properties of S100A4 affect cell motility, we determined the effect of S100A4 expression on protrusive behavior during chemotactant-stimulated motility. In addition, we prepared an antibody to the S100A4 binding site on the nonmuscle myosin-IIA heavy chain that has comparable effects on myosin-IIA assembly as S100A4. Our studies show that S100A4 promotes directional motility via a direct interaction with myosin-IIA and establish S100A4 as a critical regulator of myosin-II-mediated motility.
Materials and Methods

Cell lines. HeLa cells were maintained in DMEM containing 10% fetal bovine serum (FBS). The BAC-1.2F5 macrophage cell line was cultured in α-MEM containing 10% FBS and 10 ng/mL colony-stimulating factor-1 (CSF-1). All cells were grown at 37°C in a humidified atmosphere of 5% CO₂. The rat mammary nonmetastatic adenocarcinoma cell line MTC-GFP (20) was cultured in α-MEM containing 5% FBS.

MTC-GFP cells stably expressing S100A4 were established as follows. The rat S100A4 cDNA was amplified from an expressed sequence tag (American Type Culture Collection, Manassas, VA) and subcloned into the HindIII/ HpaI sites of the retroviral expression vector pLHCX (Clontech, Palo Alto, CA). The S100A4 sequence was confirmed by DNA sequencing. The recombinant vector and vector without insert were transfected into Phoenix packaging cells to produce recombinant virus and unrecombinant control virus, respectively, using standard protocols (21). MTC-GFP cells were infected for 24 hours with virus-containing supernatant in the presence of 8 µg/mL polybrene at 32°C. After 2 days, stably expressing cells were selected by the inclusion of 300 µg/mL of hygromycin in the medium. After 7 to 10 days of selection, the clones infected with the control pLHCX virus were pooled together (MTC-VC). S100A4-pLHCX virus–infected clones were subjected to subcloning by limiting dilution into two 96-well plates in medium containing 300 µg/mL of hygromycin. All MTC-S100A4 clones and the MTC-VC cell line were maintained in α-MEM containing 5% FBS and 300 µg/mL of hygromycin.

Antibodies. A rabbit polyclonal antibody to the S100A4 binding site on myosin-IIA was generated using a synthetic peptide to residues 1907 to 1916 (TADAMNREVSS) of the human myosin-IIA sequence. Rabbit polyclonal antibodies to myosin-IIA and myosin-IIB were prepared using synthetic peptides corresponding to the COOH termini of the two proteins, GKDAGAEEKPAE (residues 1950-1961) and SDVNETQPOQSE (residues 1965-1976), as described previously (22). A rabbit polyclonal antibody to S100A4 was prepared using recombinant human S100A4. All antibodies were produced using standard methodologies (Covance Research Products, Denver, PA). Antibodies to synthetic peptides (myosin-IIA S100A4 binding site, myosin-IIA, and myosin-IIB) were purified by affinity chromatography on a SulfoLink column (Pierce, Rockford, IL) coupled with the synthetic peptide via a COOH-terminal cysteine residue. The S100A4 antibodies were purified by affinity chromatography on an AminoLink column (Pierce) coupled with the human S100A4. The specificities of the myosin-IIA, myosin-IIB, and myosin-IIA S100A4 binding site antibodies were assessed by immunoblot and immunoprecipitation analysis using HeLa cell lysates and purified recombinant myosin-IIA and myosin-IIB rods. The specificity of the S100A4 antibody was evaluated by immunoblot analysis of NIH 3T3 and MDA-MB-231 cell lysates and purified recombinant S100A4 (data not shown).

Purified rabbit IgG was purchased from Sigma (St. Louis, MO). The phosphorysine monoclonal antibody was from Covance Research Products. The β-actin monoclonal antibody was from Sigma.

Protein purification. Rabbit skeletal muscle actin was purified from acetone powder by the method of Spudich and Watt (23). Recombinant human S100A4 and myosin-IIA and myosin-IIB rods were purified as described previously (17). Full-length myosin-IIA was prepared from outdated human platelets according to the method of Daniel and Sellers (24).

Immunoblots and immunoprecipitation. HeLa whole-cell lysates were prepared by washing monolayers with cold PBS and placing the culture plate on dry ice for 10 minutes. The cell lysate was prepared by directly scraping the frozen cells into 2 × Laemmli sample buffer (25) containing 5 µg/mL each of chymostatin, leupeptin, and pepstatin. Whole-cell lysates of MTC-S100A4 clones and MTC-VC cells were prepared as described above. Lysates for each cell line were separated on a 12% Tricine SDS-polyacrylamide gel system. Proteins were transferred to a polyvinylidene difluoride membrane and reacted with antibodies to S100A4 or β-actin, and immunoreactive proteins were detected using the SuperSignal West Pico chemiluminescent detection system (Pierce).

For myosin-II immunoprecipitations, HeLa cell lysates were prepared by scraping frozen monolayers into an extraction buffer containing 25 mMol/L Tris-HCl (pH 8.8); 100 µmol/L sodium pyrophosphate; 100 µmol/L NaF; 250 µmol/L NaCl; 10 µmol/L EGTA; 5 µmol/L EDTA; 1 mMol/L phenylmethylsulfonyl fluoride; 5 µg/mL each of chymostatin, leupeptin, and pepstatin; 1% NP40; and 10 mMol/L ATP. The soluble fraction containing myosin-II was recovered by centrifugation at 105,000 × g for 15 minutes at 4°C in a Beckman TL-100 ultracentrifuge. Total protein concentrations were determined using the Bio-Rad Detergent-Complatable Protein assay. Before the immunoprecipitation, myosin-IIA S100A4 binding site antibodies were bound to protein A-Sepharose in PBS containing 1 µg/mL bovine serum albumin (BSA): 0.5 µg of supernatant was incubated with the antibody-protein A-Sepharose for 2 hours at 4°C. Immune complexes were collected by centrifugation; the remaining supernatant was saved for analysis, and the immune complexes were washed thrice with extraction buffer and then boiled in Laemmli sample buffer. Immunoprecipitates and recovered supernatant were separated by 5% SDS-PAGE. For immunoblots of HeLa whole-cell lysates and myosin-II rods, proteins were separated by 8% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and reacted with antibodies to the myosin-IIA S100A4 binding site, myosin-IIA, or myosin-IIB. Immunoreactive proteins were detected as described above.

Myosin-IIA assays. Myosin-IIA rod assembly and disassembly assays were done as described previously (17) using 1.5 µmol/L myosin-IIA rods and 0.75 to 1.5 µmol/L S100A4 dimer or 0.75 to 4.5 µmol/L of the myosin-IIA S100A4 binding site antibody.

For ATPase assays, human myosin-IIA at a concentration of 0.229 mg/mL was phosphorylated with 10 µmol/L rabbit smooth muscle myosin light chain kinase (MLCK) in the presence of 1 µmol/L calmodulin in a buffer containing 20 mMol/L Tris-HCl (pH 7.5), 15 mMol/L KCl, 5 mMol/L MgCl₂, 0.4 mMol/L CaCl₂, 0.02% NaN₃, 0.5 mMol/L DTT, and 0.5 mMol/L ATP for 45 minutes at 30°C. The reactions were stopped by the addition of EGTA to a final concentration of 0.1 Mmol/L. Monophosphorylation of the myosin-IIA regulatory light chain was confirmed by glycerol PAGE using the method of Perrie and Perry (26). Before the start of the ATPase assay, the myosin-IIA S100A4 binding site antibody or S100A4 was incubated with the phosphorylated myosin-IIA for 45 minutes at room temperature in the presence of 0.3 mMol/L CaCl₂. Actin-activated ATPase assays were done at room temperature and contained 0.05 µmol/L phosphorylated myosin-IIA, 10 µmol/L filamentous actin (F-actin), 0.5 to 0.8 µmol/L myosin-IIA S100A4 binding site antibody or 0.4 to 0.8 µmol/L S100A4 dimer in 20 mMol/L Tris-HCl (pH 7.5), 50 mMol/L KCl, 5 mMol/L MgCl₂, 0.1 mMol/L EGTA, 0.3 mMol/L CaCl₂, and 1 mMol/L ATP. Phosphatase release was measured for 20 to 30 minutes using the EnzChek Phosphate Assay Kit (Molecular Probes, Eugene, OR).

Collagen invasion assay. The collagen invasion assay was done as described previously (27). In brief, 80,000 MTC-VC or MTC-S100A4 cells were plated on 35-mm glass-bottomed dishes (MatTek Corp., Ashland, MA) in the presence or absence of 400,000 BAC1.2F5 cells in α-MEM containing 10% FBS and 10 ng/mL CSF-1. After 18 to 24 hours, cells were overlaid with a 750- to 1,000-µm layer of 6.22 mg/mL collagen I (BD Biosciences, San Jose, CA), which was allowed to gel for 90 minutes before adding 1 mL of the medium. After 24 hours, the cells were fixed for 30 minutes with 4% formaldehyde and analyzed by confocal microscopy on a Bio-Rad Radiance 2000 Laser Scanning Confocal Microscope. Optical z sections were taken every 5 µm, starting from the base of the dish to 50 µm into the collagen gel. To quantify the invasion of the cells, the green fluorescent protein (GFP) fluorescence of the z sections from 20 to 50 µm were added and divided by the sum of the GFP fluorescence from all the z sections. Two to three different fields of cells from each dish were imaged. All data were collected from two to three independent experiments.

Micropipette assay. For light microscopy experiments, 4,000 cells were placed on poly-L-lysine-coated glass-bottomed dishes (MatTek). After ~24 hours, cells were starved in L15 medium (Life Technologies, Gaithersburg, MD) supplemented with 0.35% BSA (starvation medium) for 3 to 4 hours. A Femtotjet micromanipulator and pump (Eppendorf-Brinkman Instruments, Hamburg, Germany) were used to control the position of the micropipette and the pressure required for the chemotactant flow. To induce the formation of protrusion, an Eppendorf femtotip II micropipette was filled.
with 50% FBS and was placed ~40 μm from the edge of a quiescent cell, and a pressure of 16 hPa was exerted to induce flow. Time-lapse series were taken for 10 minutes using a ×20 objective, numerical aperture (NA) of 0.30, and a 12-bit Cooke Sensicam QFE cooled CCD camera. Images were analyzed, and protrusion was measured using ImageJ as described by Mouneimne et al. (28).

For studies examining the effects of blebbistatin on protrusive activity, the s(-) active isomer or the s(+)-control isomer (Toronto Research Chemicals, Inc., North York, Ontario, Canada) were dissolved in 100% DMSO to make a 10 mM/L stock solution. MTC-VC cells were starved for 2.5 hours and then treated with 50 μmol/L blebbistatin in starvation medium for 10 to 30 minutes before stimulation with FBS. Treatment of MTC-VC cells with the inactive isomer produced invasive activity that was similar to untreated cells (data not shown).

Microinjection. For microinjection experiments, cells were plated on poly-1-lysine-coated glass dishes as described above and starved for 1.5 to 2 hours. The myosin-IIA S100A4 binding site antibody or control rabbit IgG (Toronto Research Chemicals) was mixed with rhodamine-dextran (10,000 molecular weight; Molecular Probes) to obtain final needle concentrations of 5 mg/mL antibody and 0.5 mg/mL dextran. We estimated a 10-fold dilution of the antibody upon microinjection to yield a final intracellular concentration of 0.5 mg/mL or 3.12 μmol/L antibody, which is comparable with the S100A4 concentration in clone 7. Injected cells were identified by their fluorescence. Microinjections were done using an Eppendorf semiautomatic microinjection system and a micropipette with a 0.5-μm inner diameter and 1.0-μm outer diameter pulled on a P-97 Flaming/Brown micropipette puller (Sutter Instrument, Inc., Novato, CA). Injected cells were allowed to recover for 1 hour after the microinjection and were then subjected to the micropipette assay.

Immunofluorescence. Cells examined in the micropipette assay were fixed at specific times following the addition of chemotacticant with 4% formaldehyde in PBS for 15 to 30 minutes and then permeabilized for 10 minutes with 0.1% Triton X-100 in PBS. Fixed cells were incubated overnight at 4°C with an antibody against the COOH terminus of myosin-IIA (2 μg/mL), with the S100A4 antibody (2 μg/mL) or a phosphotyrosine antibody (1:100 dilution). The samples were incubated with Cy3-conjugated secondary antibodies for 1 hour at room temperature, rinsed with PBS, and mounted using Pro-Long Anti-Fade (Molecular Probes). Images were acquired using IPLab Spectrum software (Scanalytics, Inc., Rockville, MD) with a CoolSNAP HQ interline 12-bit, cooled CCD camera (Roper Scientific, Tucson, AZ) mounted on an Olympus IX70 microscope with a PlanApo ×40 objective (Olympus, Melville, NY), and HiQ bandpass filters (Chroma Technology Corp., Rockingham, VT). IPLab images were processed using Photoshop (Adobe Systems, San Jose, CA).

Results

To examine the regulation of cellular motility by S100A4, we used a variant of the MTC cell line derived from the 13762NF rat mammary adenocarcinoma tumor, which displays low metastatic potential (29, 30) and stably expresses GFP (20). The parental MTC cells infected with the empty vector express very low levels of S100A4 (Fig. 1A), S100A4-expressing MTC cells were established by retroviral infection, and the expression level of S100A4 in clone 7 was determined to be 3.54 μmol/L, which is comparable with the expression levels observed in CSML100 cells (10.14 μmol/L), a metastatic mouse adenocarcinoma cell line that normally expresses S100A4 (31). Clones 21 and 6 displayed reduced levels of S100A4 expression compared with clone 7.

To determine whether S100A4 expression is associated with invasive behavior, we examined the three MTC-S100A4 clones for their ability to invade a collagen I gel. Recent studies indicate that carcinoma cells and macrophages are codependent for invasion in vivo (32), and that a paracrine loop between these two cell types is both necessary and sufficient for tumor cell invasion (27). When cultured alone, <9% of the virus control and S100A4-expressing MTC cells invade the collagen gel (Fig. 1B). In the presence of the BAC-1.2F5 macrophages, ~7% of the MTC-VC cells and 26% of the MTC-S100A4 clones invade at least 20 μm into the collagen. These data show that the presence of macrophages, S100A4 expression promotes the invasion of carcinoma cells into a three-dimensional collagen matrix.

To evaluate whether the invasive behavior exhibited by S100A4-expressing carcinoma cells was due to alterations in cell motility, we used a micropipette chemotaxis assay (28). A micropipette, containing FBS, was placed near quiescent cells, and membrane protrusion was monitored by video microscopy for 10 minutes (Fig. 2E). The MTC-S100A4 cells displayed a strong front protrusion oriented towards the micropipette, which was observed within 2 to 3 minutes after the introduction of the microneedle (Fig. 2F; see Supplementary Video S1), whereas the sides of the MTC-S100A4 cells did not show any significant protrusion. For clones expressing S100A4, forward protrusions were enhanced by 22% to 34% over side protrusions (Fig. 2A-C), which is consistent with the protrusive activity observed in other highly chemotactic and metastatic carcinoma cells (28). In contrast to S100A4-expressing cells, the front and side regions of MTC-VC cells protruded nearly to the same extent (Fig. 2D; see Supplementary Video S2). Retraction of the back edge was similar in MTC-S100A4 and MTC-VC cells. Altogether, these observations suggest that S100A4 allows cells to assume a more polarized morphology during directed motility by affecting the localization of protrusions.
Previous studies have shown that antibodies recognizing epitopes near the COOH terminus of the myosin-IIA heavy chain shift the monomer-polymer equilibrium to the monomeric state and inhibit the actin-activated ATPase activity of myosin-IIA (33). These effects are remarkably similar to the activity of S100A4, which binds to the COOH-terminal tip of the myosin-IIA α-helical coiled-coil and has comparable effects on myosin-II assembly and motor activity (17, 34). To test if alterations in the directed motility of MTC-S100A4 cells are due to the regulation of myosin-IIA function by S100A4, we generated an antibody to the S100A4 binding site on the myosin-IIA heavy chain. The antibody recognizes a single polypeptide of ~200 kDa on immunoblots of HeLa cell lysates and only reacts with purified, recombinant myosin-IIA rods (Fig. 3). In addition, under native conditions, the antibody specifically immunoprecipitates myosin-IIA from whole-cell homogenates of HeLa cells (Fig. 3).

Next, we examined the ability of the S100A4 binding site antibody to modulate myosin-IIA function. At physiologic salt concentrations, ~90% of the myosin-IIA rods assembled into filaments (Fig. 4). In the presence of S100A4 (1:1 ratio of 1 S100A4 dimer to myosin-IIA rod), ~72% of the filaments were present in the supernatant, consistent with our previous observations (17). At a molar ratio of 1 or 2 antibody molecules per myosin-IIA rod, the S100A4 binding site antibody inhibited the assembly of ~47% and 84% of the myosin-IIA rods, respectively (Fig. 3C). We also assessed whether the S100A4 binding site antibody could destabilize myosin-IIA filaments in a similar manner to S100A4. At a molar ratio of 1 S100A4 dimer per myosin-IIA rod, we observed significant disassembly of the filaments with ~70% of the myosin-IIA in the supernatant (Fig. 3D), which is comparable with the extent of disassembly we observed previously in the presence of S100A4 (17).

The S100A4 binding site antibody also promoted the disassembly of myosin-IIA filaments, with 55% and 65% of the myosin-IIA in the supernatant at molar ratios of 2 or 3 antibody molecules per myosin-IIA rod, respectively. These biochemical studies show that the S100A4 binding site antibody and S100A4 have comparable effects on myosin-IIA assembly.

S100A4 has been reported to inhibit the actin-activated ATPase activity of myosin-II (34); thus, we examined whether the S100A4 binding site antibody also effects myosin-IIA motor activity. Myosin-IIA phosphorylated on Ser19 had an actin-activated ATPase of 0.40 ± 0.14 mol Pi/mol myosin head second (Fig. 3E). At a molar ratio of 8:1 and 10:1 (S100A4 or antibody to full-length myosin-IIA), the actin-activated ATPase activity was inhibited by 50%.

To determine if the S100A4 binding site antibody elicits the same effects on protrusive activity as S100A4, MTC-VC cells were microinjected with the antibody or control IgG, and membrane protrusion was evaluated in the micropipette assay. The overall pattern of protrusive activity observed in MTC-VC cells injected with the S100A4 binding site antibody was similar to that observed in the MTC-S100A4 cells. Cells injected with the S100A4 binding site antibody exhibited a predominant front protrusion, which began almost immediately after the introduction of the micropipette (Fig. 4A and B). Furthermore, the extent of the forward protrusions of cells injected with the S100A4 binding site antibody was enhanced by ~10% compared with cells injected with control IgG. This is similar to the enhancement observed in forward protrusions in S100A4-expressing cells compared with nonexpressing cells (Fig. 2A–C). However, forward protrusions formed more rapidly in cells injected with the S100A4 binding site antibody than in S100A4 expressing cells. In contrast to the differences detected in forward protrusions, the extent of the side protrusions and retraction of the back edge was similar for cells injected with either antibody. Interestingly, treatment of MTC-VC cells with the nonmuscle myosin-II ATPase inhibitor blebbistatin ablated all protrusive activity in response to chemoattractant (Fig. 4C; see Supplementary Video S3), suggesting that the observed effects on protrusive activity in S100A4-expressing cells and cells injected with the S100A4 binding site antibody do not occur via inhibition of myosin-II motor activity.

To determine if the distribution of myosin-IIA is altered in S100A4 expressing versus nonexpressing cells, MTC-VC or MTC-S100A4 cells were fixed 7.5 minutes following the addition of chemoattractant in the micropipette assay and stained with a myosin-IIA antibody. In both MTC-S100A4 and MTC-VC cells, myosin-IIA was observed in the leading edge (Fig. 5B and D). However, we observed significant myosin-IIA staining in side protrusions of MTC-VC cells, whereas in MTC-S100A4 cells the myosin-IIA was concentrated in the front protrusion that was oriented towards the micropipette. S100A4 also concentrated in the leading edge but had a broader distribution than that observed for myosin-IIA (Fig. 5F). We also compared the localization of focal...
contacts in the two cell lines with a phosphotyrosine antibody. Although qualitatively similar, focal contacts were observed in both cell types, in MTC-S100A4 cells, focal contacts predominated at the leading edge; whereas in MTC-VC cells, we observed focal contacts along the entire periphery of the cell (Fig. 5F and I).

Discussion

The motility cycle of migrating cells involves lamellipod extension, attachment of the leading lamella to the extracellular matrix, the formation of focal contacts, and finally the production of contractile force that causes retraction of the tail towards the leading lamella. Several studies have shown that S100A4 expression levels correlate strongly with motility (14, 15), which is a central element of the metastatic cascade. Moreover, the regulation of myosin-IIA by S100A4 provides a direct link between the actomyosin cytoskeleton and the modulation of cellular motility by S100A4; however, the step or steps in the motility cycle that are affected by S100A4 expression have yet to be examined. Our results show that S100A4 regulates cell polarization during directed motility by affecting the localization of protrusions through interactions with myosin-IIA, with S100A4 expressing cells displaying few side protrusions and extensive forward protrusions during chemotaxis compared with control cells. Consistent with this finding, our localization studies show that during chemotaxis, both S100A4 and myosin-IIA localize primarily to the leading edge of forward protrusions with some staining detected in side extensions. These observations are in agreement with previous studies showing enrichment of S100A4 and myosin-IIA at the leading edge of highly polarized cells (35). In addition, the distribution of focal contacts in S100A4 expressing versus nonexpressing cells differed, consistent with the different protrusive activity of these cells.

We show here that S100A4 modulates cellular motility by affecting cell polarization during chemotaxis. To determine if the observed effects of S100A4 expression on protrusive activity are due to the direct modulation of myosin-IIA function, we generated an antibody to the S100A4 binding site on the myosin-IIA heavy chain that mimics the effects of S100A4 binding on myosin-IIA assembly and motor activity. Notably, we found that the antibody elicits comparable effects on cellular protrusive activity as S100A4. Similar to S100A4, the S100A4 binding site antibody enhances forward protrusions. However, cells expressing moderate to high levels of S100A4 also suppress side protrusions, whereas cells injected with the S100A4 binding site antibody do not. Additionally, we found that the protrusive activity observed in cells injected with the S100A4 binding site antibody occurs more rapidly in response to chemoattractant than S100A4-expressing cells. These differences in cellular behavior between antibody injected and S100A4-expressing cells likely results from the different regulatory controls modulating the function of these proteins, as the interaction of S100A4 with myosin-IIA is calcium dependent (17, 34), whereas binding of the antibody to myosin-IIA is not regulated. In addition, the calcium dependency of the S100A4/myosin-IIA interaction will allow for regional activation of S100A4 that is independent of myosin-II concentration, whereas the

Figure 3. The S100A4 binding site antibody specifically recognizes myosin-IIA (MIIA) and elicits the same biochemical effects as S100A4 on myosin-IIA assembly and ATPase activity. A, immunoblots of a HeLa whole-cell lysate and purified myosin-IIA and myosin-IIB (MIIB) rods. The S100A4 binding site antibody reacts with a single band in HeLa lysates and only recognizes the myosin-IIA rods. B, a HeLa cell lysate was treated with protein A-Sepharose coupled with the S100A4 binding site antibody. Samples of the immunoprecipitate and unbound flow through (FT) were examined by immunoblot analysis with antibodies to myosin-IIA or myosin-IIB. The S100A4 binding site antibody immunoprecipitates only myosin-IIA. C, the S100A4 binding site antibody inhibits the assembly of myosin-IIA monomers. At physiologic salt concentrations, the assembled myosin-IIA rods pellet, whereas the unassembled monomers (~10%) remain in the supernatant. At 2 mol antibody per mol of myosin-IIA rod, the S100A4 binding site antibody strongly inhibits the assembly of the myosin-IIA rods in a similar manner to S100A4. D, the S100A4 binding site antibody promotes the disassembly of preexisting myosin-IIA filaments. At a molar ratio of 3 antibody molecules per myosin-IIA rod, ~64% of the myosin-IIA rods are recovered in the supernatant, which is comparable with the effects of S100A4 on filament destabilization. E, the S100A4 binding site antibody inhibits the actin-activated ATPase activity of myosin-IIA. At a molar ratio of 10 antibody molecules per myosin-IIA, the ATPase activity decreased by 50%. Columns, mean for at least three independent experiments; bars, SE.

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Antibody will accumulate in subcellular compartments that are rich in myosin-IIA (i.e., the leading edge). Interestingly, our localization studies show that myosin-IIA is enriched in the leading edge but not the sides of locomoting cells. This observation is consistent with our findings that the antibody primarily effects protrusive activity at the front of the cell. Lastly, potential differences could also result from the interaction of S100A4 with other proteins, as S100A4 has been proposed to bind several target molecules, such as F-actin, tropomyosin, and p53 (36–38).

The apparent correlation between increased directional protrusions and the regulation of myosin-IIA is consistent with previous observations. Kendrick-Jones et al. showed that a monoclonal antibody directed against the tip of the avian myosin-IIA tail, which has the same biochemical effects on myosin-IIA assembly and motor activity as S100A4, induces the formation of extensive lamellae and increased cellular motility in primary fibroblasts (33, 39). The mechanisms by which these antibodies and S100A4 increase protrusive activity and promote cellular motility are not well understood, but the observation that S100A4 can inhibit the actin-activated ATPase of myosin-IIA (34) suggests that S100A4 could function as a myosin-IIA inhibitor in vivo. However, the inhibition of myosin-IIA motor activity by S100A4 is likely an indirect effect of the ATPase assay and is related to the actin cross-linking properties of myosin-II itself. In an actin-activated ATPase assay, full-length myosin-II forms filaments that cross-link actin filaments, thus decreasing the apparent dissociation constant (K_{app}) for actin and increasing the actin-dependent ATPase activity at subsaturating actin concentrations. Under conditions in which the myosin-II filaments are partially disassembled, there is decreased actin filament cross-linking and thus an apparent decrease in the actin-activated ATPase activity, which is due to a lower local F-actin concentration rather than a direct biochemical effect on the myosin-II motor (40–43). Moreover, our observations that blebbistatin suppresses protrusive activity, whereas S100A4 enhances protrusive activity, suggests that S100A4 does not elicit...
its biochemical effects through inhibition of the myosin-IIA motor but primarily via the regulation of myosin-IIA assembly. Given all of the above considerations, we propose a model in which S100A4 enhances directed motility by promoting the turnover of myosin-IIA filaments at the leading edge.

An examination of calcium levels in migrating cells has shown that the intracellular calcium concentration is high at the rear of the cell and low at the leading edge (44, 45), a finding that is not consistent with the regulation of myosin-IIA by S100A4 at the front of locomoting cells. However, more recent studies have reported a locally high calcium wave near the plasma membrane that originates at the leading edge and propagates along the cell periphery (46, 47). These findings provide a mechanism for the activation of S100A4 at the leading edge of migrating cells and are consistent with the observation that a number of calcium-regulated proteins (e.g., MLCK and gelsolin) are active in the leading edge of motile cells (48–50). Interestingly, inhibition of MLCK, a dedicated myosin-II kinase, results in multiple protrusions and a loss of cell polarity (49). These observations suggest that MLCK and S100A4 activation of S100A4 at the leading edge of migrating cells and are consistent with the regulation of myosin-IIA by S100A4 at the front of the cell and low at the leading edge (44, 45), a finding that is not consistent with the regulation of myosin-IIA by S100A4 at the front of locomoting cells.

References


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