S100P Dissociates Myosin IIA Filaments and Focal Adhesion Sites to Reduce Cell Adhesion and Enhance Cell Migration*

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Background: Certain S100 proteins induce cell migration and metastasis but the molecular mechanism is not clear.
Results: S100P preferentially binds and disperses NMIIA fibers and subsequently affects focal adhesion sites (FAS) and cell adhesion.
Conclusion: S100P overexpression reduces the assembly of NMIIA-FAS to enhance cell migration by reducing anchoring forces.
Significance: The mechanism of the primary step of S100P-induced metastasis has been elucidated.

S100 proteins promote cancer cell migration and metastasis. To investigate their roles in the process of migration we have constructed inducible systems for S100P in rat mammary and human HeLa cells that show a linear relationship between its intracellular levels and cell migration. S100P, like S100A4, differentially interacts with the isoforms of nonmuscle myosin II (NMIIA, Kd = 0.5 μM; IIB, Kd = 8 μM; IIC, Kd = 1.0 μM). Accordingly, S100P dissociates NMIIA and IIC filaments but not IIB in vitro. NMIIA knockdown increases migration in non-induced cells and there is no further increase upon induction of S100P, whereas NMIIIB knockdown reduces cell migration whether or not S100P is induced. NMIIIC knockdown does not affect S100P-enhanced cell migration. Further study shows that NMIIA physically interacts with S100P in living cells. In the cytoplasm, S100P occurs in discrete nodules along NMIIA-containing filaments. Induction of S100P causes more peripheral distribution of NMIIA filaments. This change is paralleled by a significant drop in vinculin-containing, actin-terminating focal adhesion sites (FAS) per cell. The induction of S100P, consequently, causes significant reduction in cellular adhesion. Addition of a focal adhesion kinase (FAK) inhibitor reduces disassembly of FAS and thereby suppresses S100P-enhanced cell migration. In conclusion, this work has demonstrated a mechanism whereby the S100P-induced dissociation of NMIIA filaments leads to a weakening of FAS, reduced cell adhesion, and enhanced cell migration, the first major step in the metastatic cascade.
partially dissociates its filaments and causes their redistribution, and that these changes are accompanied by a redistribution of FAS, from the central area to the cellular periphery. The number of FAS per cell is also significantly decreased. Consequently cell adhesion is reduced and cell migration is enhanced. These changes suggest that the weakening of the anchoring forces generated through NMIIA to FAS will allow intact NMIIIB filaments to drive cell migration.

EXPERIMENTAL PROCEDURES

Cell Culture—The Rama 37 cells, a nonmetastatic benign rat mammary tumor cell line expressing undetectable levels of S100P, S100P cDNA transfected cell lines (Rama 37-S100P-1, -2; Rama 37 pool-1, -2), and the control vector alone transfected cell line (Rama 37-vector), were established and cultured as described previously (7). Human cancer cell lines, HeLa, MCF-7, and MDA-MB-231 cells, were cultured routinely (24).

Establishment of Doxycycline Inducible Cell System—The tetracycline inducible system (gifted by Dr. Adam West, National Institutes of Health) contains two plasmids, pBTE to express the regulatory element rtTA2(S)-M2 (25), and pTRE-ins to express the target protein. Two inducible clones derived from Rama 37 cells were termed Rama 37-T25 and -T28, whereas two inducible clones derived from HeLa cells were termed HeLa-A3 and -A19. The concentration of doxycycline and the induction period were optimized and 1 μg/ml of doxycycline for 24 h was used in all the inductions (supplemental Fig. S1).

Measurement of Cell Migration—Migration of cell lines was measured 24 h after the cells were seeded in Boyden chambers using 6.5-mm diameter polycarbonate membrane inserts containing 8-μm pores (Corning Costar) (26), as described previously (27). The inducing agent, doxycycline, inhibitors, or blocking peptide and antibodies were added to both the upper and lower compartments of the Boyden chambers. To standardize results between different experiments, usually the cell line controls were set to 100% migration and changes relative to this value were shown in most figures.

siRNA Transfections—Two siRNA sequences used for targeted silencing of human S100P and four siRNA sequences for each human NMII isoform, vinculin, Rac1, and RhoA were designed and synthesized by Qiagen (supplemental Table S1). S100P-expressing MCF-7 and HeLa/HeLa-A3/HeLa-A19 cell lines were used for testing S100P siRNAs, NMII siRNAs, vinculin, Rac1 and RhoA siRNAs, respectively. The RNAi Starter kit (301799), including RNAiFect, negative control siRNA, were from Qiagen. The siRNA transfection procedure followed instructions from Qiagen. At 48 h after transfection, the levels of S100P, NMIIA and -IIB (supplemental Fig. S2, A and B), vinculin, Rac1, and RhoA were measured using Western blotting and the levels of NMIIIC mRNA (supplemental Fig. 2C) by semi-quantitative RT-PCR, because there was no satisfactory antibody for NMIIIC.

Semi-quantitative RT-PCR—Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription was carried out using a cloned AMV First-strand Synthesis Kit (Invitrogen). The resultant cDNA was used as template for the amplification of actin (18 cycle) and NMIIIC (25 cycle) by PCR using the primers listed in supplemental Table S2.

Recombinant Proteins—The recombinant human S100P protein was purified as described previously (28, 29). The rC-NMIIA (C-terminal 149 amino acids), IIB (C-terminal 204 amino acids), and IIC (C-terminal 178 amino acids) were produced using the same method as used previously for rC-NMIIA (30) with the primers listed in supplemental Table S2. The deletion mutants of rC-NMIIA, -M1, -M2, -M3, -M4, and -M5 were produced by the PCR extension method (31, 32).

Western Blotting—S100P was detected using mouse anti-S100P mAb (1:50 dilution) (BD Biosciences), which showed no cross-reaction with S100A1, S100A2, or S100A4 (7). FAK was isolated using anti-FAK-conjugated agarose (Millipore, Billerica, MA) from 1 mg of protein prior to SDS-PAGE. NMIIA, -IIB, FAK, phospho-FAK (pFAK), and actin were detected using rabbit polyclonal anti-NMIIA and -IIB (Covance, Princeton, NJ), mAb-FAK and polyclonal anti-pFAK Tyr(397) (Upstate, New York).

Gel Overlay Assay—Briefly, equal amounts of proteins (3 μg) were subjected to SDS-PAGE (33). One gel was stained with Coomassie Brilliant Blue to check equal loading and the other two gels were electroblotted onto PVDF membranes, which were then incubated at 4 °C overnight with S100A4 (3 μg/ml) or S100P (3 μg/ml) in Overlay Buffer (12). The rest of the procedures were the same as those of conventional Western blotting.

Binding Assays—Binding reactions were carried out in IA/lys two-channel resonant mirror biosensor (Affinity Sensors, Saxon Hill, Cambridge, United Kingdom), as described previously (34); the rC-NMIIA, -IIB, or -IIC protein fragments were immobilized on aminosilane surfaces using BS3 (Perbio, Chester, UK). The equilibrium dissociation constant (Kd) was calculated both from the association and dissociation rate constants, and from the extent of binding at or near equilibrium (34).

Sedimentation Assay—Briefly, 5 μM rC-NMIIA, -IIB, or -IIC was incubated at 4 °C overnight in Bundling buffer (10 mm imidazole-HCl (pH 7.5), 100 mm NaCl, and 2.5 mm MgCl2)(35). To test the effect of S100P on rC-NMIIC sedimentation, 0—10 μM S100P was preincubated with rC-NMIIA, -IIB, or -IIC with 0.5 mM CaCl2 at room temperature for 40 min. Bundling buffer was then added and the NaCl and MgCl2 were adjusted to 100 and 2.5 mM, respectively. Twenty μl of mixture was removed and kept at −20 °C as a control, and the remainder was incubated overnight at 4 °C. After centrifugation at 13,600 × g for 30 min at 4 °C, 20 μl of the supernatant was subjected to SDS-PAGE together with control. Anti-His tag antibody (Sigma) was used to detect rC-NMIIA, -IIB, and -IIC by Western blotting. To test if S100P disassociates the preformed NMII filaments, the rC-NMIIA, -IIB, and -IIC were incubated in Bundling buffer for 48 h. After centrifugation, pellets were resuspended in Bundling buffer containing 0 and 3 μM S100P. After 40 min incubation, the mixtures were centrifuged and the resultant supernatants were analyzed.

Immunofluorescent Staining—Culture of the human breast cancer cell line MCF-7 and dual immunofluorescent staining were carried out as described (24). The cells were grown on the untreated glass surface of 8-well chamber slides (BD Biosciences). S100P was detected with the mAb (BD Biosciences) at
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1:50 dilution and visualized with FITC-conjugated goat antimouse antibody (Sigma). NMIIA and -IIB were detected with rabbit anti-NMIIA and -IIB IgGs (Covance) at 1:5000 dilution and visualized with Texas Red-conjugated goat anti-rabbit antibody (Molecular Probes). Actin filaments were visualized with Atto 647N-Phalloidin (Sigma). FAS were detected with mAb to vinculin (Sigma) at 1:2000 dilution and visualized as for S100P. Images were recorded using a Zeiss LSM 510 confocal microscope.

Fluorescent Lifetime Imaging—For fluorescent resonance energy transfer (FRET) analysis, the expression plasmids, pECFP-S100P and pNMIIA-EYFP, were constructed by inserting S100P and NMIIA coding sequences into pECFP-C1 vector (Clontech, Palo Alto, CA) and pEYFP-N1 vector (Clontech) at the HindIII and BamHI sites, respectively. The correct coding sequences for the two fusion proteins, ECFP-S100P and NMIIA-EYFP, were confirmed by DNA sequencing. The pECFP-S100P and pNMIIA-EYFP/pEYFP-C1 expression vectors were then co-transfected into HeLa cells. The time-resolved fluorescence decay maps were collected pixel-by-pixel from the HeLa cells co-expressing ECFP-S100P and NMIIA-EYFP fusion proteins or from HeLa cells co-expressing ECFP-S100P and EYFP by scanning confocal microscopy using time-correlated single photon counting (36) fluorescence lifetime imaging module (SPC-730) (Becker & Hickl, Berlin, Germany) and a photomultiplier tube (PMH-100-1, Hamamatsu, Bridgewater, NJ). Fluorescence lifetime imaging microscopy images were analyzed using SPImage 2.0 (Becker & Hickl). The methods for calculation of fluorescence lifetime and the distance between donor and acceptor as well as the statistical analysis are fully described elsewhere (37).

Time Lapse Confocal Microscopy—GFP-NMIIA expression plasmid (full-length) was obtained from Addgene. The HeLa-A3 was grown on glass bottom dishes (Matsunami Glass, Tokyo, Japan) and transfected with the plasmid using Lipofectamine (Invitrogen). Doxycycline (1 μg/ml) was added to the cell culture 16 h after transfection. Images of transfected cells were recorded in a Zeiss LSM 510 confocal microscope for a further 24 h.

Triton-insoluble NMIIA Detection—An equal number of HeLa and HeLa-A19 cells were cultured in the presence or absence of doxycycline for 24 h and washed with ice-cold PBS. The cells were then incubated with ice-cold cytoskeletal lysis buffer (40 mM sodium pyrophosphate, 20 mM potassium phosphate, 3 mM EGTA (pH 7.4)) and 1% (v/v) Triton X-100 for 5 min on ice. The buffer was then removed to a fresh tube and 20 μl was taken for soluble actin detection. The material remaining on the dish after two washes with the buffer was considered as the detergent-insoluble cytoskeletal fraction and harvested by adding 2× SDS gel loading buffer. The insoluble NMIIA and soluble actin (as a loading control) were detected by Western blotting.

Peptide Synthesis and Transfer—The S100P binding domain of NMIIA fused with TAT-HA2 (38) was synthesized by GeneCust Europe (Luxembourg). TAT-HA2 was used as control peptide. HeLa and HeLa-A19 cells were incubated with 5 μM peptides for 24 h. The cell migration assay was set up in the presence or absence of doxycycline.

Cell Adhesion Assay—A modified attachment assay (39) was carried out. HeLa and HeLa-A19 cells were induced with 1 μg/ml of doxycycline for 24 h. The cells were then detached by EDTA, which was neutralized by culture medium, washed with PBS, and resuspended to 4 × 10^5/ml in basal medium without serum, 50 μl of cells were seeded in each well of a 96-well microtitre plate (Costar). After incubation in a CO2 incubator at 37 °C for 5, 30, 60, or 90 min, the plate was shaken at 2000 rpm for 10–15 s and washed 3 times with PBS. The remaining cells were fixed with 5% (w/v) glutaradehyde. After washing, the cells were stained with crystal violet for 10 min. After washing with water 5 times, the plate was dried and 100 μl 10% (v/v) acetic acid was added. After 30 min incubation, the absorption was recorded at 570 nm. The cell adhesion array kit was purchased from Millipore and used according to the supplier’s instructions.

RESULTS

Regulation of Levels of Intracellular S100P Affects Cell Migration—When the rat mammary 37 (Rama 37) cells were transfected with an expression vector for S100P, the resultant transfecants, R37-S100P-1 and R37-S100P-2, and two pooled clones, pool-1 and pool-2 (7), expressed 6–14-fold of S100P mRNA and 5–12-fold of S100P protein over the pooled empty vector-transfected Rama 37 cells (R37-vector), causing an increase in cell migration of 30–70% (Fig. 1, A and B) (ANOVA test, p < 0.01). To down-regulate the levels of S100P, two specific siRNAs to the mRNA for S100P were synthesized and transfected into MCF-7 cells, a human breast cancer cell line that expressed high levels of S100P. siRNA duplexes 1 and 2 separately reduced the levels of S100P in MCF-7 cells to 10 and 80%, respectively, and led to a reduction in cell migration of about 45 and 75%, respectively (p = 0.004 and p = 0.2) (Fig. 1, C and D). To up-regulate the levels of S100P, the doxycycline-inducible cell lines, Rama 37-T25, -T28, and HeLa-A3 and -A19 were constructed from rat mammary benign and human malignant ovarian cells. After addition of 1 μg/ml of doxycycline for 24 h, the level of S100P was significantly increased by 5–10-fold (p = 0.001) and cell migration by 2–3-fold, respectively (p = 0.008) (Fig. 1, E and F). Levels of S100P and migration of Rama 37 and HeLa cells showed no significant differences with or without inducing agent (p ≥ 0.30; Fig. 1, E and F). There was a significant positive correlation between the levels of S100P and cell migration (supplemental Fig. S3).

Although there is no secretion signaling peptide in any of the S100 proteins, it is thought that the S100 proteins can be secreted and may function at low concentrations of around 100 nM (1 μg/ml) (40, 41) by activating the receptor for advanced glycation end-products (RAGE) on the cell surface (42, 43). However, S100P was not detectable in the conditioned medium of doxycycline-induced or non-induced R37-T25 and HeLa-A3 cells (Fig. 1G). When 100 nM human recombinant S100P (rhS100P) was added to Rama 37-T25 or HeLa-A3 cells (Fig. 1H), it produced no significant increase in cell migration (p = 0.23), although it significantly increased cell invasion.4 Increasing the dose of rhS100P up to 1 μM did not achieve a significant increase in cell migration (data not shown). Moreover, the

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RAGE neutralizing antibody or blocking peptide (8, 43) did not significantly inhibit cell migration enhanced by doxycycline-induced S100P (Fig. 1F) (p > 0.09). These data suggest that the up-regulation of intracellular S100P, but not the secreted S100P, is the primary cause of S100P-enhanced cell migration at least in those cell lines tested.

Preferential Interaction of S100P with NMIIa Interferes with Their Corresponding Filament Formation—The recombinant C-terminal fragments of NMIIa, -IIB, and -IIC (r-C-NMIIa, -B, and -C) were separately immobilized onto a biosensor and the extent of binding of different concentrations of S100P was measured in a buffer containing 0.5 mM Ca\(^{2+}\). The binding characterized by fast association kinetics was always homogeneous and there was no evidence for more than one binding site for S100P. The equilibrium dissociation constants (K_d) for r-C-NMIIa, -IIB, or -IIC calculated from these kinetic parameters were: 430 ± 210; 7,800 ± 4,200; and 1,000 ± 530 nM, respectively, very similar to the K_d values calculated from the extent of binding observed at equilibrium of: 500 ± 200; 8,400 ± 1,200; and 490 ± 100 nM, respectively (Table 1). Therefore, the binding affinity of S100P with r-C-NMIIa is similar to that with r-C-NMIIc and is 10–20-fold higher than that with r-C-NMIIb.

Binding of S100A4 has been mapped to amino acids 1909–1937 of NMIIa (20). Because S100P has a similar structure (44, 45), the deletion mutant polypeptides of NMIIa (M1–M5) used for mapping S100A4 binding sites (20) were employed for S100P (Fig. 2, A–C). Since mutant M1 failed to bind, but mutants M2 and M3 did bind to S100P, the region from amino acids 1909 to 1937 of the r-C-NMIIa is confirmed to be a major sequence for S100P and S100A4 binding (Fig. 2, A–C). The additional binding to M3 over M2 (Fig. 2, B and C) suggests partial overlap of this binding site with the filament assembly domain, which plays an important role in regulating NMIIa filament formation (Fig. 2A) (22, 46).

When S100P was preincubated with r-C-NMIIa or -IIC, formation of their filaments was inhibited. Thus when mixtures that contained r-C-NMIIa and S100P were centrifuged, the amount of soluble r-C-NMIIa retained in the supernatant was significantly increased by about 2-fold for 0.3 \(\mu\)M to 6-fold for 10 \(\mu\)M S100P (ANOVA test, p < 0.01) (Fig. 2, D and E). In contrast, prior incubation of S100P with r-C-NMIIb showed no significant change (p > 0.05). When S100P was preincubated with NMIIC, the amount of soluble form increased by 6-fold for 3 \(\mu\)M and 10-fold for 10 \(\mu\)M S100P; hence the formation of NMIIC filaments was also significantly inhibited by S100P (p < 0.01) (Fig. 2, D and E). When 3 \(\mu\)M S100P was added to preformed filaments of specific NMII isoforms, S100P significantly increased the amount of soluble forms of NMIIa and -IIC by 6- and 2.7-fold on average, respectively (Student’s t test, p ≤ 0.008 when compared before and after doxycycline induction. G, R37-T25 and HeLa-A3 cells were cultured in medium without FCS, but supplemented with 1 mg/ml of bovine albumin with or without doxycycline (1 \(\mu\)g/ml) for 48 h. One ml of medium with doxycycline from each cell line was collected and freeze dried. S100P was detected by Western blotting. H, effect of inhibiting RAGE receptor on cell migration of
0.007), but not those of NMIIB (p = 0.25)(Fig. 2, F and G). This result shows that S100P can partially dissociate preformed NMIIA and -IIC filaments, but not those of NMIIB.

**TABLE 1**

| Immobilized protein | $k_{\text{ass}}^a$ (mean ± S.E.) | $R^b$ | $k_{\text{diss}}^c$ (mean ± S.E.) | $K_a$ | $K_d$ | $K_d$ (mean ± S.E.) |
|---------------------|---------------------------------|------|---------------------------------|------|------|-------------------|
| rC-NMIIA            | 8900 ± 2400                     | 0.91–0.98 | 0.0039 ± 0.0007 | 430 ± 210 | 500 ± 200 |
| rC-NMIIB            | 990 ± 350                       | 0.94–0.96 | 0.0078 ± 0.0031 | 7800 ± 4200 | 8400 ± 1200 |
| rC-NMIIC            | 6800 ± 3300                     | 0.82–0.95 | 0.0068 ± 0.0016 | 1000 ± 530 | 490 ± 100 |

$^a$ The S.E. of each determination of $k_{\text{ass}}$ is derived from the deviation of 4 independent data sets from a one-site binding model, calculated by matrix inversion using the Fast-Fit software provided with the instrument (“Experimental Procedures”). No evidence was found for a two-site model of association.

$^b$ The correlation coefficient of the linear regression through the $k_{\text{ass}}$ values used for obtaining $k_{\text{ass}}$.

$^c$ The $k_{\text{diss}}$ is the mean ± S.E. of 7 values, obtained at different concentrations of S100P. No evidence was found for a two-site model of dissociation.

$^d$ The $K_d$ (kinetic) was calculated from the ratio of $k_{\text{diss}}/k_{\text{ass}}$, and the S.E. is the combined S.E. of the two kinetic parameters.

$^e$ The $K_d$ (equilibrium) was calculated from the extent of binding observed at or near equilibrium at 6 or more different concentrations of S100P in 4 independent experiments. The S.E. is the combined error of the 4 experiments.

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**FIGURE 2. Interaction of S100P with NMIIA and its effects on the formation of filaments.** A, amino acid sequence alignment of the tail region of the three NMII isoforms; the bundling domain (IIA, 1855–1903) and a major S100P-binding site (IIA, 1909–1937) are indicated. B, diagram of NMIIA mutations generated. C, gel overlay assay with S100P (upper panel) and S100A4 (middle panel) proteins to show their interaction with deletion mutants (M1, M2, M3, M4, and M5) and wild type (WT) NMIIA C-terminal fragments (“Experimental Procedures”). Lower panel shows the Coomassie Blue-stained gel of mutant and WT NMIIA C-terminal fragments used for the gel overlay assay. D, examples of Western blotting with anti-His tag antibody to soluble polyhistidine-tagged NMIIs C-terminal fragments to demonstrate the effect of different concentrations of S100P on formation of NMII filaments in vitro. B = sample taken before the filaments formed. The remaining lanes are the same volume of samples taken from the supernatants after precipitation of NMII filaments by centrifugation. E, Bundling buffer containing 0 and 3 μM S100P was used to resuspend the filaments that formed with 0 μM S100P as shown in D. After 40 min incubation, the resuspended filaments were centrifuged and the rest of the procedures were the same as those in D above. F, mean ± S.D. in arbitrary units of quantified nonfilament NMII in the supernatant plotted against micromolars of S100P from three independent experiments in D above for IIA (●), IIB (○), and IIC (▲). G, mean arbitrary units of soluble NMII peptides from 3 independent experiments were presented for control (□) and addition of 3 μM S100P (△). *, significant increase in Student’s t test, p = 0.007.

**Knocking Down NMII Isoforms Affects S100P-induced Cell Migration**—When expression of NMIIA was knocked down by over 90% using specific siRNA in HeLa-A3 cells...
co-localized with the majority of S100P-containing focal densities (Fig. 4A, panels B and E), and also formed nodules along the filaments. Some of these nodules co-localized with the majority of S100P-containing focal densities (Fig. 4A, panels C and F). To investigate if S100P physically interacts with NMIIA in mammalian cells, the plasmid expressing enhanced cyan fluorescent protein (ECFP)-S100P fusion protein and the plasmid expressing NMIIA-EYFP (enhanced yellow fluorescent protein) or EYFP alone were co-transfected into HeLa cells. The fluorescent lifetime of ECFP-S100P was observed when S100P was induced with ECFP-S100P alone. The mean fluorescent lifetime of ECFP-S100P was measured both from cells co-expressing ECFP-S100P and NMIIA-EYFP (1.12 ± 0.14 ns, mean ± S.D. from 7 different cells in two independent transfections) (Fig. 4B, panel A), and cells co-expressing ECFP-S100P and EYFP (2.38 ± 0.07 ns, mean ± S.D. from 5 different cells in two independent transfections) (Fig. 4B, panel B). A significant reduction (Student’s t test, p < 0.01) in the mean fluorescent lifetime of ECFP-S100P was observed in cells co-expressing NMIIA-EYFP compared with that of cells co-expressing EYFP (Fig. 4B, panel C). The reduction in mean fluorescence lifetime resulted from the transfer of energy from the optically excited ECFP-S100P to NMIIA-EYFP but not to EYFP. This indicates that S100P and NMIIA were in physical contact, i.e. < 5 nm apart (47, 48). These data confirmed that the in vitro interactions between S100P and NMII isoforms also occur in vivo.

S100P Induces Reorganization and Reduces Total Amount of NMIIA Filaments in Living Cells—GFP-tagged full-length NMIIA (Fig. 5A) was expressed in HeLa-A3 and -A19 cells by transient transfection. Doxycycline was added at 16 h after transfection. At that time, NMIIA filaments were visible and distributed uniformly in the majority of GFP-fluorescing cells, with only 7–10% showing a peripheral distribution (supplemental Table S3). About 5% of fluorescing cells had no obvious
filaments or only aggregated GFP-tagged NMIIA. Only cells with clear filaments were chosen for the time lapse confocal microscopy. About 60 cells in 24 locations were monitored for over 24 h in each of 3 independent experiments. About 20–30% of monitored cells either died or were lost in the field of view before 24 h and were censored. About 50–60% of the remainder with GFP-IIA fluorescent filaments underwent a significant (Student’s t test, $p = 0.039$) redistribution from an even to a more peripheral location upon induction of S100P (Fig. 5A and supplemental Table S3), the remaining 30–40% did not show
an obvious change in distribution of filaments. In contrast, when S100P was not induced in HeLa-A3 or -A19 cells or HeLa cells were treated with doxycycline, cells with fluorescent NMIIA containing filaments exhibited a similar low level of about 10% of peripherally distributed filaments (supplemental Table S3). The amount of Triton-insoluble NMIIA was also significantly reduced by about 30–40% in HeLa-A19 cells 24 h after adding 1 μM doxycycline compared with that with without doxycycline (Fig. 5, B and C) (p < 0.05), but no significant difference was observed between HeLa cells with or without doxycycline treatment. These data indicate that S100P-induced redistribution of NMIIA is accompanied by the dissociation of NMIIA filaments.

**NMIIA Peptide Blocks NMIIA-S100P Interaction and S100P-enhanced Cell Migration**—The S100P-binding domain of NMIIA (Fig. 6A) was fused with TAT-HA2 (38). TAT is the HIV Tat protein transduction domain and is used for delivery of peptides into cells (Fig. 6A) (49). Because TAT-delivered peptides are often trapped in macropinosomes, HA2, the N-terminal of the influenza virus hemagglutinin protein, a well characterized, pH-sensitive peptide that destabilizes lipid membranes at low pH (50), was also incorporated to assist the escape of trapped peptides and improve their cellular activity (38). The resultant fusion peptide specifically blocked the interaction of S100P with immobilized NMIIA (Fig. 6B) and the FITC-labeled peptide was taken up by cells after an overnight incubation (Fig. 6C). When the fusion peptide was preincubated with HeLa-A19 cells for 48 h, cell migration was significantly increased by nearly 40% (Fig. 6D). This is probably because the peptide also partially overlaps the bundling domain of NMIIA (Figs. 2A and 6A) (46). Importantly, however, the peptide nearly completely inhibited the S100P-enhanced increase in cell migration (Fig. 6D). The control peptide containing only TAT-HA2 entered cells, but did not significantly affect cell migration with or without induction of S100P (Fig. 6D). These results support the contention that S100P targets NMIIA to enhance cell migration.

**Effects of S100P Up-regulation and NMII Knockdown on FAS**—When FAS were located by immunofluorescent staining for vinculin and phalloidin staining for actin filaments, the majority of actin filaments were terminated at FAS in the HeLa cells (Fig. 7, A and B). In parental HeLa and non-induced HeLa-A3 or -A19 cells, FAS and actin filaments were evenly distributed (Fig. 7, A–C) and only 5–10% of the fluorescent cells exhibited a peripheral distribution of FAS (supplemental Table S4). When S100P was induced for 24 h, the number of FAS in the HeLa-A3 and -A19 cells was significantly reduced by 2–3-fold (p ≤ 0.012) (supplemental Table S5) producing a peripheral distribution in 40–50% of stained cells (Fig. 7, E and F) (supplemental Table S4). In parallel, actin filaments became more peripherally distributed than in HeLa (Fig. 7, E and F) or in untreated cells (Fig. 7, A–C). When NMIIA was knocked down...
in HeLa cells, FAS were reduced by 10–20-fold ($p = 0.001$) (supplemental Table S5), and virtually disappeared, but the fewer thicker actin filaments were not redistributed to the cellular peripheries (Fig. 7G). In contrast, when NMII-B (Fig. 7H) and -IIC (Fig. 7I) were knocked down, there was no obvious effect on the average numbers/cell nor distribution of either FAS or actin filaments. These data suggest that S100P-induced NMIIA dissociation caused the redistribution and reduction of vinculin-containing and actin-terminating FAS.

**Inhibition of Vinculin Production Simulates S100P Enhanced Cell Migration**—Specific siRNAs to vinculin mRNA knocked down its protein level by about 80% (Fig. 8A) and significantly increased HeLa-A19 cell migration by nearly 2-fold (Fig. 8B). However, there was no further significant increase in cell migration upon induction of S100P (Fig. 8B). Control siRNA not directed to vinculin mRNA was without any effect (Fig. 8, A and B). The vinculin-containing FAS in HeLa-A19 cells after knockdown of vinculin also became much less apparent (Fig. 8C). These data demonstrate that S100P functions via a reduction in vinculin and FAS to increase cell migration.

**Effects of S100P Induction on Cell Adhesion**—Both NMIIA filaments and their associated FAS are important for cell adhesion (51). The effect of S100P on both NMIIA and FAS will be eventually reflected by cell adhesion changes. When seeded on plastic surfaces the cellular adhesion of HeLa cells treated with doxycycline was not significantly different from that of untreated HeLa cells (Fig. 9A). Once HeLa-A19 cells were treated with doxycycline to induce S100P, cellular adhesion was significantly reduced by 30–50% at 60 and 90 min compared with that of non-induced HeLa-A19 cells (Student’s $t$ test $p = 0.02$) (Fig. 9B), indicating that induction of S100P is able to reduce significantly cell adhesion to plastic surfaces in tissue culture. Further analysis showed that induction of S100P significantly reduced cell adhesion to collagen I- and II-coated surfaces, but not to surfaces coated with collagen IV, fibronectin, laminin, tenascin, or vitronectin (Fig. 9, C and D). The reason for the differential effects is not clear.

**Inhibition of FAK Kinase Abolishes the Stimulatory Effect of S100P on Cell Migration**—To investigate whether cell migration stimulated by S100P in HeLa cells requires activation of focal adhesion kinase (FAK), the major kinase for assembly and disassembly of FAS (52, 53), an inhibitor of its phosphorylation, TAE226 (54), was added 6 h after plating the cells in a Boyden chamber. Addition of 0.5–5 μM TAE226 reduced cell migration in a dose-dependent manner in HeLa-A3 cells. S100P-enhanced cell migration was abolished when the TAE226 concentration exceeded 1 μM (Fig. 10A), at which the FAK phosphorylation (Tyr-397) was significantly inhibited (supplemental Fig.
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S100P has been shown to promote cell migration in various cell types, including breast cancer cells (55, 56). In our study, we investigated the mechanism by which S100P induces cell migration in HeLa-A19 cells.

**Methodology**

We used siRNA transfection to knockdown the expression of proteins involved in cell migration. We induced S100P expression in HeLa-A19 cells using doxycycline and monitored cell migration. We also performed Western blotting and immunofluorescent staining to assess the expression of proteins involved in cell migration.

**Results**

- **Knockdown of Vinculin:** Knockdown of vinculin using siRNA did not affect cell migration significantly (Fig. 8A). However, when vinculin was knocked down, the ratio of pFAK/FAK was significantly reduced (Fig. 8B).
- **Effect of S100P on NMIIA:** S100P-induced cell migration was significantly enhanced when vinculin was knocked down (Fig. 8C). This suggests that S100P affects cell migration by altering the activity of NMIIA, possibly through changes in FAK phosphorylation.

**Discussion**

Migration of cancer cells away from the primary tumor is the critical initial step in metastasis. We have demonstrated that S100P differentially interacts with the NMII isoforms and affects their activities, leading to enhanced cell migration. This effect may be mediated through changes in FAK phosphorylation, which is a critical regulator of cell migration.

In conclusion, our findings suggest that S100P-induced cell migration in HeLa-A19 cells is mediated by the NMIIA isoform, and that this effect is dependent on FAK phosphorylation.

**References**

1. S100 molecules and their role in cell migration.
2. Vinculin and FAK in cell migration.
3. NMII isoforms and their effects on cell migration.

**Figure Legends**

- **Figure 8A:** Effects of knockdown of vinculin on cell migration. siRNAs specific for vinculin and control siRNA from Qiagen were transfected into HeLa-A19 cells. After 48 h, the cell migration assay was set up in the presence or absence of doxycycline. The rest of the cells were grown for 24 h further to check vinculin protein by Western blotting and immunofluorescent staining using anti-vinculin antibody.
- **Figure 8B:** Typical Western blot. B, means ± S.D. of cell migration rates relative to control are shown.
- **Figure 8C:** Typical images of HeLa-A19 cells 72 h after transfection with control siRNA (panels A–C) and vinculin siRNA (panels D–F). Green dots indicate vinculin (panels A and D) and red fibers indicate NMIIA (panels B and E). Panels C and F are the superimposed images of panels A and B, and panels D and E, respectively.
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**FIGURE 9. Effect of S100P on cell adhesion.** HeLa (A) and HeLa-A19 (B) were treated with [ ] or without [ ] doxycycline for 24 h and cell adhesion assays were carried out as described under “Experimental Procedures.” The percentage of adhesion = 100 x cells retained on the surface of normal culture plate (Costar)/total cells seeded. The means ± S.D. from 3 independent experiments are presented. The cell adhesion array kit (Millipore) was also used to detect the effect of S100P induction on the adhesion of HeLa (C) and HeLa-A19 (D) cells to surfaces coated with different extracellular matrix proteins. The cell adhesion without doxycycline treatment (■) was set as 100% and the means ± S.D. of the relative adhesion after doxycycline treatment (○) are presented.

*, Student’s t test, p < 0.02.

In vivo, we demonstrate that S100P is partially co-localized with NMII filaments and their physical interaction in living cells has been confirmed using FRET. The same mutations in S100A4 that prevent binding of this molecule to a fragment of NMIIA using an optical biosensor in vitro also prevent FRET between suitably engineered, fluorescently-labeled S100A4 and NMIIA in living HeLa cells (64), confirming that interaction also does not occur using these mutants in vivo. More importantly, we have for the first time demonstrated that S100P facilitates the dissociation of NMIIA filaments in living cells. This point is also supported by the fact that the total amount of insoluble NMIIA filaments is reduced after S100P induction. In HeLa-A3 or -A19 cells, the distribution of S100P is more central and NMIIA filaments are normally distributed more uniformly before rather than after induction of S100P. The high levels of S100P in the central region induce the redistribution of NMIIA filaments to the cellular peripheries and thus may weaken their anchoring force. Inhibition of S100P stimulation of migration by a specific peptide of NMIIA containing the S100P binding region demonstrates that interaction between S100P and NMIIA is required for this effect in living cells.

The fact that dissociation of NMIIA filaments increases cell migration has also been observed in other cell systems. When blebbistatin is used to inhibit NMIIA contraction, the migration of mouse hepatic stellate cells is accelerated (66). The S1943A mutation of NMIIA increases its ability to form filaments and concomitantly reduces migration of MDA-MB-231 cells, whereas S1943D or S1943E mutations decrease its assembly and promote cell migration (67, 68). Rho and Rac kinases are profound regulators of the cytoskeleton and hence also influence cell migration (69). With Rac1, one of the important pathways is Rac1/CDC42/PAK (p21-activated kinase), which causes disassembly of stress fibers and FAS, and enhances cell migration (70). Another important pathway is Rac1/CDC42/IQ-GAP1, which forms a complex that links the actin cytoskeleton and microtubules at the leading edges and facilitates cancer cell migration (71). Thus, the Rac1 inhibitor, NSC23766 (72), which normally blocks dissociation of actomyosin IIA filaments and FAS (73), inhibits migration of S100P-expressing HeLa-A3 cells (supplemental Fig. S6A). Moreover, specific siRNAs to Rac1 knocked down over 90% of Rac1 protein in HeLa-A19 cells and significantly reduce cell migration by about 67 and 83%, respectively, and to the same basal rate in uninduced and S100P-induced cells (supplemental Figs. S6, B and C). Rac1 knockdown also caused abundant and abnormal NMIIA filaments (supplemental Fig. S6D). With Rho activation, integrin clusters are aligned through their attachment to the ends of stress fibers to form FAS. Thus inhibition of Rho-ROCK activation by Y27632 (74), which normally blocks assembly of the actomyosin IIA filaments and FAS formation (75, 76), significantly increases cell migration in our cell systems. Similarly RhoA knock down in HeLa-A19 cells by 70% using specific siRNAs reduced NMIIA filaments (supplemental Figs. S6, C and D) and significantly enhanced cell migration by nearly 2-fold attaining the same rate as in S100P-induced cells; there was, however, no further increase in specific siRNA-treated S100P-induced cells (supplemental Fig. S6B). These data strongly suggest that NMIIA filaments exert an anchoring rather than a migratory force. It appears that any mechanism that reduces NMIIA assembly, such as knockdown using specific siRNA, inhibition of Rho kinase and FAK, or causes activation of Rac1 kinase and, as shown here, up-regulation of certain S100 proteins will eventually facilitate cells to migrate away from their current location (supplemental Fig. S7).

In this study, we show that knockdown of NMIIA but not NMIIIB or -IIC dramatically reduces the number of vinculin-containing FAS, confirming reports in other cell systems (23, 77). For example, when the contractility of actomyosin is reduced by inhibition of myosin light chain phosphorylation or blebbistatin, an inhibitor of myosin II, the FAS lose vinculin and talin and consequently reduce their adhesion forces (51). How-
ever, these methods of inhibiting contractility of actomyosin do not change adhesion forces in vinculin null cells (51). These results suggest that NMIIA exerts its anchoring forces through talin and vinculin-containing FAS. FAS contains about 900 identified proteins and over half of them respond to the contractility changes of actomyosin (78), indicating the fundamental influence of NMIIA on FAS. In our cell systems we show that the S100P-induced dissociation of NMIIA filaments is another means of reducing the number of vinculin-containing FAS.

The FAS is essential for cell adhesion (79). Here we show that induction of S100P significantly reduces the number of vinculin-containing FAS. One of the important modulators of the dynamic changes of NMIIA filaments is FAK, which also regulates the effects of NMIIA contractility on FAS (77, 80). FAK signaling is critical to FAS turnover, therefore once FAS has been assembled, inhibition of FAK will prevent the loss of FAS (81). In our work, we show that TAE226, an inhibitor of FAK, significantly reduces the S100P-induced loss of FAS once it is added after cells adhere to culture surfaces. As a result, S100P-enhanced cell migration is also inhibited. Moreover inhibition of vinculin production by specific siRNAs both replicates and nullifies the stimulatory effect of S100P. Therefore this combined data suggests that S100P-enhanced cell migration also requires the disassembly of vinculin-containing FAS.

It is more than a decade since the interaction of S100A4 with a major motor protein NMIIA in vitro (12, 20, 82, 83) and in vivo (37) has been identified, but it was not clear how this interaction affects cell migration. Previous studies used S100A4 stable expression cells and its effects cannot be explicitly demonstrated. With the development of S100P-inducible cell lines, the dynamic changes of NMIIA filaments and associated FAS as well as cellular adhesion upon induction of S100P become much more obvious than that in stable expression cells and have been clearly demonstrated in this study. Accordingly, cell migration is much more strongly enhanced upon S100P induction than that in cells that stably express similar levels of S100P. These data suggest that oscillation of S100P in cancer cells may be more effective in promoting cell migration and metastasis. In
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summary, our new contribution here is the establishment of the direct cause-effect relationship between overexpression of S100P and cell migration as well as the underlying mechanism. This novel mechanism consists of a signal pathway involving dissociation of NMIIA filaments and a consequent weakening of FAS anchoring forces coupled with the unopposed locomotory action of unaffected NMIIB filaments to drive the cells forward. This work lays the basis for why certain S100 proteins cause metastasis in rodent and human cancers.

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