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Increase in Motility of Breast Cancer Cells

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Characterization of the Metastasis-associated Protein, S100A4

ROLES OF CALCIUM BINDING AND DIMERIZATION IN CELLULAR LOCALIZATION AND INTERACTION WITH MYOSIN*

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Elevated S100A4 protein expression is associated with metastatic tumor progression and appears to be a strong molecular marker for clinical prognosis. S100A4 is a calcium-binding protein that is known to form homodimers and interacts with several proteins in a calcium-dependent manner. Here we show that S100A4 localizes to lamellipodia structures in a migrating breast cancer-derived cell line and colocalizes with a known S100A4-interacting protein, myosin heavy chain IIA, at the leading edge. We demonstrate that S100A4 mutants that are defective in either their ability to dimerize or in calcium binding are unable to interact with myosin heavy chain IIA. An S100A4 mutant that is deficient for calcium binding retains the ability to form homodimers, suggesting that S100A4 can exist as calcium-free or calcium-bound dimers *in vivo*. However, a calcium-bound S100A4 monomer only interacts with another calcium-bound monomer and not with an S100A4 mutant that does not bind calcium. Interestingly, despite the calcium dependence for interaction with known protein partners, calcium binding is not necessary for localization to lamellipodia. Both wild type and a mutant that is deficient for calcium binding colocalize with known markers of actively forming leading edges of lamellipodia, Arp3 and neuronal Wiskott-Aldrich syndrome protein. These data suggest that S100A4 localizes to the leading edge in a calcium-independent manner, and identification of the proteins that are involved in localizing S100A4 to the lamellipodial structures may provide novel insight into the mechanism by which S100A4 regulates metastasis.

Cancer mortality most often results directly from metastatic spread to distal, vital organs. The isolation and characterization of molecular markers that can identify patients at a high risk for metastatic spread and consequently a poor prognosis is of obvious importance. There is increasing evidence that a member of the S100 family of proteins, S100A4 (also known as pEL-98, *mts1*, p9Ka, CAPL, calvasculin, and *Fsp1*), is one such molecular marker for metastatic potential with high prognostic significance. An increase in S100A4 protein expression has been correlated with a worse prognosis for patients with differ-

ent types of cancer including colorectal, gallbladder, bladder, esophageal, breast, and nonsmall lung cancer (1–7). S100A4 has also been found to be more highly expressed in malignant tumor samples than in either benign tumor cells or normal tissue (3, 8–11). Causal evidence for the role of S100A4 in metastasis comes from studies that show a metastatic phenotype can be either induced by ectopic expression (12, 13) or inhibited by reduced expression (14, 15). However, transgenic mice overexpressing S100A4 do not develop tumors, suggesting that S100A4 is not tumorigenic *per se* (16). Instead, S100A4 can more appropriately be categorized as a potential inducer of metastasis in a given tumorigenic background. This categorization is supported by the observation that the progeny from mice overexpressing S100A4 crossed with mice overexpressing the *HER2/Neu* oncogene develop tumors that metastasize more frequently and more rapidly than tumors in the parental *neu* mice (17).

Studies to determine the mechanistic basis for S100A4 function have shown a potential role for S100A4 in several different facets of tumor progression including motility, invasion, and apoptosis (18–20). It has also been reported that S100A4 can be secreted and once extracellular can affect angiogenesis, cell differentiation, and migration (21–23). S100A4 has been shown to be localized along stress fibers, concentrated in the perinuclear region, or at membrane protrusions (24–26). Because S100A4 has no known enzymatic activity, interactions with other proteins, both intracellularly and extracellularly, are undoubtedly critical. The putative role of S100A4 in motility has been strengthened through the characterization of its interaction with a critical cytoskeletal component of motility, non-muscle myosin. *In vitro* biochemical assays have shown that S100A4 can bind specifically to nonmuscle myosin heavy chain IIA (MHC-IIA),¹ disrupt myosin self-assembly, and inhibit protein kinase C or casein kinase II-dependent phosphorylation of the heavy chain (27–32). S100A4 was reported recently (26) to interact with liprin β 1 and to inhibit *in vitro* phosphorylation of liprin by the same two protein kinases, protein kinase C and casein kinase II. The tumor suppressor protein p53 has also been identified as an S100A4-interacting protein and may provide a link between S100A4 and apoptosis (20). Other reported S100A4-interacting proteins include tropomyosin, methionine aminopeptidase, and CCN3 (cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed) (33–35).

The proteins in the S100 family share the ability to bind to calcium through the use of two EF-hand motifs (36). Calcium-dependent regulation of S100A4 involves a conformational

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¹ The abbreviations used are: MHC-IIA, nonmuscle myosin heavy chain IIA; mAb, monoclonal antibody; pAb, polyclonal antibody; HA, hemagglutinin; VSV, vesicular stomatitis virus; N-WASP, neuronal Wiskott-Aldrich syndrome protein.

shift that occurs upon binding to calcium. This conformational shift is necessary for S100A4 interaction with its known protein partners. Biochemical analysis of the calcium binding event has shown that the two EF-hand motifs bind calcium noncooperatively (37). Based on early studies of the S100A1 and S100B proteins, it is believed that the S100-specific N-terminal EF-hand motif (site I) has a lower calcium binding affinity than the canonical C-terminal EF-hand motif (site II) (38, 39). However, a recent study (40) demonstrated that for at least S100A4, the N-terminal EF-hand motif actually fills with calcium first. It remains unclear whether there is a dissectible, functional relevance to calcium binding at each of the EF-hand motifs. In addition, whether this calcium-dependent regulation of S100A4 extends to other properties such as intracellular localization remains unclear.

S100 proteins have been shown to also share the ability to homodimerize and/or heterodimerize with other S100 family members (36). S100A4 itself was shown previously to both homodimerize (41) and heterodimerize with S100A1 (42). Homodimerization is reportedly enhanced by the presence of calcium (41). However, it remains unclear whether calcium binding at either EF-hand motif is essential for homodimerization. It is also unknown whether homodimerization is actually necessary for interaction with the known S100A4 protein partners and by extension, functionality.

To address the functional dependence of S100A4 on calcium binding and dimerization, we analyzed the effects of site-directed mutagenesis in the two calcium-binding domains and dimerization interface. These studies demonstrate that S100A4 interaction with MHC-IIA is dependent on calcium binding, specifically at site II. Homodimerization of S100A4 can occur in the complete absence of calcium, but homodimerization is necessary for interaction with MHC-IIA. Despite the calcium dependence for association with known partner proteins, S100A4 is targeted to the leading edge of lamellipodia in migrating breast cancer cells in a calcium-independent manner, suggesting an additional level of regulation of S100A4 that is independent of calcium.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibodies were used: mAb anti-HA tag (12CA5; produced and purified by the Antibody Facility of Cold Spring Harbor Laboratory), mAb anti-VSV tag (V-5507; Sigma), mAb anti-T7 tag (69522-1; Novagen), pAb anti-MHC-IIA (PRB-440P; Covance), pAb anti-MHC-IIB (PRB-445P; Covance), pAb anti-Arp3 (07-272; Upstate Biotechnology), and pAb anti-N-WASP (a kind gift from M. W. Kirschner, Harvard Medical School, Boston, MA) (43). pAb anti-S100A4 (AP1178) was generated as follows. A peptide corresponding to the C-terminal 16 amino acids of human S100A4 (NH₂-CNEFFEGFPD-KQPRKK-CO₂H) was obtained (Research Genetics) and conjugated to keyhole limpet hemocyanin (Pierce). The peptide-keyhole limpet hemocyanin conjugate was injected into rabbits (Covance), and antiserum was obtained and affinity-purified using a peptide column generated with PreACT activated resin (Novagen), as per the manufacturer's instructions.

Secondary antibodies goat anti-mouse and anti-rabbit IgG conjugated to Alexa Fluors were purchased from Molecular Probes, Inc. (Eugene, OR) as was Texas Red-conjugated phalloidin. All chemicals and reagents were obtained from Sigma, unless otherwise noted, and all cell culture reagents were obtained from Invitrogen.

Cell Culture—MDA-MB-231 cells were from ATCC (HTB-26), and 293 cells were from the Tissue Culture Facility (Cold Spring Harbor Laboratory). All cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Expression Vectors, Transient Transfections, and Retroviral Transduction—Human S100A4 cDNA was isolated from an IMAGE Consortium cDNA clone (clone number 3543900) by PCR, sequenced, and inserted into derivatives of the expression plasmid pCG (44). pCGN, pCGV, and pCGT give rise to N-terminal translational fusion protein with the HA epitope, VSV epitope, and T7 epitope, respectively. All

transient transfections were done using FuGENE6 (Roche Applied Science) according to the manufacturer's instructions. The T7-tagged S100A4 was subsequently subcloned into the retroviral vector pBabe (45) and transfected into Phoenix amphotropic packaging cells to produce recombinant viruses (46). MDA-MB-231 cells were infected with virus-containing supernatants in the presence of 4 µg/ml polybrene, followed by selection and maintenance in the presence of 1 µg/ml puromycin.

Immunoprecipitation and Western Blot Analysis—Cells grown in 60-mm culture plates were harvested in 500 µl of immunoprecipitation buffer containing 1% Triton X-100, 20 mM imidazole, 300 mM KCl, 5 mM MgCl₂, 3% glycerol, and EDTA-free Complete (Roche Applied Science) protease inhibitor mixture. Lysates were centrifuged briefly to remove cell debris. 50 µl of the supernatant was added to an equal volume of 2× Laemmli sample buffer and represents whole cell lysate prior to immunoprecipitation. The remaining supernatant was pre-cleared using protein A-agarose beads (Roche Applied Science) and then incubated for 1 h at 4 °C with antibodies to either the HA tag or S100A4. The samples were then incubated with protein A-agarose beads for 1 h at 4 °C. The beads and immunoprecipitated protein were recovered by mild centrifugation, washed five times with immunoprecipitation buffer, and then boiled in Laemmli sample buffer. Immunoprecipitated samples and whole cell lysate samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). Immunoreactive bands were detected by chemiluminescence (PerkinElmer Life Sciences) following incubation of the nitrocellulose with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies.

Prior to harvesting the cells, *in vivo* calcium levels were altered by incubating cells with 5 µM ionomycin or Me₂SO as a control for 3 min at room temperature. Calcium conditions were maintained throughout the immunoprecipitation by the addition of either 5 mM CaCl₂ or 10 mM EGTA to the immunoprecipitation buffer used for both harvesting the cells and for all wash steps.

Sequence Alignment and Site-directed Mutagenesis—Sequence alignment and analysis were performed with ClustalW software. Single and double point mutations were introduced into the full-length human S100A4 cDNA using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Desired mutations were confirmed by sequence analysis for all constructs.

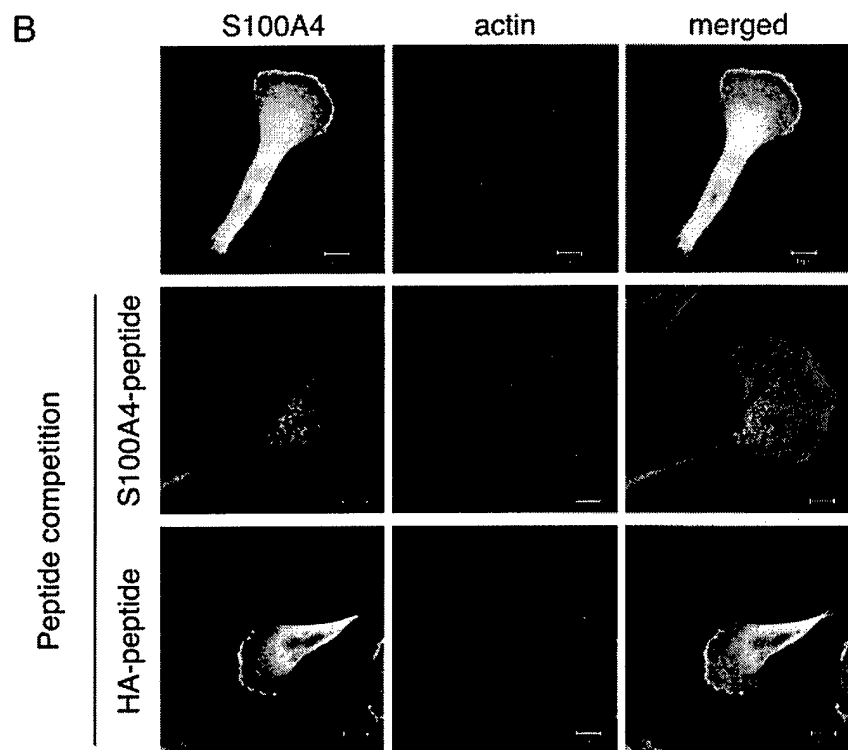
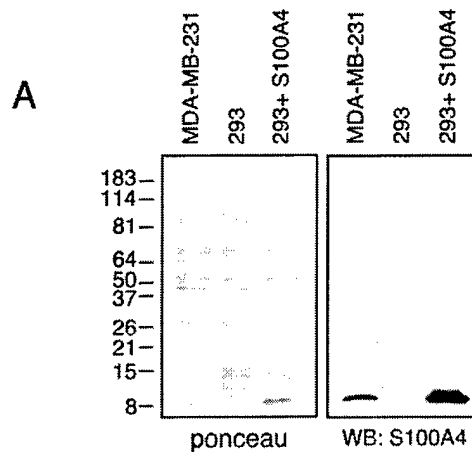
Immunofluorescence—Glass coverslips were pre-coated with 5 µg/cm² collagen IV (BD Biosciences) in 0.05 M HCl and blocked with 0.5% bovine serum albumin. Cells grown on these pre-coated coverslips were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% Triton X-100 and then blocked with 5% bovine serum albumin at room temperature. Samples were incubated with primary antibodies against S100A4 (1:500), N-WASP (1:200), Arp3 (1:100), T7 tag (1:500), and MHC-IIA (1:5000) at room temperature for 1 h. This was followed by incubation with Alexa Fluor-conjugated secondary antibodies and Texas Red-conjugated phalloidin at room temperature for 1 h. Images were collected by confocal microscopy (Zeiss) and processed using LSM software.

Peptide competition for S100A4 antibody was done by pre-incubation for 30 min with control peptide (HA-tagged peptide) or antigenic peptide at room temperature (2.5 µg of peptide per 100 µl of affinity-purified S100A4 antibody diluted 1:500).

Recombinant Protein Production and Ca⁴⁵ Overlay—Wild type and mutant human S100A4 cDNAs were subcloned into the pET-19b vector (Novagen) using *Nde*I and *Bam*HI sites to create pET-19b-S100A4, which produces a His-S100A4 fusion protein. *Escherichia coli* were transformed with these constructs, and expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Four h post induction, the bacteria were harvested by centrifugation, and proteins were extracted using BugBuster (Novagen) 10× protein extraction reagent diluted to 1× using extraction buffer containing 300 mM NaCl and 50 mM sodium phosphate, pH 7.0. Cell debris was removed by centrifugation, and the supernatant was applied to a TALON (Clontech) metal affinity resin column and allowed to incubate for 1 h at 4 °C. The column was then washed extensively with extraction buffer before eluting purified His-S100A4 from the resin with extraction buffer containing 150 mM imidazole.

Recombinant proteins were then subjected to a calcium binding assay that has been described previously (47). Recombinant S100A4 proteins, calmodulin (Sigma), and lysozyme (Sigma) were resolved by SDS-PAGE and transferred to nitrocellulose. The membrane was soaked in equilibration buffer (60 mM KCl, 5 mM MgCl₂, and 10 mM imidazole, pH 6.8) and then incubated in equilibration buffer containing 1 mCi/liter Ca⁴⁵ (Amersham Biosciences) for 10 min. The membrane

FIG. 1. S100A4 antibody specificity and immunolocalization of endogenous S100A4 protein in MDA-MB-231 cells. *A*, whole cell lysate from MDA-MB-231, 293, and 293 cells transiently transfected with wild type S100A4 were separated by SDS-PAGE and transferred to nitrocellulose. Transferred proteins were visualized using Ponceau stain before Western blot (WB) analysis with peptide affinity-purified antiserum for S100A4 (AP1178). *B*, MDA-MB-231 cells plated on collagen IV ($5 \mu\text{g}/\text{cm}^2$)-coated coverslips were stained for actin using phalloidin and for S100A4 with AP1178. Peptide competition of AP1178 with either control (HA-epitope) peptide or antigenic peptide was done by pre-incubating AP1178 with $2.5 \mu\text{g}$ of peptide per $100 \mu\text{l}$ of AP1178 diluted 1:500. Bar, $10 \mu\text{m}$.



was then rinsed twice with 50% ethanol for 5 min. Autoradiography of the Ca^{45} -labeled proteins was performed by exposing the dried membrane to Eastman Kodak Co. BioMax MR film for 24 h.

RESULTS

Intracellular S100A4 Localization—We began our study of the human S100A4 protein by generating an affinity-purified rabbit antiserum (AP1178) against the far C-terminal 16 amino acids of the human S100A4 protein. AP1178 detects a single 10-kDa band in extracts from cells known to express endogenous S100A4 (MDA-MB-231) or 293 cells transiently transfected with the human S100A4 gene (Fig. 1A). Using this affinity-purified S100A4 antibody, we examined the intracellular localization of endogenous S100A4 in MDA-MB-231 cells. These highly motile and invasive carcinoma cells have been shown to form tumors and metastasize aggressively *in vivo*. Because expression of S100A4 has been linked to these facets of tumor progression and is highly expressed in these cells, MDA-MB-231 cells are an appropriate breast cancer model system in which to study S100A4. These cells exhibit a diverse array of morphologies when plated directly on to glass

coverslips. In contrast, when plated on coverslips pre-coated with collagen, the cells more uniformly exhibit a spread but polarized morphology consistent with migrating cells. We used AP1178 to study localization of endogenous S100A4 in cells grown under these conditions and observed the previously reported perinuclear enrichment. However, we also noticed a consistent pattern of intense staining at the leading edge of cells with prominent lamellipodia (Fig. 1B). To determine the specificity of this staining pattern we carried out a peptide competition assay. Pre-incubation of AP1178 with the S100A4 peptide resulted in the complete loss of intense staining at both the perinuclear region and at the leading edge of lamellipodia (Fig. 1B). In contrast, a nonspecific peptide (HA-epitope peptide) failed to abolish the staining pattern at these intracellular sites (Fig. 1B).

To further confirm this intracellular localization, we established MDA-MB-231 sublines stably expressing a T7-tagged wild type S100A4 protein. Immunofluorescent staining of the parental cells with the T7 antibody yields background staining limited to the nucleus with virtually complete sparing of any

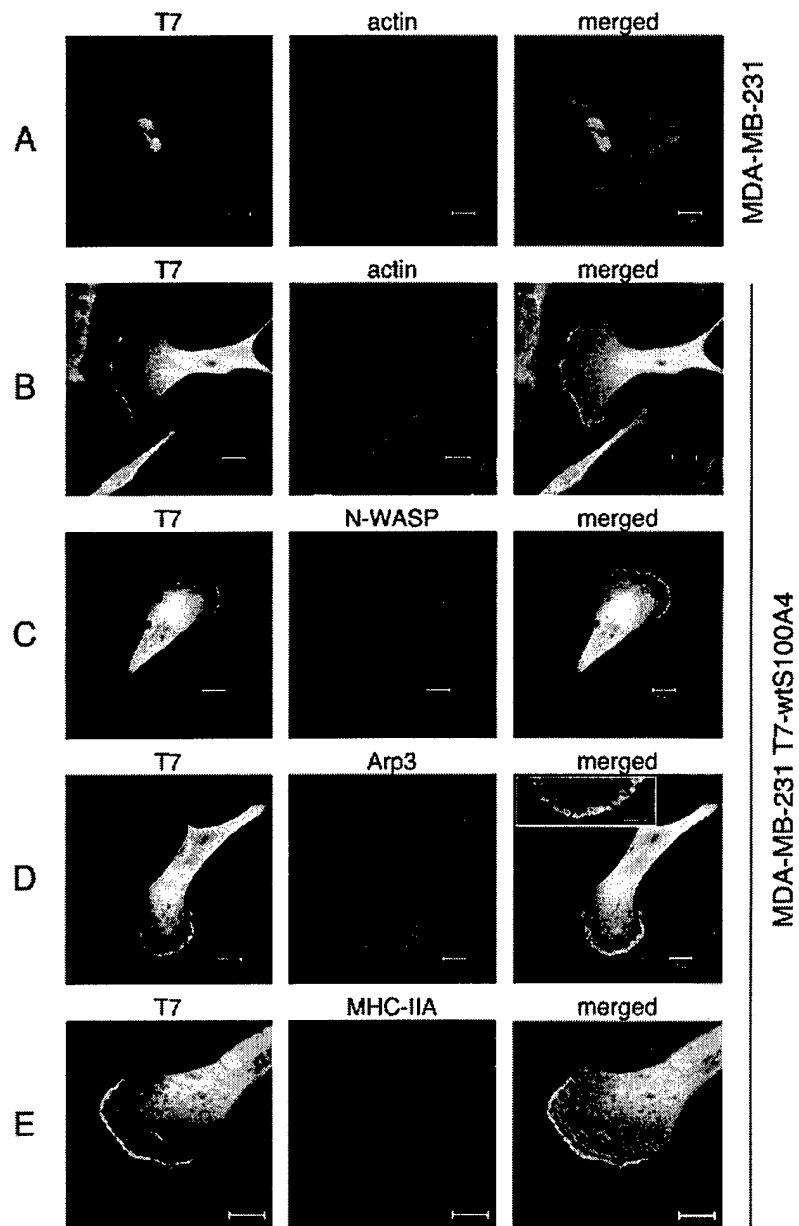


FIG. 2. Colocalization of wild type S100A4 with known markers of leading edge formation. Parental MDA-MB-231 cells (A) and cells stably expressing T7-tagged wild type (*wt*) S100A4 (B–E) were plated on collagen IV-coated coverslips. Cells were then stained for exogenous T7-tagged protein using an anti-T7 mAb (A–E) and co-stained for actin with phalloidin (A and B), anti-N-WASP pAb (C), anti-Arp3 pAb (D), or anti-MHC-IIA pAb (E). Bar, 10 μ m. Inset in D was taken at $\times 3$ magnification. Bar, 5 μ m.

cytoplasmic background (Fig. 2A). The T7-tagged wild type protein localizes to the same perinuclear and leading edge locations as observed for the endogenous protein (Fig. 2B).

The particular cellular localization that we observed for S100A4 is reminiscent of the cellular localization of several proteins that are known to be functionally important for lamellipodial formation. To more precisely characterize the localization of S100A4 at the leading edge, we compared the localization of S100A4 to two proteins known to localize to the leading edge, N-WASP and Arp3 (48, 49). We found the wild type S100A4 to almost perfectly colocalize with N-WASP in MDA-MB-231 cells at the very tip of the leading edge of lamellipodia (Fig. 2C). Wild type S100A4 also colocalized to the same leading edge region as the Arp3 complex, but higher magnification microscopy clearly showed that they are in distinct compartments within that region (Fig. 2D). We were unable to determine whether the N-WASP colocalization was similarly restricted to the region and not to a specific subcompartment because of limitations in the resolution seen with the N-WASP and S100A4 antibodies. The regional colocalization, however, confirms S100A4 localization at the very tips of dynamic lamelli-

podia as indicated by the presence of both N-WASP and the Arp2/3 complex. Given the hypothesized functional importance of the interaction of S100A4 with MHC-IIA, we also compared the localization of S100A4 with MHC-IIA. We found MHC-IIA to be more widely distributed in the cell but did find it to colocalize with S100A4 at the leading edge (Fig. 2E).

Calcium Binding-dependent MHC-IIA Interaction and Homodimerization—The specific localization of endogenous S100A4 to the leading edges of lamellipodia raises the question of how S100A4 is targeted to that region. Because calcium binding and dimerization are two properties shared by most S100 proteins, these properties are obvious candidates for involvement in the observed localization. We began our evaluation of these candidates using biochemical techniques. First, we evaluated S100A4 calcium binding by testing the reported dependence on calcium binding for S100A4 interaction with a known partner protein, MHC-IIA. Using AP1178 to immunoprecipitate the endogenous S100A4 from MDA-MB-231 cells, we specifically pulled down MHC-IIA only in the presence of calcium (Fig. 3A). Although the IIB isoform of myosin heavy chain is also expressed in these cells, S100A4 does not appear

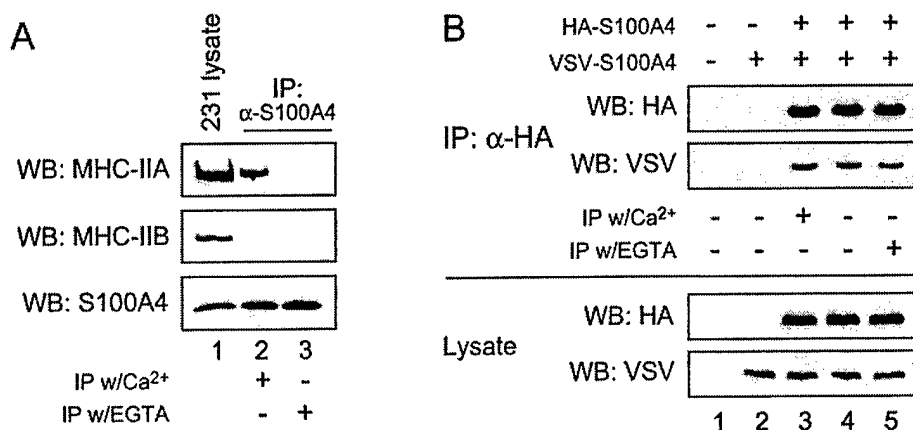


FIG. 3. S100A4 interacts with myosin heavy chain IIA and homodimerizes. *A*, AP1178 was used to immunoprecipitate the endogenous S100A4 from MDA-MB-231 cell lysate in the presence of 5 mM $CaCl_2$ (lane 2) or 10 mM EGTA (lane 3). The whole cell lysate and immunoprecipitates (IP) were analyzed by Western blot (WB) using isoform-specific antibodies for either MHC-IIA or IIB, as well as with AP1178 for S100A4. *B*, 293 cells were transiently co-transfected with an HA-tagged S100A4 construct and a VSV-tagged S100A4 construct. The HA-tagged protein was specifically immunoprecipitated with an HA tag monoclonal antibody (12CA5). Either 5 mM $CaCl_2$ (lane 3) or 10 mM EGTA (lane 5) was added during all steps of the immunoprecipitation. Whole cell lysate before immunoprecipitation and the immunoprecipitates were analyzed by Western blot using monoclonal antibodies against either the HA or VSV tags.

to interact with this isoform (Fig. 3A). This result supports previous reports that MHC-IIA is an S100A4 partner protein and that this interaction is calcium-dependent.

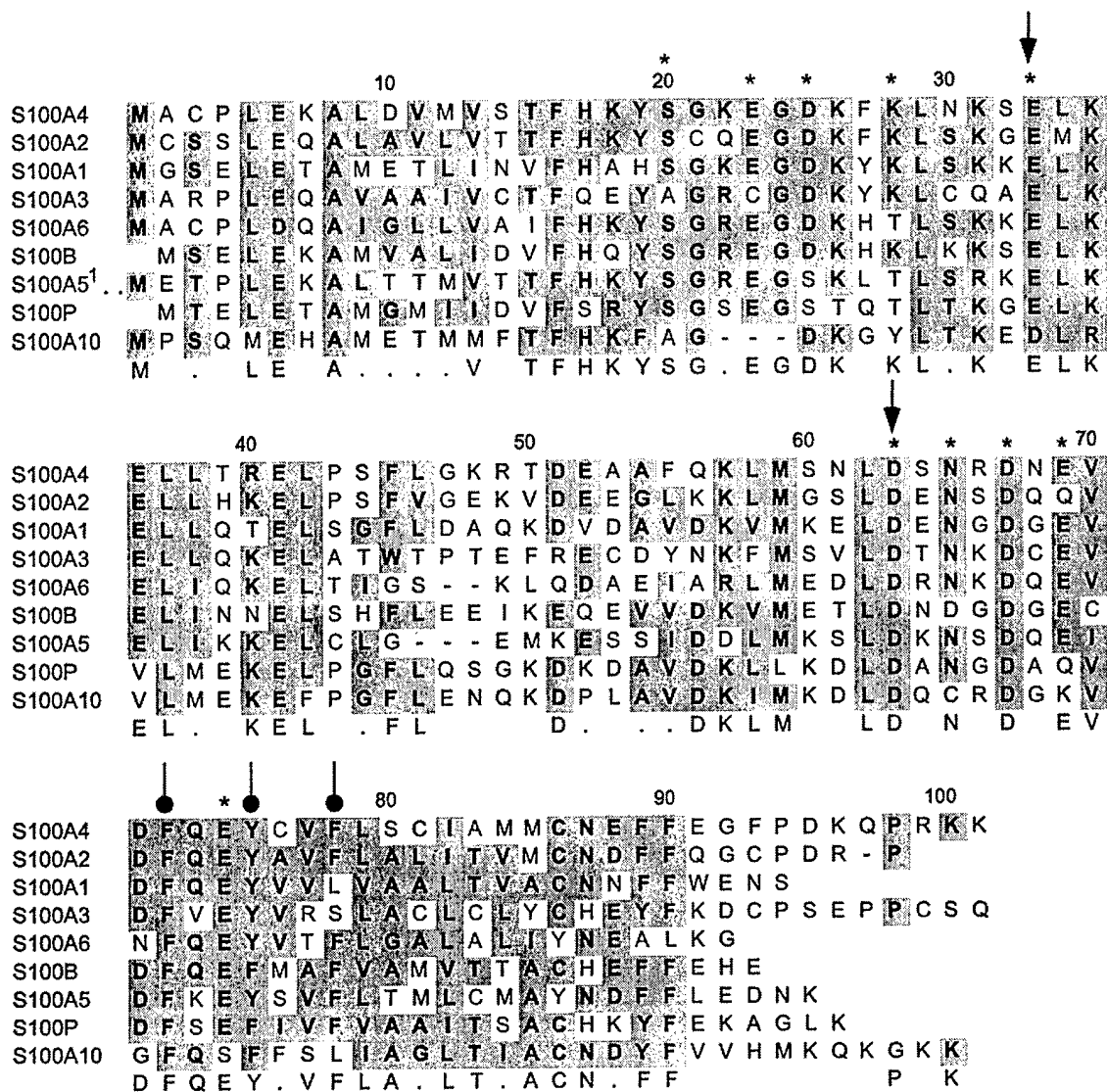
Next, we assessed S100A4 homodimerization using an assay based on our previous work on tropomyosin dimerization that utilizes co-immunoprecipitation of differently tagged constructs (50, 51). We co-transfected expression vectors encoding HA- and VSV-tagged human S100A4 into 293 cells, which do not express endogenous S100A4. Immunoprecipitation of the HA-tagged protein using a monoclonal antibody against the HA tag (12CA5) yielded co-immunoprecipitation of the VSV-tagged protein (Fig. 3B). This demonstrates that S100A4 can homodimerize *in vivo*. As a negative control the same antibody failed to immunoprecipitate the VSV-tagged protein in the absence of any HA-tagged S100A4 (Fig. 3B). In our assay, homodimerization was detected regardless of the presence or absence of calcium in the immunoprecipitation buffers, as controlled by the addition of 5 mM $CaCl_2$ or 10 mM EGTA, respectively (Fig. 3B). These data suggest that calcium binding is in fact not essential for a stable homodimer.

Construction of S100A4 Mutants Deficient in Calcium Binding or Dimerization—To further evaluate the roles of calcium binding and dimerization in S100A4 localization and function, we sought to develop a set of S100A4 mutant constructs deficient in these properties. To begin with, we attempted to generate a calcium binding-deficient mutant S100A4. It has been reported that changing single amino acids critical to the coordination of calcium in EF-hand motifs can affect calcium binding (53). Two of these critical residues are the acidic residues almost always found at the X and -Z coordination site of EF-hand motifs, a pattern that is true for S100A4 and the other S100 family members (Fig. 4). Changing either of these coordinate acidic residues to its corresponding amine can abolish calcium binding (53). Based on this report, we used site-directed mutagenesis to convert both the -Z position glutamic acid 33 to glutamine and the X position aspartic acid 63 to asparagine to generate a mutant unable to bind calcium at site I and site II (*mutCaI+II*) (Fig. 4). To study the relative functional importance of each of the calcium-binding sites, we also generated mutants unable to bind calcium at either site I (*mutCaI*) or site II (*mutCaII*). In addition, based on previous studies on homodimerization of both S100A4 (41) and the closely related S100A6 (52), we used site-directed mutagenesis to create three separate potential dimerization-deficient

mutants, changing highly conserved hydrophobic residues at positions 72 (*mutDimI*), 75 (*mutDimII*), and 78 (*mutDimIII*) (Fig. 4). Each mutant construct was sequenced to confirm that only the desired mutation(s) were introduced.

We analyzed these mutants to determine whether the specific changes in amino acids resulted in the expected changes in their biochemical properties. First, we assessed the effect of the mutations on dimerization using the co-immunoprecipitation assay described for Fig. 3B. HA-tagged wild type or mutant S100A4 constructs were co-transfected with VSV-tagged wild type S100A4 into 293 cells. Immunoprecipitation of HA-tagged *mutDimI* and *mutDimII* did not pull down any VSV-tagged wild type protein indicating that the single amino acid change introduced did prevent dimerization and therefore can be considered dimerization-deficient (Fig. 5A). In contrast, *mutDimIII* retained the wild type ability to dimerize, suggesting that this residue is not in fact critical for S100A4 dimerization (Fig. 5A). It is worth noting that we consistently observed lower amounts of the dimerization-deficient mutants when expressed by transient transfection. We believe that this is consistent with the hypothesis that these single amino acid changes do not necessarily affect dimerization by directly interfering with the dimerization interface. Instead, it has been proposed that these residues are critical for the stable monomeric conformation necessary for dimerization (41). One possible explanation therefore for the reduced amount of detectable *mutDimI* and *mutDimII* protein by Western blot is because of a reduced overall stability in the protein that ultimately affects the half-life of the protein. However, the fact that we can immunoprecipitate a significant amount of *mutDimI* or *mutDimII* provides strength to the observation that we do not co-immunoprecipitate a dimer partner.

To evaluate the effect of our mutations on calcium binding, we tested specific mutant constructs in a standard calcium binding assay (47). We generated recombinant wild type and mutant S100A4 protein from *E. coli* and confirmed specific expression of recombinant S100A4 proteins through Western blotting with our S100A4-specific antibody (data not shown). These recombinant proteins were then separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with radioactive Ca^{45} . Ca^{45} binding is detected using autoradiography, revealing a dark band where there is a protein like calmodulin that can bind to calcium (Fig. 5B). In contrast, proteins like lysozyme that do not bind calcium appear as a white shadow against a gray background that results from Ca^{45} adhering



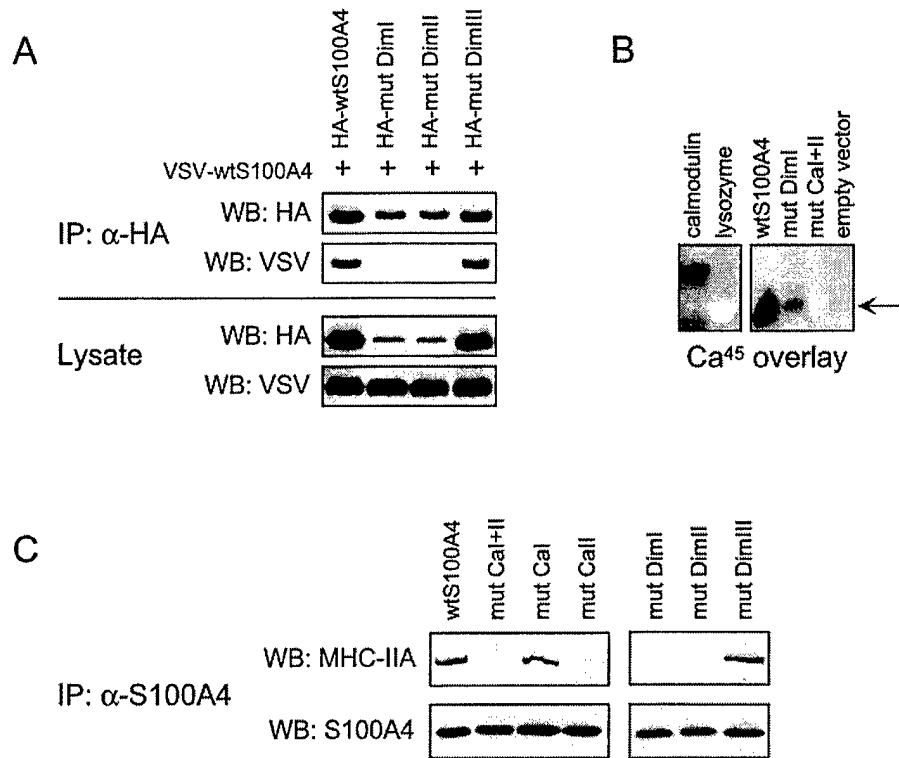
mut Cal	-	E33Q
mut CalI	-	D63N
mut Cal+II	-	E33Q,D63N
mut DimI	-	F72Q
mut DimII	-	Y75K
mut DimIII	-	F78A

FIG. 4. S100A4 sequence alignment. ClustalW was used to align human S100A4 with eight other S100 family members. Gray shading identifies residues that share either identity or similarity. Asterisks indicate residues that coordinate calcium. Arrows with triangle arrowheads indicate residues that were mutated to affect calcium binding. Arrows with circle arrowheads indicate residues that were mutated to affect dimerization. 1, the first 18 amino acids of S100A5 are MPAAWILWAHSHSELHTV.

nonspecifically to the protein-free areas of the gel (Fig. 5B). We selected one of the dimerization-deficient mutants, *mutDimI*, and the doubly calcium binding-deficient mutant, *mutCaI+II*, and tested them for the ability to bind calcium relative to the wild type protein. As was expected, wild type S100A4, which has been shown previously to bind calcium, reveals a dark band (Fig. 5B). *MutDimI* also appears on the film as a dark band, albeit less intensely than the wild type protein (Fig. 5B). In contrast, *mutCaI+II* appears as a white band against the gray

background (Fig. 5B), suggesting that it is deficient for calcium binding. It has been shown previously that the two EF-hand motifs bind calcium in a noncooperative manner, suggesting that the calcium binding affinity of each EF-hand motif is independent of binding calcium at the other EF-hand motif. Therefore, had our mutagenesis failed to abolish calcium binding at either site, *mutCaI+II* should still have bound some calcium and have appeared as a dark band in this assay under excess Ca^{45} conditions.

FIG. 5. Characterization of mutant S100A4 constructs. Mutant S100A4 proteins were tested for the ability to homodimerize, bind calcium, and interact with MHC-IIA. **A**, HA-tagged wild type (*wt*) or HA-tagged dimerization mutant (*mut*) S100A4 were co-transfected with VSV-tagged wild type into 293 cells. 12CA5 was used to specifically immunoprecipitate (IP) the HA-tagged protein from the cell lysate, and dimerization was assessed by Western blot (WB), as described in the legend to Fig. 3B. **B**, His-tagged purified recombinant S100A4 proteins, recombinant calmodulin, and lysozyme were subjected to SDS-PAGE and then transferred to nitrocellulose. The nitrocellulose was incubated in a solution containing 1 mCi/liter ^{45}Ca followed by autoradiography for 24 h. **C**, wild type or mutant S100A4 constructs were transiently transfected into 293 cells. AP1178 was used to immunoprecipitate the S100A4 proteins in the presence of 5 mM CaCl_2 . Immunoprecipitates were analyzed by Western blot using AP1178 to confirm precipitation of exogenously expressed S100A4 proteins and anti-MHC-IIA pAb to assess co-immunoprecipitation.



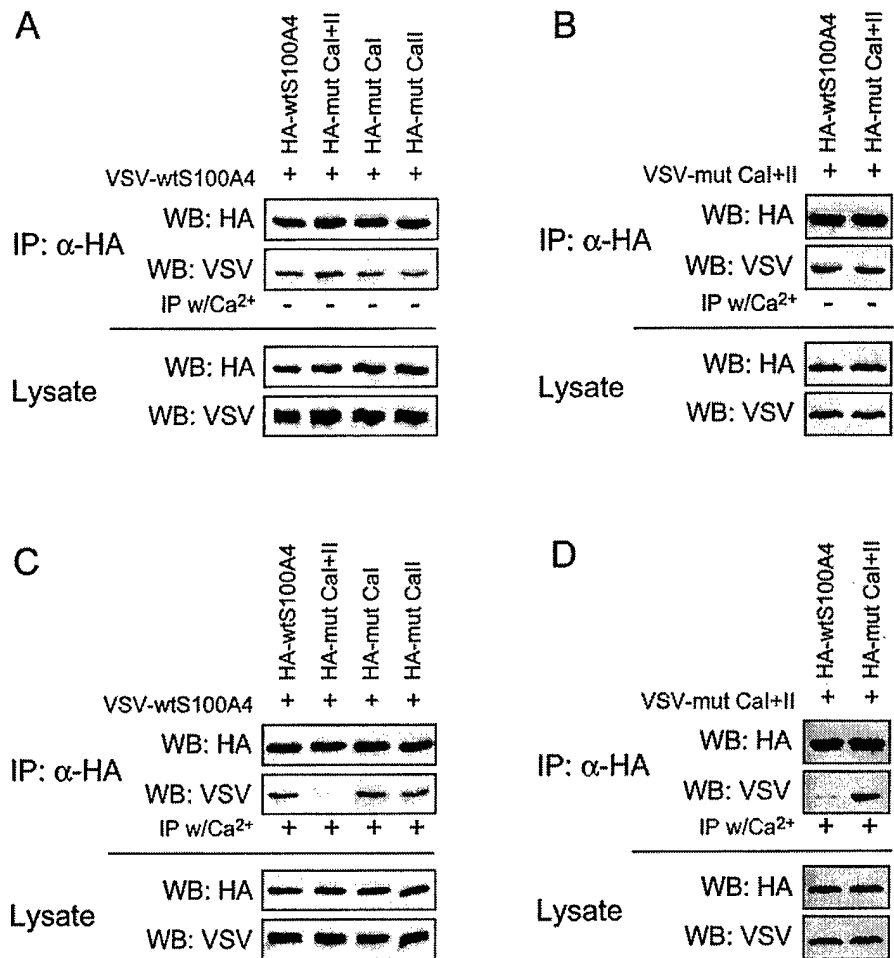
Calcium Binding- and Dimerization-dependent S100A4 Interaction with MHC-IIA—Having characterized the effect of the mutations on the basic biochemical features of S100A4 itself, we next examined the importance of calcium binding and dimerization on the interaction with a target protein such as MHC-IIA. We transfected wild type and mutant S100A4 into 293 cells and performed immunoprecipitations with our S100A4-specific antibody. Consistent with our previous data for endogenous S100A4 in MDA-MB-231 cells (Fig. 3A), MHC-IIA was co-immunoprecipitated with the exogenous wild type S100A4 protein from 293 cells (Fig. 5C). As was expected, the mutant deficient in calcium binding at both EF-hand motifs (*mutCaI+II*) failed to pull down MHC-IIA in the presence of calcium (Fig. 5C). This further confirms that the interaction of S100A4 with MHC-IIA is calcium-dependent. Interestingly, although the mutant deficient for calcium binding at site II (*mutCaII*) failed to pull down MHC-IIA, the mutant deficient for calcium binding at site I (*mutCaI*) retained the ability to interact with MHC-IIA (Fig. 5C). This result suggests that binding calcium to site II, but not site I, is sufficient to allow S100A4 to interact with MHC-IIA. Thus it appears site I and site II have separable functions with respect to MHC-IIA binding. Immunoprecipitation of either of the two dimerization-deficient mutants, *mutDimI* or *mutDimII*, failed to pull down MHC-IIA even in the presence of calcium (Fig. 5C). This result indicates that dimerization is in fact a functionally relevant feature of S100A4 with respect to its interaction with MHC-IIA. Accordingly, *mutDimIII*, which behaved like the wild type protein for dimerization, also retained the ability to interact with MHC-IIA (Fig. 5C).

Calcium Binding-dependent Conformational Effect on Dimerization—A previous experiment suggested that the addition of either calcium or EGTA does not affect S100A4 dimerization (Fig. 3B). We repeated this dimerization assay using HA-tagged wild type or HA-tagged calcium binding-deficient mutants to pull down VSV-tagged wild type S100A4 without adding calcium or EGTA to the buffers. Under these conditions, mutating the calcium-binding sites did not appear to affect

dimerization (Fig. 6A). This result supports our initial finding that S100A4 homodimer formation is possible in the absence of calcium (Fig. 3B). In fact, HA-tagged *mutCaI+II* can dimerize with VSV-tagged *mutCaI+II* (Fig. 6B). However, when we tested the calcium binding-deficient mutants for the ability to dimerize with wild type S100A4 in the presence of calcium, we were surprised to find that *mutCaI+II* and wild type S100A4 do not form a dimer under these conditions (Fig. 6, C and D). Each mutant singly deficient in calcium binding was still able to dimerize with the wild type protein in the presence of calcium (Fig. 6C). Taken together, these results suggest that even though S100A4 is able to dimerize in the absence of calcium, a completely calcium-free monomer (*mutCaI+II*) is unable to form a stable dimer with a calcium-bound wild type monomer. It is noteworthy that a *mutCaI+II* monomer can dimerize with another *mutCaI+II* monomer in the presence or absence of calcium (Fig. 6, B and D), because this demonstrates that the absence of a stable interaction between *mutCaI+II* and MHC-IIA (Fig. 5C) is not because of an additional deficiency in dimerization.

Calcium Binding-independent Intracellular Localization—The biochemical assays described above demonstrate the importance of both calcium binding and dimerization for S100A4 interaction with the partner protein, MHC-IIA. To determine whether these properties also play a role in localization, we attempted to generate MDA-MB-231 sublines stably expressing T7-tagged mutant S100A4 proteins. Although we did obtain sublines stably expressing T7-tagged *mutCaI+II*, we failed to generate any sublines stably expressing either of the two dimerization mutants. As described previously (41), the particular mutations we made may not directly affect the dimerization interface and instead affect the stability of the monomer. Based on this hypothesis, it is not that surprising that we were unable to generate any sublines that would stably express these mutant proteins. Immunofluorescent labeling of cells stably expressing T7-tagged *mutCaI+II* surprisingly revealed that the calcium binding-deficient protein localized to the same regions as the wild type protein (Fig. 7A). Because wild type

FIG. 6. Dimerization of calcium binding-deficient mutants. Mutant S100A4 proteins deficient in calcium binding at either one or both of the EF-hand motifs were tested for the ability to dimerize. *A*, HA-tagged wild type (*wt*) or HA-tagged calcium binding mutant (*mut*) S100A4 were co-transfected with VSV-tagged wild type into 293 cells. In the absence of any calcium, 12CA5 was used to specifically immunoprecipitate (*IP*) the HA-tagged protein from the cell lysate, and dimerization was assessed by Western blot (*WB*), as described in the legend to Fig. 3*B*. *B*, HA-tagged wild type or HA-tagged *mut*CaI+II were co-transfected with VSV-tagged *mut*CaI+II into 293 cells. Dimerization was assessed as described for *A*. *C* and *D*, dimerization was assessed as described for *A* and *B*, respectively, except that the immunoprecipitations were done in the presence of 5 mM CaCl₂.



S100A4 and N-WASP appear to tightly colocalize at the leading edge, we used the N-WASP localization as a tool to indirectly compare localization of the wild type and *mut*CaI+II proteins. *Mut*CaI+II closely colocalizes with N-WASP (Fig. 7*B*), strongly suggesting that the wild type and *mut*CaI+II S100A4 proteins localize to precisely the same region of the cell. *Mut*CaI+II also colocalizes to the same region as Arp3, but like the wild type S100A4 protein, does not appear to be in the exact same compartment within that region (Fig. 7*C*). Based on these observations, it seems that the cellular localization of the wild type S100A4 protein is calcium-independent.

DISCUSSION

Although it is generally believed that all of the S100 family members bind calcium and to some degree either homo- or heterodimerize, the particular details of how these characteristics play functional roles remain unclear. In the present study, we generated a set of mutants and conducted a detailed analysis on the phenotypic relevance of the calcium binding and dimerization biochemical properties of S100A4. Our studies with mutants deficient for dimerization or calcium binding allowed us to reveal previously unidentified aspects in the regulation of dimerization, interaction with protein partners, and intracellular localization.

Previous biochemical studies on S100A4 had shown that the addition of calcium promotes dimerization. In an initial assay with wild type S100A4, we could not detect a difference in the co-immunoprecipitation of a dimer regardless of the presence or absence of calcium. This assay is limited, however, in terms of sensitivity to relative degrees of dimer stability. The protein was transiently expressed, and there are inherent difficulties

in using immunoprecipitation to obtain quantitative results. Nevertheless, this assay suggested that a dimer could form in the absence of calcium, and we found that a double calcium binding-deficient mutant was even able to form a dimer with another double calcium binding-deficient mutant (Fig. 5*B*). Surprisingly, however, the double calcium binding-deficient mutant was unable to form a stable dimer with the wild type protein in the presence of calcium (Fig. 5*C*). Like most calcium-binding proteins, S100A4 undergoes a conformational change when it binds to calcium. Therefore, the data suggests that binding calcium induces a conformational change in a wild type S100A4 monomer that allows it only to exist as a dimer with another calcium-bound monomer. We thus propose that calcium plays a distinct role in dimer formation. Although two calcium-free monomers can dimerize, as will two calcium-bound monomers, a calcium-bound monomer and a calcium-free monomer will not form a stable "heterodimer." Because each single calcium binding-deficient mutant can still dimerize with the wild type protein in the presence of calcium, it seems that filling either EF-hand is sufficient to induce the particular conformational change that allows stable dimerization with a wild type calcium-bound monomer.

Previous studies suggested that changing a single amino acid could affect dimerization. Our assay for dimerization shows that changing a single residue (F72Q or Y75K) can abolish dimerization. However, we could not confirm the earlier finding that changing phenylalanine 78 to an alanine affects dimerization (41). In fact, this particular "mutant," *mut*DimIII, was still able to interact with MHC-IIA. This is in contrast with the two dimerization-deficient mutants that failed to interact

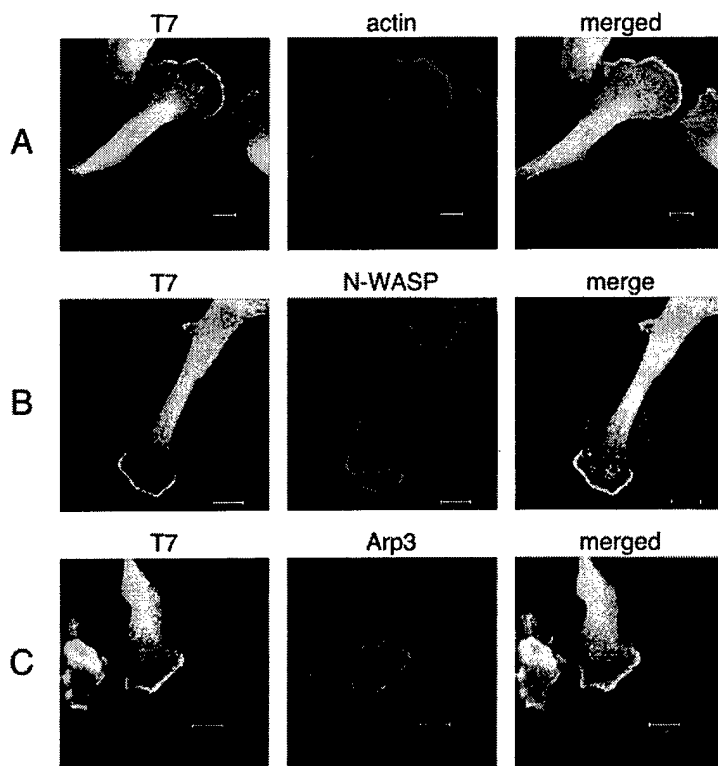


FIG. 7. Calcium-independent intracellular localization. MDA-MB-231 cells stably expressing T7-tagged *mutCaI+II* S100A4 (A–C) were plated on collagen IV coated coverslips. Cells were then stained for exogenous T7-tagged protein using an anti-T7 mAb (A–C) and costained for actin with phalloidin (A), anti-N-WASP pAb (B), and anti-Arp3 pAb (C). Bar, 10 μ m.

with MHC-IIA, a result we believe to be a direct consequence of its inability to dimerize. However, it does remain possible that each of the single amino acid changes that affected dimerization also directly influenced the interaction of S100A4 with MHC-IIA. Such a tight association between dimerization and partner protein interaction would actually serve to support an argument for the importance of the dimer state of S100A4. Nevertheless, to our knowledge, our results provide the first evidence that supports a functional significance for S100A4 homodimerization.

We have demonstrated that a single amino acid change, specifically in each EF-hand motif, is sufficient to abolish calcium binding for S100A4. The amino acids in an EF-hand motif that allow for calcium coordination are relatively well conserved. The particular residues we chose to mutate are especially well conserved between EF-hand motifs in general and specifically with the S100 proteins (Fig. 3). Therefore, it is highly probable that making similar mutations in other S100 proteins would result in a similar loss of calcium binding ability. As discussed earlier, the double calcium binding-deficient mutant (*mutCaI+II*) allowed us to elucidate calcium regulation of dimerization. This mutant has also allowed us to clearly confirm calcium dependence for the interaction of S100A4 with the MHC-IIA protein. Furthermore, experiments using the single calcium binding-deficient mutants revealed the functional importance of the two individual EF-hand motifs. Based on the data that calcium binds to site I first (40), we suggest that *mutCaII*, which allows site I to still bind to calcium, resembles the wild type protein in the presence of a hypothetical calcium concentration only sufficient to fill the higher affinity site at the N-terminal EF-hand. Dutta *et al.* (40) proposed two possible scenarios for the functional relevance of each EF-hand motif for S100A4 interaction with MHC-IIA. Their data suggested that if MHC-IIA bound to S100A4 through the C terminus region of S100A4, it could be possible that binding calcium to just the N-terminal EF-hand motif might be sufficient to induce the conformational change necessary for this protein-protein interaction. If the linker region between the two EF-

hand motifs of S100A4 is involved in this S100A4-MHC-IIA interaction, filling the C-terminal EF-hand motif with a second calcium might be necessary. Our data clearly support the latter scenario. *MutCaII* can bind to calcium at site I but cannot bind to MHC-IIA suggesting that filling site I is not sufficient for the S100A4-MHC-IIA interaction. *MutCaI* can bind calcium at site II, and now this seems sufficient to allow for the S100A4-MHC-IIA interaction. Taken together, the data from *mutCaI* and *mutCaII* suggest that filling site I is neither necessary nor sufficient for S100A4 interaction with MHC-IIA whereas filling site II appears to be both necessary and sufficient. Furthermore, the observations of Dutta *et al.* (40) of a distinct conformational change upon binding calcium at site I is consistent with our result that although *mutCaII* cannot interact with MHC-IIA, it does dimerize with the wild type monomer, which we have already argued undergoes a conformational change that does not allow it to interact with a calcium-free monomer. Based on the dimerization and MHC-IIA results for the single calcium binding-deficient mutants, we propose the following model for the effect of filling each of the two EF-hand motifs. An S100A4 monomer that binds a single calcium, filling site I, adopts an intermediate conformation that permits dimerization with a calcium-filled monomer but does not permit interaction with MHC-IIA. To adopt the ultimate conformation that allows interaction with target proteins such as MHC-IIA, the second lower affinity site, site II, must also be filled with calcium. It seems reasonable that the lower affinity site would be the true regulatory site as this would limit the "activation" of S100A4 to conditions with sufficient calcium levels to fill both sites.

To gain further insight into how dimerization and calcium affect S100A4 function, we examined its intracellular localization. S100A4 is widely believed to influence metastasis through an effect on motility, and thus elucidating the intracellular localization of S100A4 in motile cells is important. A generally accepted hypothesis for the mechanism of cell motility is that generation of the leading edge is driven by actin polymerization. We observe MHC-IIA at the leading edge, and there is

increasing evidence that myosin contractility also plays a role at this region (54). Consequently, the striking colocalization of the endogenous S100A4 protein with N-WASP and Arp2/3 complex to the leading edges of highly motile MDA-MB-231 cells is not only reasonable but rather intriguing. The broadly accepted hypothesis for the mechanism of S100A4 effect on motility is through a dynamic regulation of myosin polymerization and phosphorylation. Taken together, the potential for S100A4 to influence myosin dynamics, combined with its localization to the leading edge of motile cells where myosin may play a critical role in motility, provides a suggestive mechanistic clue to the intracellular function of S100A4. Equally intriguing is our data that the double calcium binding-deficient mutant (*mutCaI+II*) also localizes to this region of the cell. We confirmed that *mutCaI+II* localizes to the same intracellular location as the wild type by taking advantage of the close colocalization of N-WASP with both wild type and calcium binding-deficient mutant S100A4 proteins. This result suggests that S100A4 is not actually targeted to the membrane region by a calcium-dependent interaction with another protein. It is generally assumed that the ability to bind calcium translates to a calcium dependence for function. Clearly, the fact that S100A4 interacts with all the known protein partners in a calcium-dependent manner supports this assumption. However, this is partly a product of the experimental design used to identify these protein partners, because the absence of calcium is often used as the negative control to rule out nonspecific binding proteins. In addition, we do not believe that this localization of *mutCaI+II* is through heterodimerization with the endogenous wild type protein. *MutCaI+II* can only dimerize with the wild type protein when the wild type protein is not bound to calcium, and the wild type protein cannot bind to calcium-dependent protein partners in this state. However, this result does not preclude the likely possibility that wild type S100A4, once targeted to the leading edge, could then be activated by calcium and then bind to target proteins such as myosin and liprin in that region. Nevertheless, to our knowledge, the calcium-independent localization of S100A4 to the leading edge region of lamellipodia is the first calcium-independent property of S100A4.

This calcium-independent localization may be the result of a calcium-independent interaction between S100A4 and an unknown partner protein. S100A4 was previously biochemically shown to have two hydrophobic patches that are potential docking sites for partner proteins. One is only exposed upon calcium binding, but the other is constitutively accessible and may provide the interface through which S100A4 is able to interact with a currently unknown protein partner (37). There is also another S100 family member, S100A1, which has been shown to interact with multiple calcium-dependent and -independent protein partners (55). We therefore hypothesize that regulation of S100A4 function is comprised of two steps. First, S100A4 is targeted to the leading edge of lamellipodia through calcium-independent interactions with as yet unknown partner proteins. Calcium binding acts as a second signal, inducing a conformational change in S100A4 that allows functional interaction with known protein partners like MHC-IIA and liprin β 1. Based on this hypothesis, the identification of calcium-independent S100A4 protein partners is clearly necessary and will hopefully provide further insight into how S100A4 functions to affect metastatic progression in cells.

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