Metastasis-associated Mts1 (S100A4) Protein Modulates Protein Kinase C Phosphorylation of the Heavy Chain of Nonmuscle Myosin*

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Mts1 protein (S100A4 according to a new classification) has been implicated in the formation of the metastatic phenotype via regulation of cell motility and invasiveness. Previously we have demonstrated that Mts1 protein interacted with the heavy chain of nonmuscle myosin in a calcium-dependent manner. To elucidate the role of the Mts1-myosin interaction, we mapped the Mts1-binding region on the myosin heavy chain molecule. We prepared proteolytically digested platelet myosin and a series of overlapped myosin heavy chain protein fragments and used them in a blot overlay with Mts1 protein. Here we report that the Mts1-binding site is located within a 29-amino acid region, at the C-terminal end of the myosin heavy chain (between 1909–1937 amino acids). Two-dimensional phosphopeptide analysis showed that Mts1 protein inhibits protein kinase C phosphorylation of the platelet myosin heavy chain at Ser-1917. We hypothesize that Mts1 protein regulates cytoskeletal dynamics of the metastatic cells through modulation of the myosin phosphorylation by protein kinase C in calcium-dependent fashion.

Metastatic progression is a multi-step process starting with genetic alterations in tumor cells. Various genes have been implicated as important determinants of the metastatic phenotype (1–5). The mts1 gene (according to the new nomenclature named S100A4) was revealed as one of them (6, 7). Its expression correlates with the metastatic phenotype of different mouse and human tumors (7, 8). Induction of the mts1 expression in some nonmetastatic cell systems led to metastasis (9–11). While a reduction in the level of the Mts1, achieved by antisense or ribozyme techniques, suppressed the metastatic capability of tumor cells (10, 12, 13). Recently, mts1 transgenic animals were generated, and it was demonstrated in two different animal models that the presence of the mts1 transgene led to the formation of more invasive primary tumors and the appearance of metastasis (14, 15).

The mts1 gene encodes a 10-kDa calcium-binding protein belonging to the S100 family proteins. These proteins form homodimers and contain two calcium-binding conservative EF-hand domains, which differ by their ability to bind calcium (for reviews, see Refs. 16 and 17). The biological role of S100 proteins including Mts1 is poorly understood. The binding of calcium results in a conformational change within the protein, leading to the exposure of the hydrophobic patches that enable S100 family members to interact with a target proteins and to transmit a biologically important signal (16, 17).

One proposed role of the Mts1 protein in inducing metastatic phenotype is its implication in the regulation of the cytoskeletal dynamics and cell motility (13, 18, 19). In support of this suggestion, Mts1 was detected in a normal, highly motile cell type (8) where it is diffusely distributed in the cytoplasm and is partly associated with stress fibers (9, 20–22). Specifically, the Mts1 protein was associated with different cytoskeletal proteins such as F-actin and tropomyosin (20, 23). In addition, it was shown that Mts1 protein interacts with the heavy chain of nonmuscle myosin II in a calcium-dependent fashion (22, 24).

Nonmuscle myosin II is a chromomechanical protein that participates in cell division, motility, secretion, and capping (for reviews, see Refs. 25–27). This hexameric protein consists of two heavy chains (about 200 kDa) and four light chains (15–22 kDa). Dimers of heavy chains are folded in stable structures with two globular heads and extended α-helical coiled-coil tails. The head domain of the myosin heavy chain (MHC) possesses ATPase activity and contains actin and light chains binding sites (25). The tail domain of the heavy chain contains phosphorylation sites for protein kinase C (PKC) and casein kinase II and is involved in the regulation of the filament formation (reviewed in Refs. 28 and 29). There are two types of MHC isoforms (A and B), which are encoded by two different genes and have cell-type-specific expression (30–32). MHC-A is mainly expressed in intestinal epithelium, thymus, spleen, fibroblasts, and macrophages; MHC-B is predominantly expressed in brain and testis.

In the present study, we have mapped the region of the MHC-A that interacts with the Mts1 protein. This region is located at the C-terminal end of the MHC rod and contains the site of the phosphorylation by PKC. We report here that in vitro Mts1 protein modulates the phosphorylation of MHC by PKC.

EXPERIMENTAL PROCEDURES

Cell Line—Human metastatic osteosarcoma OHS (33) cells were propagated in a Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Preparation of the Myosin—Myosin was isolated from outdated platelet concentrates as described (34). The fractions were collected after a Sepharose 4B column, subjected to SDS-PAGE, and analyzed by Coo-
massie Blue staining. The myosin-containing fractions were pooled and concentrated by centrifugal concentrators ( Pall Filtron).

**Proteolytic Digestion of the Myosin**—The myosin (20 μg) was digested with sequencing grade modified trypsin (Promega) at various myosin-to-enzyme ratios (w/w): 2500/1, 1000/1, 500/1, 200/1, and 100/1. All digestions were carried out in the buffer (0.5 mM NaCl, 10 mM Mops, pH 7.0, 0.1 mM EDTA, 1 mM diithiothreitol) at 37 °C for 5 min and terminated by the addition of an equal volume of 2× SDS-gel sample buffer and boiling.

**Synthesis and Expression of the Human MHC-A Fragments**—Total RNA from human OSH cells was isolated as described (35). RT-PCR was conducted with poly(A)+ RNA purified on an oligo(dT) column (Promega) using Stratascript reverse transcriptase (Stratagene). The antisense primer (GGCTTATCGGCAGG) corresponding to the MHC-A sequence (+5882 to +5908) (32, 36) was used for cDNA synthesis. Four overlapping MHC-A fragments were PCR amplified using the following sets of primers: hmyo1, GGGTATCCGCGAGGCGAGGTCT (to +1055 to +1084); hmyo2, GGGTATCCGAGGTAGTCCTCCAG (to +971 to +997) and CAGGACCCGCAAGGAAGAAC (to +2300 to +2323); hmyo3, GGCGTACGGCGCTGGGGAGAGGAGCAAGAC (to +5743 to +5760); and CTGGTTCGTCACCGCCCTACAC (to +4195 to +4218) and GGCTTACCGGGATGATGTCGACCAG (to +5822 to +5849). The myodel-2 (5707); and GCCGATGCCATGAACCGCGA (1 to +5363 to +5380); and hmyo4–3D, GCCGATGCAGCTCAGTGAGGC (1 to +5720 to +5743); hmyo4–3B, GGCGTTCGAGGGTACCCAGGC (to +5363 to +5377); hmyo4–3A-G with oligonucleotide GCGGATCCAGATCCGAGGCC (to +5303 to +5317) and 3′ end primers for hmyo4–3A to AGTGCGTCCCTCCAG (to +5720 to +5743); hmyo4–3B, GGCTTACCGGGATGATGTCGACCAG (to +5743 to +5760); hmyo4–3E, TCTGGTCGAGGGTACCCAGGC (to +5843 to +5857); hmyo4–3F, CAGTGGTCCAGGGG (to +5788 to +5800); and hmyo4–3G, CGGGATCCATGGTCCCGG (to +5817 to +5830).

Internal deletions of hmyo4 were obtained by PCR using hmyo4 plasmid DNA as a template. Primers were designed as follows: hmyo4–1, CCGATGGCCGTGCTGACGCTG (to +4295 to +4309) and GGCGTTCGTCACCGCCCTACAC (to +4853 to +4873); hmyo4–2, CGGCGGATGCCAGGAGGAAGCGGAGG (to +4757 to +4771) and GGCTTACCGGGATGATGTCGACCAG (to +5822 to +5849); hmyo4–3A-G with oligonucleotide GCGGATCCAGATCCGAGGCC (to +5303 to +5317) and 3′ end primers for hmyo4–3A to AGTGCGTCCCTCCAG (to +5720 to +5743); hmyo4–3B, GGCTTACCGGGATGATGTCGACCAG (to +5743 to +5760); hmyo4–3E, TCTGGTCGAGGGTACCCAGGC (to +5843 to +5857); hmyo4–3F, CAGTGGTCCAGGGG (to +5788 to +5800); and hmyo4–3G, CGGGATCCATGGTCCCGG (to +5817 to +5830).

RESULTS

**Mts1 Protein Binds to the Tail Domain of MHC-A**—To map the Mts1 binding region on the MHC molecule, we performed blot overlay assay with proteolytically digested platelet myosin. The kinetics of the myosin cleavage by trypsin is shown in Fig. 1A. Mts1 protein interacted in blot overlay assay in the presence of calcium with several proteolytic fragments ranging approximately from 140 to 68 kDa (Fig. 1B). Interaction was abolished in the presence of EGTA (data not shown). The same fragments were detected when the experiment was repeated with digested platelet myosin which was first precipitated at 50 μM NaCl, and the pellet fractions were dissolved and analyzed (data not shown). Since all myosin fragments interacting with the Mts1 protein were capable of precipitation at low salt concentration, they contained C-terminal regions of the MHC (29).

To confirm the interaction of the Mts1 protein with the MHC tail, cDNA clones which overlapped the whole MHC-A molecule were prepared by RT-PCR (see “Experimental Procedures”) using poly(A)+ RNA from human osteosarcoma OSH cells. The schematic map of synthesized human MHC-A protein fragments is given in Fig. 2A. Mts1 protein interacted in blot overlay assay in the presence of calcium only with the Hmyo4 protein fragment (1384–1961 aa) derived from the proximal part of the MHC-A tail domain (Fig. 3, lanes 1–4). EGTA blocked Mts1-myosin interaction (data not shown).

**Mapping of the Mts1 Binding Site on the MHC-A Tail**—To ascertain the location of the Mts1 binding site, protein fragments, which overlapped the 68-kDa region of the human MHC-A tail, were prepared (Fig. 2B). We found that the region essential for the Mts1 binding was located to within the C-terminal 56 amino acids (Fig. 3, lanes 5–8), as was demonstrated by blot overlay assay in the presence of calcium (but not EGTA, data not shown).

To map the C-terminal border of the Mts1 binding site, we constructed a new series of plasmids based on the hmyo4–3B. These constructs (Fig. 2C) expressed recombinant Hmyo4–3B with expanded deletions near the C terminus. As shown in Fig. 3, lanes 8–13, the Mts1 protein interacted with increasing efficiency with Hmyo4–3B (1762–1961 aa), Hmyo4–3E (1762–1946 aa), and Hmyo4–3G (1762–1937 aa). To a much lesser extent, the Mts1 protein interacted with Hmyo4–3D (1762–1930 aa) and Hmyo4–3F (1762–1927 aa) and did not interact with Hmyo4–3C (1762–1914 aa). Therefore, the C-terminal border of the region that is essential for the binding of the Mts1 does not extend beyond Arg-1937.

To map the N-terminal border of the Mts1 binding site on the

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**FIG. 1. Identification of the Mts1-binding fragments in myosin digests by blot overlay.** Platelet myosin (lane 1) was digested with trypsin at the following myosin-to-trypsin ratios (w/w): lanes 2–7, 2500/1, 1250/1, 500/1, 200/1, and 100/1. Proteolytic fragments were resolved in 4–20% SDS-PAGE, stained with Coomassie Blue (A), or blotted. Blot overlay was performed with Mts1 protein in the presence of 1 mM CaCl2 (B).
Mts1 Modulates Phosphorylation of the Nonmuscle Myosin

MHC molecule, we prepared a series of deleted variants of the Hmyo4 protein (1384–1961 aa). Recombinant proteins contained the following deletions: Myodel-1 (1870–1882 aa), Myodel-2 (1896–1908 aa), Myodel-3 (1909–1926 aa), Myodel-4 (1926–1939 aa), and Myodel-5 (1941–1953 aa) (Fig. 4A). The interaction of these proteins with the Mts1 was analyzed by blot overlay assay (Fig. 4B). As we expected, since the deletion in Myodel-5 was located between Arg-1937 and the C terminus, this protein interacted with the Mts1 with the same affinity as the undeleted Hmyo4 protein did (Fig. 4B, lanes 5 and 6). Furthermore, deletions comprising the 1870–1908 aa region in Myodel-1 and Myodel-2 proteins did not affect interaction with the Mts1 (Fig. 4B, lanes 1, 2, and 6). On the contrary, Myodel-3 (deleted area 1909–1926 aa) and Myodel-4 showed no binding affinity to the Mts1 (Fig. 4B, lanes 3 and 4). Therefore, aa residues preceding Asp-1909 are not essential for the Mts1 binding. In summary, the region which is required for the Mts1 binding site of the MHC covers Ser-1917, which is revealed as a unique PKC phosphorylation site on the MHC (39). There are several PKC phospho-

Mts1 Protein Inhibits Phosphorylation of the MHC by PKC—

The Mts1 binding site of the MHC covers Ser-1917, which is phosphorylated by PKC in vitro and in vivo (39, 40). Therefore, we investigated whether the binding of the Mts1 protein to MHC may affect the phosphorylation of the MHC by PKC. Platelet myosin (1 μM) was phosphorylated by PKC in the absence or presence of the Mts1 protein and analyzed by

PKC (S\textsuperscript{1917}) and casein kinase (S\textsuperscript{1944}) phosphorylation sites on the MHC. Addition of the Mts1 protein to the reaction mixture showed minor variation in the efficiency of the MHC phosphorylation (Fig. 5B, compare lanes 1 and 2). However, the addition of the 9 μM Mts1 protein showed nearly 50% inhibition of the MHC phosphorylation (Fig. 5B, compare lanes 1 and 3). No phosphorylation occurred in the absence of PKC, calcium, or phosphatidylserine, indicating that substrates did not have kinase activity and reaction was calcium-phospholipid-dependent (data not shown).

Mts1 protein was phosphorylated by PKC in vitro (Fig. 5A, lanes 2 and 3), although in vivo this modification was not detected in human osteosarcoma cells (data not shown). To prove that phosphorylation of the Mts1 protein did not result in the substrate inhibition of the MHC, we used an excess amount of the enzyme. The observed level of the PKC autophosphorylation was not inhibited by the phosphorylation of the added Mts1 protein (Fig. 5B).

The excess amount of the Mts1 protein (up to 27 μM) did not block phosphorylation of the MHC (1 μM) completely (data not shown). Presumably, in vitro, there are several PKC phosphorylation sites on the MHC apart from Ser-1917, which was revealed as a unique PKC phosphorylation site on the MHC (39).

To determine whether Mts1 protein affects PKC phosphorylation of the MHC at Ser-1917, phosphopeptide analysis was performed. Platelet myosin (1 μM) was phosphorylated by PKC in the absence or presence of the Mts1 protein (3 μM, 9 μM) and subjected to SDS-PAGE followed by transfer to the nitrocellulose membrane by electroblotting. Bands corresponding to the MHC were excised and treated by trypsin, and equal amounts of radioactive materials from all samples were loaded on cellulose thin-layer plates. Analysis of the tryptic maps revealed

**Fig. 3.** Mts1 interacts with a C-terminal part of the MHC. The equimolar amounts of the recombinant MHC protein fragments in bacterial lysates were run on a 12% SDS-PAGE. The gel was either stained with Coomassie Blue (A) or blotted (B). Blot overlay was performed in the presence of 1 mm CaCl\textsubscript{2}. Lanes: 1, Hmyo1; 2, Hmyo2; 3, Hmyo3; 4, Hmyo4; 5, Hmyo4–1; 6, Hmyo4–2; 7, Hmyo4–3A; 8, Hmyo4–3B; 9, Hmyo4–3C; 10, Hmyo4–3D; 11, Hmyo4–3E; 12, Hmyo4–3F; and 13, Hmyo4–3G.
several phosphopeptides (Fig. 5, C, E, and F). To identify the phosphopeptide that contained Ser-1917, we included in parallel a synthetic peptide (AMNREVSSLKNKLRRGDL) corresponding to the 1910–1927 MHC amino acid sequence (32, 36) and containing only one site for PKC phosphorylation at Ser-1917. The control peptide was phosphorylated by PKC and subjected to the same procedure as MHC (see “Experimental Procedures”). Tryptic digestion of the synthetic phosphopeptide gave only one spot on the two-dimensional phosphopeptide map (Fig. 5D) whose position corresponded to M1 phosphopeptide (Fig. 5, compare C and D). Analysis of the phosphopeptide maps showed the Mts1-mediated activation of the phosphorylation of the MHC M2 peptide (Fig. 5, E and F). On the contrary, phosphorylation of the Ser-1917 (M1 peptide) was completely blocked in the presence of 9 mM Mts1 protein (Fig. 5F). No influence of the Mts1 protein on the M3 peptide phosphorylation was observed (Fig. 5, C, E, and F).

DISCUSSION

Previous studies have provided direct evidence that Mts1 protein binds to the heavy chain of nonmuscle myosin in the presence of calcium (22, 24). Since the role of this interaction was not clear, it was of interest to identify and characterize the Mts1 binding region of the MHC. For this purpose we used two methods to prepare fragments of MHC. First, platelet myosin was digested by trypsin, and second, we applied RT-PCR to obtain MHC peptides overlapping the whole MHC-A. We demonstrated by blot overlay technique that Mts1 protein interacted with the C terminus of the MHC-A rod in a calcium-dependent manner. Using two series of the recombinant MHC proteins with expanded deletions from C terminus or internal deletions in the C-terminal end of the tail domain, we mapped the Mts1 binding site between the 1909–1937 amino acids of the MHC.

Analysis of residues 1909–1937 of the MHC predicted two amphipathic α-helices with a four-residue linker, which contained hydrophobic and basically charged regions (data not shown). The side chain of the hydrophobic residues within the binding site can form extensive contacts with Mts1 hydrophobic patches that are exposed in the presence of calcium and forms a complex in a manner similar to that observed in calmodulin. Calmodulin and some members of the S100 family bind target peptides with similar structural motifs via a combination of hydrophobic and electrostatic interactions (16, 41–46).
Basic amphiphilic α-helices, which were noted as target sequences for EF-hand calcium-binding proteins, overlap with or are adjacent to the PKC phosphorylation sites (16, 41–43). PKC phosphorylation of these substrates is inhibited in the presence of interacting proteins such as calmodulin, S100A1, S100B, and S100C (16, 41–43). Mts1 protein binds to the region on the MHC molecule that contains Ser-1917, a residue involved in MHC filament formation (29, 39, 40), and it was proposed that PKC phosphorylation of the MHC controls assembly/disassembly of myosin filaments (40). Murakami et al. (47) have demonstrated that the assembly of the MHC filament formation (29, 39, 40), and it was proposed that PKC phosphorylation of the MHC controls assembly/disassembly of myosin filaments (40). Murakami et al. (47) have demonstrated that the assembly of the MHC filament formation. The influence of the Mts1 binding on the phosphorylation of the platelet myosin by PKC was studied by direct in vitro assay followed by tryptic phosphopeptide analysis. It was found that Mts1 protein inhibited phosphorylation of the MHC at the Ser-1917.

The biological role of the PKC phosphorylation of vertebrate nonmuscle MHC is not entirely clear. The PKC phosphorylation site is located close to the non-helical tail region that is involved in MHC filament formation (29, 39, 40), and it was proposed that PKC phosphorylation of the MHC controls assembly/disassembly of myosin filaments (40). Murakami et al. (47) have demonstrated that the assembly of the MHC was inhibited by the PKC phosphorylation in vitro. This effect was observed when brain MHC-B, but not macrophage MHC-A, was used. In addition, there is an indirect indication that PKC phosphorylation of the MHC may influence disassembly of the myosin filaments in vivo. The stimulation of the PKC activity in the rat basophilic leukemia cells was accompanied by phosphorylation of the myosin heavy and light chains and dissociation of the myosin filaments in the cell cortex (48). Such reorganization of the myosin filaments could induce changes in cell shape and motility (48–50).

Use of the myosin fluorescent analogues linked with imaging techniques revealed the migration of bead-like structures containing myosin in locomoting cells (51). It was proposed that stress fibers at the periphery of the cell continuously moved to the perinuclear region with disappearance following. Immunofluorescent staining showed that Mts1 protein is co-localized with nonmuscle myosin as an integral part of stress fibers and is concentrated in the perinuclear region (21, 22). Recently, a correlation between the level of the Mts1 protein and cell motility was found (13, 18, 19). Taken together these data allowed us to suggest that Mts1 protein regulates cell motility via the control of the MHC-A phosphorylation during the myosin assembly/disassembly process.

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