Non-muscle Myosin Heavy Chain as a Possible Target for Protein Encoded by Metastasis-related mts-1 Gene*

(Received for publication, March 21, 1994, and in revised form, June 1, 1994)

Marina V. Kriaievskaï, Mauricio Neira Cardenas, Mariam S. Grigorian, Noona S. Ambartsumian, Georgi P. Georgiev, and Eugene M. Lukandan

From the Department of Molecular Cancer Biology, Danish Cancer Society, Strandboulevarden 49, 7.1, DK-2100 Copenhagen, Denmark and the Institute of Gene Biology, Moscow 117334, Russia

The mts-1 gene is associated with the expression of the metastatic phenotype of tumor cells. The protein product of the mts-1 gene belongs to the Sl100 family of Ca2+-binding proteins, which has been cloned independently by several other groups from different sources and have been named pel-98, 18A2, p9Ka, 42A, CAPl, and calvuscin (10-15). The protein encoded by the mts-1 gene shows a high degree of homology to the Sl100 subfamily of Ca2+-binding proteins, which have been reported to be involved in regulation of cell growth and differentiation (16). Some of these proteins show interaction with cytoskeleton proteins (17), and in some cases it leads to inhibition of microtubule polymerization (18). The Mts-1 protein is thought to function as a positive regulator of metastasis of tumor cells (19, 20).

In the present work, we have searched for intracellular molecules that can be associated with Mts-1 protein. Using different techniques, we show that the Mts-1 protein is associated with a heavy chain of non-muscle myosin.

* This work was supported by a grant from the Danish Cancer Society, the Russian Foundation for Basic Research (to G.P.G.); and a FEBS fellowship (to M. N. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Metastatic dissemination of malignant tumor cells is a complex process that involves a multitude of steps, including migration of primary tumor cells through the stroma and invasion of the cells into lymphatic and blood vessels. The tumor cells survive in the vasculature and arrest, invade, and finally grow at distant secondary sites (1, 2). In order to complete the malignant process successfully the tumor cells must express a unique set of genes. Many genes have been described that are associated with the metastatic process (3-7). Using the technique of differential screening of cDNA libraries we have isolated the mts-1 gene,† which is expressed at high levels in different mouse metastatic cell lines but not expressed in nonmetastatic cells (8). mts-1-specific RNA has also been observed in normal tissues (thymus, spleen) and activated macrophages, T-lymphocytes, and neutrophils (8, 9). Several analogues of the mts-1 gene have been cloned independently by several other groups from different sources and have been named pel-98, 18A2, p9Ka, 42A, CAPl, and calvuscin (10-15). The protein encoded by the mts-1 gene shows a high degree of homology to the Sl100 subfamily of Ca2+-binding proteins, which have been reported to be involved in regulation of cell growth and differentiation (16). Some of these proteins show interaction with cytoskeleton proteins (17), and in some cases it leads to inhibition of microtubule polymerization (18). The Mts-1 protein is thought to function as a positive regulator of metastasis of tumor cells (19, 20).

In the present work, we have searched for intracellular molecules that can be associated with Mts-1 protein. Using different techniques, we show that the Mts-1 protein is associated with a heavy chain of non-muscle myosin.

MATERIALS AND METHODS

Cell Lines and Metabolic Labeling—The mouse mammary adenocarcinoma cell lines CSML-0 and CSML-100 (8) and human osteosarcoma OHS (21) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For metabolic labeling, cells growing on a 6-cm dish were incubated for 1 h at 37 °C in 2 ml of methionine-free medium supplemented with 2.5% fetal bovine serum and containing 0.5 mcI of [35S]methionine (50 mcI/mmol, Amerham Corp.).

Preparation of Monoclonal Antibodies—Mice were immunized with affinity-purified recombinant mouse Mts-1 protein (22) or with synthetic peptide (NEFEGCPDEPRKK) coupled to bovine serum albumin by glutaraldehyde (22). The peptide corresponding to the COOH terminus of the Mts-1 protein was synthesized by KEM-EN-TEC A/S.

Cell fusion, cloning, and analysis of hybridomas were performed as described (23). The isotype of mAb2 was determined using a mouse monoclonal antibody isotyping kit (Life Technologies, Inc.) according to the manufacturer’s instructions.

Antibodies—Rabbit antiserum against myosin (M-7648, Sigma); anti-myosin light chain mAb (M-4401, Sigma); anti-vimentin mAb (V-5255, Sigma); anti-ribonucleotide reductase mAb (MAS 378, Sera-lab); mouse IgG (P260, DAKO); and peroxidase-conjugated goat anti-rabbit Ig (P448, DAKO) were used. Anti-smooth muscle myosin mAb was a gift of Dr. M. Glukhova (24).

Immunoprecipitation and Immunoblotting—Immunoprecipitation was performed according to the standard protocol (25). Cell lysate, immunoprecipitation, and washing were carried out in buffer A (0.5% Nonidet P-40, 100 mM NaCl, 50 mM Tris-HCl, pH 8.0). Cell extracts were precleared on Protein A-Sepharose CL-4B beads (Sigma). All incubations were performed for 1 h at 4 °C. Immunoprecipitated proteins were collected on Protein A-Sepharose beads, washed, eluted in gel loading buffer (23), and separated by two-step SDS-PAGE (6-15% gels). Gels were dried and exposed to Kodak XAR-5 film, or proteins were transferred to Immobilon-P (Millipore) and analyzed by the standard Western blotting procedure (23). An enhanced chemiluminescence (ECL) detection system (Amersham Corp.) was used for developing filters.

Sucrose Gradient Analysis—Cell lysate from CSML-100 cells (107 cells) in 200 µl of lysis buffer A was layered onto a 5-ml linear sucrose gradient (5-30%). Ultrasrifugation was carried out at 41,000 rpm for 17 h at 4 °C in the Beckman SW 60.1 rotor. 21 fractions were collected, and proteins of each fraction were separated on SDS-PAGE followed by immunoblotting analysis.

Blot Overlay Analysis—The blot overlay technique was described as (25) with modifications. Proteins were separated by two-step SDS-PAGE (6 and 15% gels) and blotted. Separation of heavy chains of...
FIG. 1. Co-immunoprecipitation of the Mts-1-myosin complex. A, immunoprecipitation from CSM-1-0 (lanes 1–7) and CSM-1-100 (lanes 8–12) [35S]methionine-labeled cell lysates. Lanes: 1 and 8, anti-Mts-1 mAb HM-4 (IgG); 3 and 9, mouse IgG; 4 and 10, anti-ribonucleotide reductase IgG; 5 and 11, rabbit anti-serum against myosin heavy chain; 6 and 12, anti-Mts-1 mAb A8/7. Lanes 2 and 7, immunoprecipitation with anti-Mts-1 HM-4 and A8/7, respectively, obtained from CSM-1-0 cell extracts mixed with recombinant Mts-1 protein (0.1 μg). Arrows indicate myosin heavy chain (MHC), 20- and 17-kDa myosin light chains (MLC), and Mts-1 protein. The molecular masses for markers are indicated in kDa. B, C, and D, immunoblotting of the immunoprecipitates. Lysates of CSM-1-100 cells were immunoprecipitated with anti-Mts-1 mAb HM-4 (lane 1) or with mouse IgG, as a negative control (lane 2). Staining with antibodies against Mts-1 protein (HM-4) (B), pan-myosin heavy chain (C), and 20-kDa myosin light chain (D) is shown.

RESULTS AND DISCUSSION

Mouse anti-mouse Mts-1 mAb were raised against the Mts-1 COOH-terminal peptide (HM-4) or recombinant Mts-1 protein (A8/7). These antibodies specifically recognize Mts-1 protein in enzyme-linked immunosorbent assay and react with epitopes of SDS-denatured and cytologically fixed forms of the Mts-1 protein.

To identify Mts-1 associated proteins, [35S]methionine-labeled metastatic CSM-1-100 (Mts-1 positive) and nonmetastatic CSM-1-0 (Mts-1-negative) mouse mammary adenocarcinoma cells were lysed, and proteins were immunoprecipitated with anti-Mts-1 mAb HM-4 and analyzed in SDS-PAGE. Four major bands were detected in immunoprecipitates from CSM-1-100 cells (Fig. 1A, lane 8). In competitive experiments an excess amount of the peptide used to raise mAb HM-4 completely abolished the immunoprecipitation (data not shown). The band at about 11 kDa, corresponding to the Mts-1 protein, was stained with anti-Mts-1 mAb HM-4 in an immunoblot assay (Fig. 1B). Three other major bands with molecular masses of approximately 200, 20, and 17 kDa were observed. The asso-
tion of Mts-1 with these proteins was also observed after immunoprecipitations with mAb A8/7 against recombinant Mts-1 protein (Fig. 1A, lane 12). So, antibodies against different epitopes of Mts-1 protein coprecipitated Mts-1 protein as a complex with 200-, 20-, and 17-kDa proteins, confirming the specificity of immunoprecipitation.

Precipitation of the complex with anti-Mts-1 antibodies from lysates of nonmetastatic CSML-0 cells, in which the mts-1 gene is not expressed, was not observed (Fig. 1A, lanes 1 and 6).

To identify the nature of the 200-kDa protein, its amino acid composition was determined. The amino acid composition of the 200-kDa protein was similar to that of the heavy chain of brain neuronal myosin from rat (EMBL/GenBank accession number S98128).

To confirm the association of Mts-1 protein with myosin complex, independent immunoprecipitation was performed with antibodies against myosin. The Mts-1 protein was detected in immunoprecipitates from metastatic CSML-100 cells (Fig. 1A, lane 11). These results indicated the association between Mts-1 protein and myosin by immunoprecipitation with antibodies against both proteins.

The presence of myosin-Mts-1 protein complex in immunoprecipitates was demonstrated in immunoblot assay. CSML-100 cell lysates were immunoprecipitated with anti-Mts-1 antibodies or mouse antibodies against bacterial protein as a negative control, and the filters were probed with antibodies against the heavy chain or light chain of myosin. These antibodies detected the 200- or 20-kDa myosin chains in the anti-Mts-1 precipitates but not in control precipitates (Fig. 1, C and D).

To determine whether precipitation of myosin by anti-Mts-1 antibodies was indeed dependent on the presence of the Mts-1 protein in cell lysates, reconstitution experiments were performed. CSML-0 cell lysates were mixed with recombinant Mts-1 protein and immunoprecipitated with anti-Mts-1 antibodies. In this case, the anti-Mts-1 mAb (HM-4 and A8/7) immunoprecipitated myosin complex or only the heavy chain of myosin in association with added Mts-1 protein from lysates of nonmetastatic cells (Fig. 1A, lanes 2 and 7). The same pattern of the precipitated proteins was observed with anti-myosin antibodies (Fig. 1A, lane 5). The 13-kDa band is the result of nonspecific precipitation. The same band was obtained with rabbit control serum (data not shown).

The association of Mts-1 with myosin was also observed in other cell lines expressing Mts-1 protein. Immunoprecipitation from human osteosarcoma cell line OHS demonstrated a pattern of proteins similar to that which was detected in immunoprecipitates from CSML-100 cells (data not shown).

To confirm the association between Mts-1 protein and myosin, we determined whether the Mts-1 protein could be copurified with the myosin. Lysates from metastatic CSML-100 cells were separated in a sucrose gradient, and proteins of each fraction were analyzed by immunoblotting with anti-myosin heavy and light chain antibodies (Fig. 2, A and B) or anti-Mts-1 mAb (Fig. 2C). The 200- and 20-kDa proteins were stained with anti-myosin antibodies in fractions 5–9. Anti-Mts-1 antibodies gave an immunopositive ~11-kDa band with proteins isolated from two zones of sucrose gradient. The largest amount of Mts-1 protein was found in fractions 14–20, and a small amount was cosedimented with myosin in fractions 5–9 (Fig. 2C).

This finding strongly supports the suggestion that Mts-1 protein was associated with myosin complex.

Direct evidence for an association between the Mts-1 protein and myosin complex was obtained by the blot overlay technique. Recombinant Mts-1 protein bound to the 200-kDa heavy chain of myosin complex in a Ca²⁺-dependent manner (Fig. 3, lanes 1 and 2). The binding was abolished in the presence of EGTA (Fig. 3, lanes 3 and 4). We demonstrate here that nonmetastatic CSML-0 cells and their metastatic counterpart CSML-100 cells contain non-muscle as well as smooth muscle types of myosins (Fig. 4, A and B, lanes 1 and 3). However, anti-Mts-1 antibodies immunoprecipitated only the non-muscle type of myosin from CSML-100 cells (Fig. 4, A and B, lane 2). Preferable Ca²⁺-dependent binding of the Mts-1 protein to the heavy chain of non-

![Image](https://example.com/image)
Muscle myosin was detected by the blot overlay technique (Fig. 4C).

In view of the data obtained on association of the Mts-1 protein with the myosin complex we analyzed the intracellular distribution of Mts-1 protein using immunofluorescence staining of CSML-100 cells with anti-Mts-1 mAb HM-4 preabsorbed with relevant peptide. Magnification, ×800.

**REFERENCES**