Direct interaction between metastasis-associated protein 1 and endophilin 3

Yuko Aramaki[a], Katsuhiro Ogawa[a], Yasushi Toh[b], Takahiro Ito[c], Nobuyoshi Akimitsu[c], Hiroshi Hamamoto[c], Kazuhisa Sekimizu[c], Kimihiko Matsusue[d], Akira Kono[a], Haruo Iguchi[a], Soichi Takiguchi[a,*]

[a] *Institute for Clinical Research, National Kyushu Cancer Center, 3-1-1 Notame, Minami-ku, Fukuoka 811-1395, Japan*
[b] *Department of Gastroenterologic Surgery, National Kyushu Cancer Center, 3-1-1 Notame, Minami-ku, Fukuoka 811-1395, Japan*
[c] *Department of Developmental Biochemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[d] *Department of Hygiene Chemistry, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka 814-0180, Japan*

Received 16 March 2005; revised 16 May 2005; accepted 23 May 2005

Available online 13 June 2005

Edited by Felix Wieland

Abstract The yeast two-hybrid system was used to search for partners of mouse metastasis-associated protein 1 (Mta1). Screening of a cDNA library prepared from mouse embryo yielded positive clones coding for endophilin 3. The site of interaction was suggested to be the SH-3-binding domain of Mta1 and SH-3 domain of endophilin 3. This interaction was confirmed by GST pull-down assay in vitro and immunoprecipitation in vivo. The Mta1 and endophilin 3 transcripts were highly expressed in testis and brain. But, Mta1 localized mainly in nucleus and to a lesser extent in cytoplasm while endophilin 3 localized mainly in cytoplasm. If Mta1 functions in cytoplasm, it might be involved in the regulation of endocytosis mediated by endophilin 3.

Keywords: Endocytosis; Yeast two-hybrid system; Cancer metastasis; NuRD complex; Histone deacetylase; Chromatin; Estrogen receptor

1. Introduction

Metastasis-associated protein 1 (Mta1) was originally isolated by differential screening of a cDNA library using a rat mammary adenocarcinoma metastatic system. Northern blot analysis showed that the mRNA expression level of the mta1 gene was higher in highly metastatic cell lines than in a non-metastatic cell line [1]. It was also discovered that the expression level of human MTA1 correlates with the metastatic potential of several human cancer cell lines and tissues [2,3].

Metastasis-associated genes (MTAs) represent a rapidly growing novel gene family. At present, there are three different members (MTA1, MTA2, and MTA3) and six reported isoforms (MTA1, MTA1s, MTA1-ZG29p, MTA2, MTA3, and MTA3L). MTA1, MTA2 and MTA3 are components of the nucleosome remodeling and deacetylation complex (NuRD), which is associated with adenosine triphosphate (ATP)-dependent chromatin remodeling and transcriptional regulation. MTA proteins, as a part of NuRD, are thought to modulate transcription by influencing the status of chromatin remodeling [4]. MTA1s is the C-terminal truncated form of MTA1, localizes in the cytoplasm, sequesters the estrogen receptor (ER) in the cytoplasm, and enhances non-genomic responses of ER [5].

A recent study has shown that the stimulation of breast cancer cells with growth factors induces the expression of MTA1 and its interaction with and repression of the transactivation function the ER-α, leading to enhanced anchorage-independent growth in vitro and hormone independence [6]. Furthermore, MTA1 and MICoA were identified as partners of MTA1 using a yeast two-hybrid system [7,8]. They may interact with MTA1 and modulate the transactivation functions of ER-α.

We have made extensive use of the yeast two-hybrid system to search for additional binding partners of Mta1. Here we demonstrate interaction between Mta1 and endophilin 3 in vitro and in vivo, and present a possible cytoplasmic function for Mta1.

2. Materials and methods

2.1. Construction of plasmids

Mouse Mta1 cDNA [9] fragments were obtained by PCR from a mouse 11-day embryo cDNA library (Clontech, Palo Alto, CA). The upstream primers were tagged with a *Bam*HI site and the downstream primers were tagged with a *Sal*I site. The sequences of the PCR primers (restriction enzyme sequences are underlined) are 5′-CGGGATCCGTATGGCCGCCAACATGTAC-3′ and 5′-ACGCGTCGACTCAAGGCTGCCGCACGGAG-3′ for Mta1-F1, 5′-CGGGATCCGTAGCCTGCACATGAGTGC-3′ and 5′-ACGCGTCGACTCAGCGTAGGATCTCACGGC-3′ for Mta1-F2, 5′-CGGGATCCCCATGGCATGCTGCCCG-3′ and 5′-ACGCGTCGACCTAGTCCTCAATAACAATGG-3′ for Mta1-F3, and 5′-CGGGATCCTACAAACCCATTGCCCTGC-3′ and ACGCGTCGACCTAGTCCTCAATAACAATGG-3′ for Mta1-SH3BD. The PCR-amplified fragments were digested with *Bam*HI and *Sal*I and ligated into *Bam*HI, *Sal*I-digested pAS2-1 (Clontech). Then their sequences were confirmed.

The *Eco*RI, *Bgl*II-tagged Mta1-F3 fragment was obtained by PCR from a mouse 11-day embryo cDNA library (Clontech) using a pair of primes (restriction enzyme sequences are underlined); 5′-CGGAATTCCGCCATGGCATGCTGCCCG-3′ and 5′-GAAGATCTTCCTAGTCCTCAATAACAATGG-3′. The PCR-amplified fragment was digested with *Eco*RI and *Bgl*II and ligated into *Eco*RI, *Bgl*II-digested pAcGHLT-B (PharMingen, San Diego, CA). Then

*Corresponding author. Fax: +81 92 551 4585.
E-mail address: sctakigu@nk-cc.go.jp (S. Takiguchi).

0014-5793/$30.00
doi:10.1016/j.febslet.2005.05.069

the sequence was confirmed. For in vitro translation assay, two-hybrid positive cDNA fragments in a pGAD10 vector were cloned inframe into a pcDNA3.1/HisA expression vector (Invitrogen, Carlsbad, CA). For the mammalian expression construct of Mta1-F3, a *Bam*HI, *Sal*I-tagged Mta1-F3 fragment was cloned into *Bam*HI, *Xho*I-digested pcDNA3 (Invitrogen). The myc tag was added to the N-terminus of Mta1-F3 by PCR.

2.2. Yeast two-hybrid screen and assay

The yeast two-hybrid screen was performed using the Matchmaker GAL4 Two Hybrid System 2 (Clontech). A mouse 11-day embryo library in pGAD10 was purchased from Clontech. The pAS2-1-Mta1 bait plasmids and the cDNA library were transfected by the lithium acetate method into the Y190 yeast strain (Clontech). Selection for HIS3 reporter gene activation was performed on histidine, tryptophan, and leucine-deficient agar plates with 25 mM 3-aminotriazole (Sigma Chemical, St. Louis, MO). Colonies appearing after 7–10 days at 30 °C were assayed for β-galactosidase activity utilizing the colony-lift assay. For quantitative analysis, the colonies were grown in liquid medium and assayed for β-galactosidase activity using *O*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The plasmid DNA of positive clones was isolated with phenol and glass beads as suggested by the maker of the system. Positive two-hybrid protein interactions were verified by transfection of the plasmids back into the Y190 yeast strain together with the original bait or with selected controls. The plasmids of positive colonies were amplified in *Escherichia coli* DH5α and sequenced.

2.3. GST pull-down assay

Purified GST-fusion proteins were generated using a *Baculovirus* expression vector system (Pharmingen). The Mta1-F3 transfer vector, pAcGHLT-B-Mta1-F3, was used for the production of GST-Mta1-F3 fusion protein. Purified GST fusion protein (2 μg) bound to glutathione–Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ) was incubated with 20 μl of ^{35}S labeled methionine protein with moderate shaking at 4 °C for 2 h in 200 μl of binding buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), 0.1% Nonidet P-40, and 1 mM DTT. The ^{35}S labeled probes were generated from two-hybrid positive cDNA fragments in a pcDNA3.1/HisA expression vector using TnT Coupled Reticulocyte Lysate Systems (Promega, Madison, WI). The bound ^{35}S labeled protein was washed three times with binding buffer and the beads were collected by centrifugation. The bound protein was eluted in SDS sample buffer, subjected to SDS–PAGE, and detected by autoradiography.

Additionally, 4 μg of GST or GST-Mta1-F3 bound to glutathione–Sepharose 4B beads was prepared. Highly metastatic mouse melanoma K-1735 M2 cells (a gift from I.J. Fidler) [10,11] grown in 150-mm dishes to 70–80% confluency were scraped into lysis buffer containing 20 mM Tris (pH 7.4), 250 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, and protease inhibitor cocktail (Pharmingen). The cell lysate was incubated on ice for 20 min, vortexed, and centrifuged at $100\,000 \times g$ for 35 min. The supernatant was precleared with glutathione beads and incubated with the prepared glutathione beads at 4 °C for 2 h. The beads were washed three times with NP40 lysis buffer and boiled in SDS sample buffer. The proteins were separated by SDS–PAGE and subjected to immunoblotting with anti-endophilin 3 or anti-PACSIN 2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

2.4. Immunoprecipitation

K-1735 M2 cells grown in 100-mm dishes to 70–80% confluency were lysed with 1 ml of ice-cold RIPA buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Pharmingen). Cell lysates containing amounts of equal protein were immunoprecipitated with anti-Mta1 or pre-immune antibodies (Santa Cruz Biotechnology) using Protein G–Sepharose 4 Fast Flow (Amersham Biosciences). The pellet was washed three times with RIPA buffer and boiled in SDS sample buffer. The proteins were separated by SDS–PAGE and subjected to immunoblotting with anti-endophilin 3, anti-Mta1 or anti-PACSIN 2 antibody (Santa Cruz Biotechnology).

For the reconstitution of the interaction between Mta1 and endophilin 3 in transfected cells, COS7 cells were transfected with pcDNA3-myc-Mta1-F3 and pcDNA3.1-Xpress-endophilin 3 (175-end). Immunoprecipitation was performed as described above with anti-myc antibody (Cell Signaling Technology). The proteins were separated by SDS–PAGE and subjected to immunoblotting with anti-myc and anti-Xpress antibodies (Invitrogen).

2.5. Cell fractionation

Cell fractionation was done by the mini-extracts method as described with modifications [12]. Briefly, K-1735 M2 cells grown in 100-mm dishes to 70–80% confluency were collected into 400 μl of buffer containing 10 mM HEPES–KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1% Nonidet P-40, 1 mM DTT, and protease inhibitor cocktail (Pharmingen). The cell suspensions were vortexed and centrifuged at $2500 \times g$ for 1 min. The supernatant containing cytoplasm was transferred to a fresh tube and the nuclear pellet was resuspended in 100 μl of buffer containing 50 mM HEPES–KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 20% glycerol, 1 mM DTT, and protease inhibitor cocktail (Pharmingen).

2.6. Immunoperoxidase cell staining

K-1735 M2 cells grown on sterile cover slips overnight at 37 °C were washed with PBS, fixed for 10 min in 1% formalin in PBS, and washed three times in PBS. Staining was performed with anti-Mta1 or anti-endophilin 3 antibodies raised against a peptide located near the carboxyl terminus of Mta1 or endophilin 3 (Santa Cruz Biotechnology) using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) as suggested by the manufacturer of the kit.

3. Results

3.1. Identification of endophilin 3 and Spag5 as Mta1-interacting proteins in the yeast two-hybrid screen

A mouse 11-day embryo library was screened with the pAS2-1-Mta1 baits to elucidate any Mta1-interacting proteins. The yeast two-hybrid screen was performed under stringent conditions. More than 10^6 transformants were screened with each bait which derived from four different parts of Mta1 (Fig. 1A). When pAS2-1-Mta1-F1 and pAS2-1-Mta1-F2 were used as baits, several clones grew on the selection plates but none of them were positive for β-galactosidase activity. When pAS2-1-Mta1-F3 and pAS2-1-Mta1-SH3BD were used as baits, one and three β-galactosidase-positive clones which harbored pGAD10-Spag5 (residue 867–897 + 915-end) and pGAD10-endophilin 3 (two clones of residue 175-end and a clone of residue 189-end) prey plasmids were obtained, respectively. Positive two-hybrid protein interactions were verified by transfection of the plasmids back into the host together with the original bait or with selected controls. As a result, positive interaction was observed between endophilin 3 (residues 175-end) and Mta-SH3BD or Mta1-F3, and between Spag5 and Mta1-F3 (Fig. 1B). Mta1 and endophilin 3 seem to interact through their SH3-binding domain (residue 697–705) and SH3 domain (residue 286–346), respectively.

3.2. Mta1 interacts with endophilin 3 in vitro

To independently confirm the results obtained with the two-hybrid assay, we performed GST pull-down assays. A GST-Mta1-F3 fusion protein was produced using the *Baculovirus* expression vector system and bound to glutathione–Sepharose beads. ^{35}S labeled methionine endophilin 3 (residue 175-end) or Spag5 (867–897 + 915-end) were applied to the beads and after extensive washing, the proteins were eluted from the beads, size fractionated by SDS–PAGE and visualized by autoradiography. GST-Mta1-F3 protein specifically interacted with endophilin 3, whereas GST alone failed to interact with endophilin 3. No specific interaction could be observed