CMTM5-v1, a four-transmembrane protein, presents a secreted form released via a vesicle-mediated secretory pathway

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The CKLF-like MARVEL transmembrane domain-containing family (CMTM) is a novel family of proteins linking classical chemokines and the transmembrane 4 superfamily (TM4SF).

Our earlier studies indicated several CMTM members (such as CKLF1 and CMTM2) have a secreted form. This is the first report of the secreted form of CMTM5-v1, the major RNA splicing form of CMTM5, which is produced as small vesicles (<100 nm diameter) and floats at a peak density of 1.19 g/ml on continuous sucrose gradients. CMTM5-v1 has no obvious co-localization with CD63 or Golgi complex. In addition, breflidin A but not wortmannin can inhibit the secretion of CMTM5-v1. Our results suggest that CMTM5-v1 might be secreted via a different vesicle-mediated secretory pathway, which will be helpful for the studies of vesicle-mediated secretion and MARVEL domain-containing proteins. [BMB reports 2010; 43(3): 182-187]

INTRODUCTION

Human CKLF-like MARVEL transmembrane-domain-containing family (CMTM) is a novel family of proteins encoded by 9 genes, CKLF and CMTM1 - CMTM8 (1, 2). Of these, CKLF1, CMTM1 and CMTM2 are more related to chemokines, while CMTM8 is more related to TM4SF11, with a level of sequence homology of up to 39% at the overall amino acid level. The characteristics of other CMTM members are intermediate between those of CMTM1 and CMTM8. They have important roles in the immune system, and in the male reproductive system, and they participate in tumorigenesis (3-12).

TM4SF includes several types of proteins possessing the four transmembrane-helix structure, such as the classical TM4SF (tetraspanin) and MARVEL-domain containing proteins. TM4SF members usually have no signal peptide, but several reports have shown that both tetraspanins, such as CD63, CD9, and CD81, and MARVEL-domain containing proteins like MAL and CMTM2, have a secreted form(8, 13, 14).

Soluble secretory proteins typically contain N-terminal signal peptides and are usually secreted through the classical or endoplasmic reticulum (ER)/Golgi-dependent secretory pathway. However, an increasing number of secretory proteins, such as the above-mentioned TM4SF members, devoid of signal peptide have been reported, and some of them can be exported without the help of the ER/Golgi system. At least three distinct types of nonclassical protein secretion can be distinguished in mammalian cells: (a) direct translocation across the plasma membrane; (b) membrane flip-flop mechanism; (c) membrane vesicles (15). Membrane vesicles are complex structures composed of a lipid bilayer that contains transmembrane proteins and encloses soluble hydrophilic components derived from the cytosol of the donor cell. There are various types of secreted membrane vesicles, including exosomes, exosome-like vesicles, membrane particles, microvesicles and ectosomes. They have distinct structural and biochemical properties that depend on their intracellular site of origin (16).

Here, we report that secreted CMTM5-v1 can be sedimented by sequential high-speed centrifugation, is associated with small vesicles (<100 nm diameter), and floated on continuous sucrose gradients at a peak density of 1.19 g/ml. CMTM5-v1 has no obvious co-localization with the CD63 or Golgi complex. In addition, the secretion of CMTM5-v1 can be inhibited by the classical secretion pathway inhibitor breflidin A (BFA), but not by wortmannin. More importantly, the above characteristics of CMTM5-v1 are not completely consistent with any of the reported types of vesicles, which indicates it might represent a novel vesicle-mediated secretory pathway. Therefore, this study will be helpful for the studies of vesicle-mediated secretion and MARVEL domain-containing proteins.

RESULTS

Identification of the secreted form of CMTM5-v1

Our earlier studies showed that CMTM5 is widely expressed in normal tissues and has at least six RNA splicing forms, named CMTM5-v1 to CMTM5-v6. To further study the expression profile of CMTM5, we used a real-time PCR assay that selectively amplified CMTM5-v1, v2 and v6, which have the same molecu-
ular size. CMTM5-v1 is the major RNA splicing form of CMTM5; therefore, it can be inferred that CMTM5-v1 is expressed predominantly in brain, prostate and small intestine across 16 normal human tissues (Fig. 1A).

CMTM5 has the second highest level of expression in the prostate. In order to know the expression pattern of CMTM5 in prostate, we detected it in human normal prostate and prostate hyperplasia tissues with Immunohistochemistry assay. As illustrated in Fig. 1B, CMTM5 is mainly located in the prostate lumen. Of 112 cases, 80 cases (71.4%) had secreted products in the prostate lumen, and 41 cases (51.3%) of these were CMTM5-positive.

5A10 is one of the three PC-3 clones with stable expression of CMTM5-v1 and 5B1 is one of the two vector-transfectants (6). In order to further verify that CMTM5 has a secreted form, we cultured PC-3 clones 5A10 and 5B1 with conditioned medium and collected the supernatants with sequential centrifugation. As shown in Fig. 1C, CMTM5-v1 can be secreted in PC-3 clone with stable expression of CMTM5-v1.

Characterization of the secreted form of CMTM5-v1

The secreted form of CMTM5-v1 was identified in the prostate lumen and in a prostate carcinoma-derived cell clone. We investigated the vesicles with both electron microscopy and linear sucrose density-gradient centrifugation. First, electron microscopy observation revealed that the secreted CMTM5-v1 was located in small vesicles <100 nm in diameter (Fig. 2A). Second, secreted form of CMTM5-v1 floated on a linear sucrose density-gradient and at a peak density of 1.19 g/ml (Fig. 2B). CD63, a classical extracellular vesicle marker was not de-
CMTM5-v1 has no obvious co-localization with CD63 or Golgi complex
The intracellular distribution of CMTM5-v1 was studied by confocal microscopy in PC-3 cells. As shown in Fig. 3, CMTM5-v1, CD63 and Golgi complex were found in cytoplasmic granule-like structures. However, dual labelling of CMTM5-v1 and either CD63 or Golgi tracker showed that neither CD63 nor Golgi complex had obvious degree of co-localization with CMTM5-v1. Further subcellular localization analysis indicated that CMTM5-v1 had no obvious co-localization with the endoplasmic reticulum or mitochondria (data not shown).

CMTM5-v1 secretion is inhibited by BFA but not by wortmannin
Bioinformatics analysis indicated that CMTM5-v1 does not have a typical signal peptide, and the results presented above indicated that CMTM5-v1 was released as small vesicles. To gain further insight into the mechanism of CMTM5-v1 secretion, we treated PC-3 cells with BFA (2.5 μg/ml) or wortmannin (100 nM). BFA is an inhibitor of the classical secretion pathway (17), while wortmannin is an inhibitor of phosphatidylinositol 3-kinase (PI3K), which inhibits the formation of multivesicular bodies/late endosomes (18). Western blot showed that BFA can reduce the secretion amount of CMTM5-v1 in vesicles, but wortmannin had no obvious effect on CMTM5-v1 secretion. Control experiments indicated that the treatment with BFA or wortmannin did not affect the intracellular amount of CMTM5-v1 in cell lysates (Fig. 4).

DISCUSSION
This study shows that CMTM5-v1 is secreted via a vesicle-mediated secretory pathway, because conditioned medium with secreted CMTM5-v1 could be sedimented by sequential high-speed centrifugation. The presence of CMTM5-v1 in vesicles was confirmed by electron microscopy and linear sucrose density-gradient centrifugation. CMTM5-v1 is secreted as small vesicles (<100 nm in diameter) and floats at a peak density of 1.19 g/ml on a sucrose gradient. Comparing the characteristics of CMTM5-v1-containing vesicles and the other types of vesicles shows the former cannot be classified as any type of known vesicle. The size and density of CMTM5-v1-containing vesicles is similar to that of exosomes. However, the lack of co-localization of CMTM5-v1 with the CD63 or Golgi complex and the inhibition by BFA but not wortmannin suggest that CMTM5-v1 secretion is different from exosomes. The size of CMTM5-v1-containing vesicles is also similar to that of membrane particles, but their densities are different. Although secretion of most non-classical secreted protein cannot be inhibited by BFA, a recent study showed that BFA could selectively inhibit the secretion of TNFR1 exosome-like vesicles, which resemble CMTM5-v1 (19). In summary, secreted CMTM5-v1 is not completely identical with all kinds of vesicle that have been reported, so we hypothesized it might be secreted via a novel vesicle-mediated secretion pathway.

The MARVEL domain has been identified in proteins of the myelin and lymphocyte (MAL), physins, CMTM, gyrins and occludin families. Most MARVEL-domain proteins are involved in membrane apposition and vesicle-trafficking events (20). Our earlier studies demonstrated that CMTM5 exerts tumor
suppressor functions, with frequent epigenetic inactivation by promoter methylation (6). Restoration of CMTM5-v1 leads to the suppression of cell growth and induces apoptosis in cervical carcinoma cells and pancreatic carcinoma cells mainly through a caspase-dependent pathway (7, 12). However, in prostate carcinoma cell lines, restoration of CMTM5-v1 could inhibit the proliferation and migration of PC-3 cells, and initiate apoptosis of Du145 cells, but not PC-3 cells (data not shown). So the apoptosis-inducing effect of CMTM5-v1 may be cell-type specific, which needs further study. It has been reported that prostasomes belong to membranous vesicles, which are produced by epithelial cells of prostate gland and has inhibiting effects on the growth of prostate carcinoma cells especially Du145 cells (21, 22). Therefore, it can be inferred that CMTM5-v1-containing vesicles might have more obvious effects, such as inhibiting the proliferation and migration of PC-3 cells and initiating apoptosis in Du145 cells.

This is the first report that CMTM5-v1 is secreted via a different vesicle-mediated secretory pathway, which will be helpful for studies of vesicle-mediated secretion and MARVEL domain-containing proteins. Further work is required to elucidate the mechanism and the function of CMTM5-v1-containing vesicles.

MATERIALS AND METHODS

Cell culture and infection

Human prostate cancer cell line PC-3 (ATCC; Manassas, VA, USA) and stably transfected PC-3/CMTM5-v1 cells (6) were grown in RPMI 1,640 medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone), 4 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were grown at 37°C in a humidified 5% (v/v) CO₂ atmosphere and used for assays during the exponential phase of growth. Cells were infected with CMTM5-v1 adenovirus (ad-CMTM5-v1) or ad-null (vector-containing adenovirus, defined as MOCK) as described (7). A multiplicity of infection of 30 was used, and the efficiency of infection was monitored by ad-pEGFP-N1. Cells with greater than 90% infection efficiency were used for further analysis.

Reagents

BFA and wortmannin were purchased from Sigma-Aldrich (St. Louis, MO, USA), IRDye™-800-conjugated secondary antibodies against mouse and rabbit IgG were purchased from LI-COR Bioscience (Lincoln, NE, USA). Monoclonal antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO). The antibody against CD63 was from BD Biosciences (USA). Polyclonal antibody against CMTM5-v1 was prepared as described (7). Protease Inhibitor Cocktail tablets were obtained from Roche Applied Science (Switzerland).

Real-time reverse transcription (RT) PCR

Reverse transcription was done using the ThermoScript First-Strand Synthesis kit (Invitrogen Technologies). The human multiple tissue cDNA panel was purchased from Clontech (USA). Amplifications were done with the SYBR Green PCR Master Mix Kit (Applied Biosystems, USA) in a total volume of 20 μl. Specific primers for CMTM5 were:

5’-CCCTCCTCACCTCCACAAAG-3’
5’-CAGAGATGGAGCCCGTGA-3’

Template cDNA was denatured at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The quantification data were analyzed with ABI Prism 7,000 SDS software. The expression levels for target genes were measured as fluorescent signal intensity and normalized to the internal standard gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The experiment was done three times to assess reproducibility.

Tissue specimens and immunohistochemistry

Human normal prostate and prostate hyperplasia tissue chips (Shanxi Chaoying Biotechnology, Xian, Shanxi, China) were deparaffinized and rehydrated. Antigen retrieval was done by heating a solution in 0.01 M citrate buffer (pH 6.0) twice for 5 min. The tissue chips were incubated with 3% (v/v) H₂O₂ at room temperature for 10 min, rinsed twice and blocked in PBS plus 10% normal goat serum for 20 min. The slides were then incubated at 4°C overnight with a rabbit anti-CMTM5-v1 polyclonal antibody (1 : 50 (v/v) in PBS). After thorough washing, Dakocytomation Envision™ System HRP (DakoCytomation, USA) was applied for 30 min. After rinsing in PBS, all slides were visualized with 0.05% (w/v) 3,3′-diaminobenzidine, and then counter-stained with hematoxylin. Rabbit IgG was used as a negative control.

Isolation of secreted vesicles from conditioned medium by sequential centrifugation

Vesicles were isolated from the conditioned medium of PC-3 cells or stably transfected PC-3/CMTM5-v1 cells incubated in HEK 293 serum-free medium (SAFC Biosciences™, USA) for 48 h. The medium was centrifuged to remove cell debris at 2,000 g for 10 min and then at 10,000 g for 30 min. Vesicles were sedimented by ultracentrifugation at 100,000 g for 70 min in an SW40 rotor, washed once in PBS and then sedimented again by ultracentrifugation at 100,000 g for 70 min. Vesicles isolated from one or two 100 mm diameter tissue culture dishes were analyzed by SDS-PAGE and Western blot.

Protein extraction and Western blot analysis

Vesicles isolated from one or two 100 mm diameter tissue culture dishes were suspended in loading buffer. Whole cell lysates were suspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) TritonX-100) in the presence of a protease inhibitor mixture. After incubation on ice for 30 min, lysates were centrifuged at 18,000 g for 20 min at 4°C. The supernatant was removed and the protein concentration was measured using the BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's in-
Sucrose density-gradient centrifugation
Vesicles collected as described above were suspended in 1 ml of 2.5 M sucrose, 20 mM Hepes, pH 7.4, and floated into an overlayed linear sucrose density gradient (2.0 M-0.5 M sucrose, 20 mM Hepes, pH 7.4) at 100,000 g for 16 h in a Beckman SW40 rotor as described. Fractions (400 μl) were collected from the bottom of the tube and the density was determined by refractometry. Each fraction was diluted with 5 ml of PBS, and then centrifuged in an SW40 rotor for 1 h at 100,000 g. Finally, concentrated vesicles were analyzed by SDS-PAGE and Western blot.

Immunoelectron microscopy
Vesicles were collected as described above and suspended in 2% (v/v) paraformaldehyde and then adsorbed for 20 min on Formvar-carbon-coated electron microscopy grids (Nickel; Gilder Grids, UK) floating on 5 μl drops of Parafilm. Grids with adhered vesicles were rinsed in PBS and blocked in 5% (v/v) BSA. After the wash and block, the grids were incubated with primary antibody overnight at 4°C. The grids were washed with 0.5% BSA and transferred to protein A-gold conjugates (10 nm; Electron Microscopy Sciences) diluted in 0.1% BSA and 0.5% BSA and transferred to protein A-gold conjugates. The grids were washed with PBS, fixed with 1% (v/v) glutaraldehyde, stained with uranyl oxalate for 20 min at room temperature. Grids were washed with PBS, fixed with 1% (v/v) glutaraldehyde, stained with uranyl oxalate solution for 5 min and examined with an electron microscope operated at 80 kV. Control labeling was performed identically.

BFA and wortmannin block assay
PC-3 cells were infected with ad-CMTM5-v1 and MOCK, and incubated in HEK 293 serum-free medium (SAFC Biosciences™, USA). After 24 h, 2.5 μg/ml of BFA, 100 nM wortmannin or DMSO/ethanol used as negative control, were added to the cell culture supernatant, and the cells were incubated at 37°C for another 24 h. Finally, the cell lysate and concentrated vesicles were harvested for Western blot.

Immunofluorescence staining and microscopy
PC-3 cells grown on coverslips were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min, then permeabilized with 0.1% Triton X-100 at room temperature for 10 min. After washing three times with PBS and blocking for 30 min in a blocking buffer (10% BSA in PBS), the coverslips were incubated with the indicated primary antibodies at 4°C overnight. The cells were washed three times with PBS followed by incubation at 37°C for 1 h with the corresponding secondary antibodies. The secondary antibodies were TRITC-conjugated goat anti-mouse and FITC-conjugated anti-rabbit immunoglobulin. The cells were then washed three times with PBS. Samples were observed with an Olympus laser-scanning confocal microscope (Olympus Fluoview FV300).

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