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MARCH-III Is a Novel Component of Endosomes with Properties Similar to Those of MARCH-II

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MARCH comprises a recently identified family of transmembrane RING-finger proteins which is implicated in diverse biological functions, such as immune regulation, protein quality control, and membrane trafficking. We previously identified MARCH-II, as a binding partner of syntaxin 6, which plays a role in endosomal protein transport. In this paper, we describe the cloning and characterization of MARCH-III which is the closest homolog of MARCH-II. It is broadly expressed at relatively high levels in spleen, colon, and lung. An immunofluorescence study of HeLa cells demonstrated that MARCH-III is present in peripheral vesicles partially colocalized with transferrin receptor. Overexpression of MARCH-III resulted in the redistribution of TGN46 and strong inhibition of transferrin uptake. Immunoprecipitation studies revealed that MARCH-III is associated with syntaxin 6 and MARCH-II. Mutational analyses revealed that the PDZ-binding motif and RING finger are essential for the subcellular localization of MARCH-III and the inhibitory effect on transferrin uptake. The location, associated molecules, and effects of overexpression suggest that MARCH-III is involved in the regulation of vesicular trafficking in endosomes.

Key words: membrane trafficking, PDZ, RING finger, syntaxin 6, transferrin.

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; GST, glutathione *S*-transferase; MARCH, membrane-associated RING-CH; RT-PCR, reverse transcription–PCR; Tf, transferrin; TGN, *trans*-Golgi network; TMR, tetramethylrhodamine.

The membrane associated RING-CH (MARCH) family comprises a structurally related protein family, the members of which consist of an N-terminal RING-CH finger followed by C-terminal transmembrane spans (*1*). Genes encoding MARCH proteins are found in many eukaryotes from yeast to man and plants. Currently, there are at least nine known *MARCH* genes (*MARCH-I–IX*) in the human genome (*1*). The RING-CH finger is a C4HC3-type double Zn^{2+}-binding structure found in proteins of eukaryotes and viruses (*2–5*). Like other RING fingers [*e.g.*, RING-H2 (C3H2C3) and RING-HC (C3HC4)], this domain associates with ubiquitin-conjugating enzymes (E2) and possesses E3 ubiquitin ligase activity (*1*, *2*, *6–10*). Several MARCH members have been shown to be ubiquitin ligases and are proposed to function in a variety of cellular processes. MARCH-VIII [originally termed cellular modulator of immune recognition (c-MIR)] targets the B7-2 (CD86) costimulatory molecule for ubiquitination, leading to rapid endocytosis and degradation of B7-2 (*9*). Similar effects are observed on major histocompatibility complex (MHC) class I on expression of MARCH-IV and MARCH-IX (*1*). Thus, MARCH-IV, -VIII, and -IX are thought to participate in immune regulation. MARCH-VI (TEB4) and its yeast ortholog Ssm4p/Doa10p have been shown to be transmembrane ubiquitin ligases of the endoplasmic reticulum (ER) involved in ER-associated protein degradation (*8*, *10–12*). MARCH-VII (axotrophin) seems to play multifunctional roles in the regulation of immune tolerance and neuronal development (*13*). We have independently identified MARCH-II as a novel endosomal protein bound to syntaxin 6, a SNARE protein of the *trans*-Golgi network (TGN) and endosomes (*14*, *15*). We showed that overexpression and knockdown of the *MARCH-II* gene result in a change in the localization of syntaxin 6 and in perturbation of the endocytic protein transport to the TGN, which is similar to the effect caused by a syntaxin-6 mutant (*16*). Therefore, MARCH-II is involved in the regulation of endosomal trafficking, which is a novel function of the MARCH family. Vesicular trafficking events are controlled by a diverse array of proteins including (i) coat proteins (*e.g.*, clathrin and adaptin), (ii) SNARE proteins, and (iii) small GTPases (*e.g.*, Rab and Arf families), many of which act in a compartment-specific manner (*17–20*). In addition, the number of their regulators that modulate the activities of core trafficking machineries is growing. Since MARCH proteins are localized to specific intracellular organelles such as the ER, TGN, endosomes, lysosomes, plasma membrane, and nuclear membrane (*1*, *8–10*, *14*, *21*), they might function in particular membrane transport pathways.

Here, we identified MARCH-III, which exhibits the highest sequence similarity with MARCH-II among the MARCH family. We show that MARCH-III is localized to early endosomes, and binds to MARCH-II and syntaxin 6. Its overexpression affected the distribution of TGN46 and inhibited Tf uptake. Both the PDZ-binding motif and the RING finger were found to be important for the subcellular

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localization of MARCH-III to the site of its function. MARCH-III is likely a regulator of the endosomal transport pathway.

MATERIALS AND METHODS

Construction of Plasmids—The full-length coding region of MARCH-III was obtained from rat small intestine by reverse transcriptase–PCR (RT-PCR) using the primer pair of 5′-TATGAGTGTGGAAGCCATTA-3′ and 5′-TATTTAAGTAAACAGCTCACTGCG-3′. The PCR product (1.47 kb) was subcloned into the *Eco*RV site of pBluescript II SK$^-$ (Stratagene, La Jolla, CA, USA), yielding pBS-MAR3. A mammalian expression vector for MARCH-III was constructed by cloning the *Xho*I–*Eco*RI insert from pBS-MAR3 into the same sites of pcDNA3 (Invitrogen, Carlsbad, CA, USA). The expression vectors for MARCH-II and FLAG-syn6 were described previously (*14*). Green fluorescent protein (GFP)-MAR3 and Myc-MAR3 were constructed by cloning the fragment encoding amino acid residues 2–253 of MARCH-III into pEGFP-C2 (BD Biosciences Clontech, Palo Alto, CA, USA) and pCMV-Myc (BD Biosciences Clontech), respectively. GFP-MAR3ΔPDZ was constructed by cloning the fragment encoding amino acid residues 2–249 of MARCH-III into pEGFP-C2. His-MAR3$_N$ was constructed by cloning the fragment encoding amino acid residues 2–70 of MARCH-III into pRSET (Invitrogen). Glutathione *S*-transferase (GST)-fusion proteins of the RING fingers of MARCH-III and MARCH-II (GST-MAR3$_{RING}$ and GST-MAR2$_{RING}$) were constructed by cloning the fragments encoding amino acid residues 45–144 of MARCH-III and 41–137 of MARCH-II, respectively, into pGex-4T (Amersham Biosciences, Piscataway, NJ, USA). Hexahistidine and Xpress-tagged E2 enzymes were constructed using the coding regions of human E2 into pRSET (Invitrogen). All point mutations were introduced by means of primer-based site-direct mutagenesis.

RT-PCR Amplification—Total RNA prepared from various cell lines was used to synthesize oligo(dT)-primed first strand cDNA with SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). The products of first strand cDNA synthesis were directly amplified by PCR using *ExTaq* DNA polymerase (Takara, Kyoto, Japan) using a MARCH-II-specific primer pair (5′-ATGACGACAGGTGACTGTTGC-3′ and 5′-CCGAATCTTCAGGCGGACTTTC-3′) or a MARCH-III–specific primer pair (5′-ATGACAACCAGTCGCTGCAGT-3′ and 5′-TTCTAAGCCACTCCACTAACGGCCT-3′).

Cell Culture, Transfection and Internalization Assay—HeLa and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transient transfection was performed using LipofectAMINE 2000 reagent (Invitrogen) and Opti-MEM I (Invitrogen) according to the manufacture instructions. For transferrin (Tf) uptake experiments, at 24 h after transfection, cells were incubated in Opti-MEM I containing 25 μg/ml tetramethylrhodamine (TMR)-labeled Tf (Molecular Probes, Eugene, OR, USA) at 37°C for 30 min. The cells were then rinsed three times in ice-cold PBS, fixed, and subjected to fluorescence microscopy.

Antibodies—Polyclonal antibodies to MARCH-III (anti-MAR3$_N$#487) were raised against His-MAR3$_N$ fusion proteins. The antibodies were affinity-purified with HiTrap NHS-activated HP (Amersham Biosciences) coupled with His-MAR3$_N$. The polyclonal antibodies to MARCH-II (anti-MAR2$_C$#41) were described previously (*14*). The antibodies to the following antigens were purchased: EEA1 (BD Transduction Laboratories, Lexington, KY, USA); Tf receptor (Zymed Laboratories, South San Francisco, CA, USA); calreticulin (Affinity Bioreagents, Cambridge, UK); LAMP1 and ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); HA and Myc (Roche Diagnostics, Mannheim, Germany); and FLAG and GST (Sigma, St. Louis, MO, USA).

Preparation of Rat Liver Membranes—A Golgi-enriched fraction was prepared from rat liver as described previously (*14*). The membranes were sedimented by centrifugation at 40,000 rpm in an SW41 rotor (Beckman Coulter, Fullerton, CA, USA), and then extracted with HEPAD buffer (20 mM Hepes, pH 7.3, 2 mM EDTA, 100 mM KCl, 0.5 mM ATP, 2 mM dithiothreitol, and protease inhibitors) containing 0.5% Triton X-100. The extract (0.5 mg protein) was loaded onto a linear 10–35% glycerol gradient in HEPAD buffer containing 0.5% Triton X-100 and then centrifuged for 18 hours at 40,000 rpm in an SW41 rotor. Ten fractions (fractions #1–#10) were collected from the top of the tube. The proteins in fraction #9 were precipitated with 80% acetone and subsequently subjected to Western blot analysis.

Fluorescence Microscopy—HeLa cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and then blocked with 1% fetal bovine serum for 30 min at room temperature. Cells were incubated with anti-MAR2$_C$#41 (1:1000), anti–MARCH-III (1:200 to stain endogenous MARCH-III or 1:1,000 for overexpressed MARCH-III), anti-Myc (1:1000), anti-FLAG (1:2000), anti-HA (1:1,000), anti-EEA1 (1:500), anti-Tf receptor (2 μg/ml), anti-TGN46 (1 μg/ml), anti-LAMP1 (1:500), or anti-calreticulin (1:200) for 30 min at room temperature. After washing with PBS, the cells were incubated with appropriate secondary antibodies conjugated with Alexa Fluor 488 (1:1,000; Molecular Probes), Alexa Fluor 546 (1:1,000; Molecular Probes), or Alexa Fluor 594 (1:3,000; Molecular Probes). Signals were observed under an Axioskop epifluorescence microscope (Carl Zeiss, Thornwood, NY, USA) equipped with an ORCA-ER cooled charge-coupled device digital camera (Hamamatsu Photonics, Hamamatsu), a CSU10 confocal scanner unit (Yokogawa Electric, Tokyo, Japan), and an argon/krypton ion laser. For Figs. 5C, 6 and 7, signals were captured under an IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a SenSys cooled CCD digital camera (Photometrics, Huntington Beach, CA, USA) or an Axiovert 200M epifluorescence microscope (Carl Zeiss) equipped with an ApoTome optical sectioning device (Carl Zeiss).

Northern Blot Analysis—Total RNA was prepared from rat tissues by the acid guanidinium thiocyanate/phenol/chloroform method and then electrophoresed on a denaturing formaldehyde/agarose gel. After separation, the RNA was transferred to a Hybond-N$^+$ nylon membrane (Amersham Biosciences). The filter was hybridized with a rat MARCH-III cDNA probe labeled with [α-^{32}P]dCTP

in PerfectHyb hybridization buffer (Toyobo, Osaka, Japan) at 68°C overnight. The filter was washed with 1× SSC (0.15 M NaCl and 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at 68°C, and then exposed to an imaging plate (Fujifilm, Tokyo, Japan). Signals were detected with a BAS2000 bio-imaging analyzer (Fujifilm).

Immunoprecipitation—HeLa cells were grown on a 6-well plate, and then transfected with Myc-MAR3, HA-MAR2, or FLAG-syn6. At 36 h after transfection, the cells were lysed in PBS containing 1% Triton X-100 and proteinase inhibitors. The lysates were cleared by high-speed centrifugation and then incubated with 20 μl of anti-FLAG affinity gel (Sigma) or with anti–HA-agarose beads (Sigma) for 2 h at 4°C. After washing 10 times with 1 ml of PBS containing 1% Triton X-100, the bound materials were eluted with 100 μl of 0.1 M glycine-HCl (pH 2.5) containing 1% Triton X-100.

In Vitro *Ubiquitination Assay*—Purified GST-$MAR3_{RING}$, GST-$MAR2_{RING}$, or GST-$MAR3_{RING}$mut (1 μg each) was incubated with a mixture comprising 0.1 μg of rabbit E1 (Boston Biochem, Cambridge, MA, USA), 0.5 μg of purified E2, 1 μg of ubiquitin (Sigma), and 1 mM creatine phosphokinase (Sigma) in an ATP-regeneration buffer (25 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1 mM $MgCl_2$, 5 μM $ZnCl_2$, 0.3 mM dithiothreitol, 2 mM ATP, and 1 mM creatine phosphate) for 5 h at 30°C.

RESULTS

Primary Structure and Expression in Rat Tissues and Cultured Cells—The nucleotide and amino acid sequences of MARCH family members were found in the GenBank database by means of Blast searches using a MARCH-II cDNA sequence as a query. For further analysis, we selected MARCH-III that is the most closely related to MARCH-II. A cDNA fragment containing the entire coding region of MARCH-III was obtained from rat small intestine by RT-PCR. The coding sequence encoded a protein of 253 amino acid residues (Fig. 1A), with a calculated molecular mass of 28.5 kDa, which exhibits high identity with MARCH-II (63.2%) and moderate identity with other MARCH proteins (<29%). As described by Bartee *et al.* (*1*), MARCH-III contains two deduced transmembrane spans flanked by a long N-terminal and a short C-terminal cytoplasmic tail. As other noteworthy sequence features, MARCH-III contains a RING-CH finger in the N-terminal cytoplasmic region and a PDZ-binding motif at the C-terminus (Fig. 1A). The tissue expression of MARCH-III mRNA was determined by Northern blot analysis of total RNA isolated from various rat tissues. A 2-kb transcript was predominantly found in lung, colon, and spleen, with substantially lower expression in brain, stomach, intestine, kidney, and testis (Fig. 1B). To examine the expression of MARCH-III and MARCH-II mRNAs in a variety of cultured cells including NRK (rat kidney epithelial), HeLa (human cervix epithelial), NIH/3T3 (mouse embryonic fibroblast), and AtT-20 (mouse pituitary tumor) ones, RT–PCR analysis was performed using the specific primer pairs for MARCH-II and MARCH-III. The results shown in Fig. 1C revealed that all cells examined contain both messages at comparable levels.

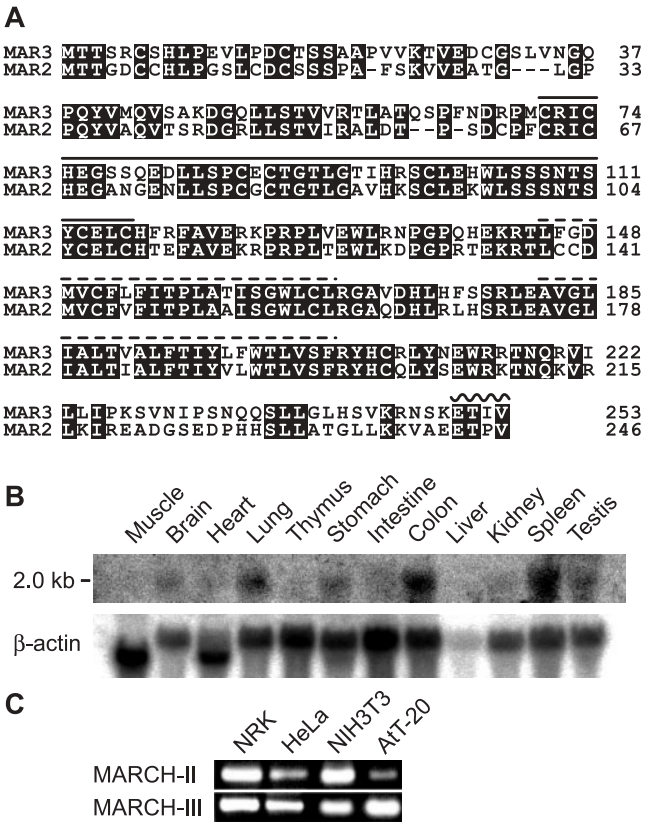

Fig. 1. **Primary structure and expression profile of MARCH-III.** (A) Multiple sequence alignment of rat MARCH-III (MAR3) and rat MARCH-II (MAR2) (accession numbers AB048840 and AB048838, respectively). Identical amino acid residues are shaded. The RING-CH finger domain, hydrophobic domains, and PDZ-binding motif are indicated by a bold line, dashed lines, and a wavy line, respectively. (B) Blots containing 20 μg of total RNA from each tissue were hybridized with ^{32}P-labeled cDNA probes specific for rat MARCH-III (top panel). The stripped filter was rehybridized with a β-actin probe (bottom panel). (C) Amplification of MARCH-II and MARCH-III cDNA fragments by RT-PCR from total RNAs of the indicated cell lines. The products should not be construed as a quantitative representation of the expression in each cell line.

Ubiquitin Ligase Activity of the RING Finger—Previous studies have demonstrated that the RING-CH fingers of MARCH-I, -II, -IV, -VI, and -VIII, and Ssm4p/Doa10p possess E3 ubiquitin ligase activity (*1*, *8–10*). To examine the ability of the RING-CH finger of MARCH-III to catalyze polyubiquitin chain synthesis, we performed *in vitro* ubiquitination assay with recombinant GST-fusion proteins of the MARCH-III RING finger (GST-$MAR3_{RING}$). GST-$MAR3_{RING}$ was incubated with ubiquitin-activating enzyme (E1), various human ubiquitin-conjugating enzymes (E2), ubiquitin, and an ATP-regenerating system. When samples were analyzed by Western blotting with anti-ubiquitin antibodies, high-molecular-weight ubiquitinated products were detected in the presence of the E2 proteins, ubcH5C, ubcH6, and ubcH9 (Fig. 2A). Such ubiquitination was abolished when the first two Zn^{2+}-binding cysteine residues of GST-$MAR3_{RING}$ were substituted with serine residues (C71S and C74S, GST-$MAR3_{RING}$mut) (Fig. 2B). The RING finger of MARCH-III exhibits a high

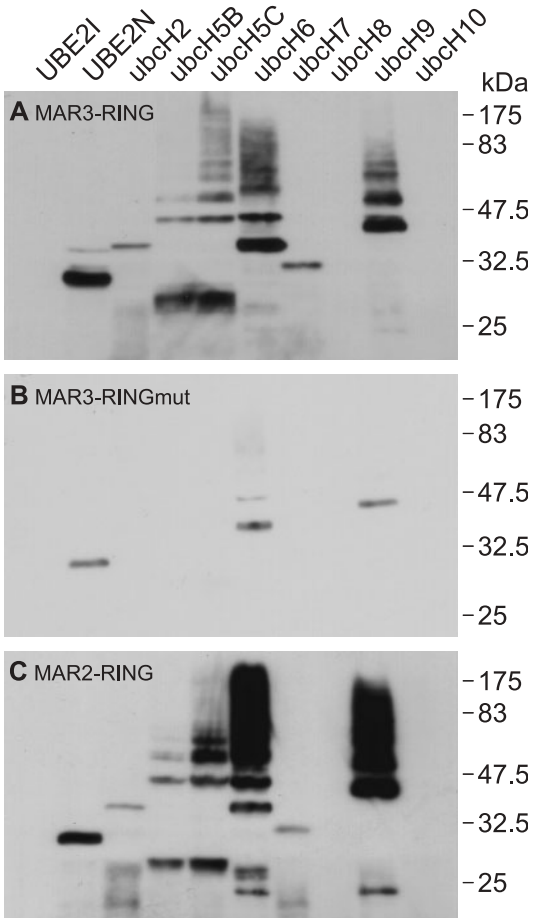

Fig. 2. **Ubiquitin ligase activity of the RING finger of MARCH-III.** An *in vitro* ubiquitination assay was performed with E1, E2, and either GST-MAR3$_{RING}$ (A), GST-MAR3$_{RING}$mut (B), or GST-MAR2$_{RING}$ (C). The reaction samples were separated by 12.5% SDS–polyacrylamide gel electrophoresis and then immunoblotted with anti-ubiquitin antibodies.

degree of identity (80.4%) in sequence with that of MARCH-II. To determine whether or not MARCH-II has similar E3 activity, the ubiquitination assay was performed with the MARCH-II RING finger (GST-MAR2$_{RING}$). GST-MAR2$_{RING}$ exhibited the same specificity for the E2 enzymes as GST-MAR3$_{RING}$ (Fig. 2C).

Intracellular Localization—To examine the protein expression of MARCH-III, a rabbit polyclonal antibody was raised against a recombinant protein of the N-terminal region of MARCH-III (residues 2–70). Upon Western blot analysis, the affinity-purified antibody specifically detected a single band at 28 kDa for 293T cells transfected with MARCH-III (Fig. 3A, lane 6), but did not cross-react with MARCH-II (Fig. 3A, lane 5). Western blot analysis with this antibody revealed the expression of an endogenous MARCH-III protein with the same molecular weight in rat liver microsomal membranes (Fig. 3B). To examine the intracellular localization of endogenous MARCH-III proteins, HeLa cells were processed for indirect immunofluorescence microscopy with anti–MARCH-III antibodies. Laser confocal images showed faint staining in cytoplasmic vesicular structures (Fig. 3C). Double staining with anti-MARCH-III and anti–Tf-receptor antibodies showed that MARCH-III was partially colocalized with Tf receptor (Fig. 3D), suggesting that MARCH-III is localized to a subset of early endosomes.

Redistribution of TGN46 on Overexpression of MARCH-III—To address the function of MARCH-III, we analyzed the effect of its overexpression. HeLa cells were transfected with an expression vector for MARCH-III, and then double-stained with antibodies to MARCH-III and organelle marker proteins. The staining pattern of overexpressed MARCH-III proteins was similar to that of the endogenous protein and partially overlapped that of Tf receptor (Fig. 4, A–A″). Although very little colocalization was observed, the staining of MARCH-III differed substantially from that of other endosomal markers, EEA1 (early endosomes, Fig. 4, B–B″) and LAMP1 (late endosomes, Fig. 4, C–C″). Interestingly, cells overexpressing MARCH-III exhibited a

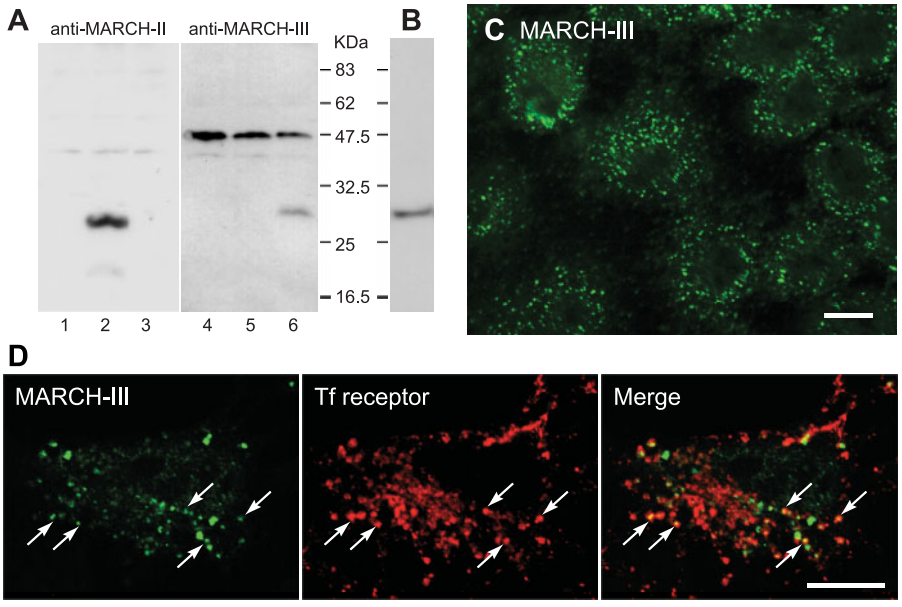

Fig. 3. **Western blot and immunofluorescence analyses of MARCH-III.** (A) 293T cells were mock transfected (lanes 1 and 4) or transfected with MARCH-II (lanes 2 and 5) or MARCH-III (lanes 3 and 6). Whole cell lysates (20 μg of protein) were subjected to Western blot analysis with antibodies to MARCH-II (lanes 1–3) and MARCH-III (lanes 4–6). (B) Western blot analysis of rat liver microsomal membranes (20 μg of protein) with anti–MARCH-III antibodies. (C) HeLa cells were fixed, permeablized, and then stained with anti–MARCH-III antibodies followed by Alexa 488–conjugated anti-rabbit IgG. (D) A double-labeling study was performed with antibodies to MARCH-III and Tf receptor. Signals were detected under a confocal microscope, and those for MARCH-III and Tf receptor are represented by green and red, respectively. Arrows indicate the colocalization of the two proteins. Bars, 10 μm.

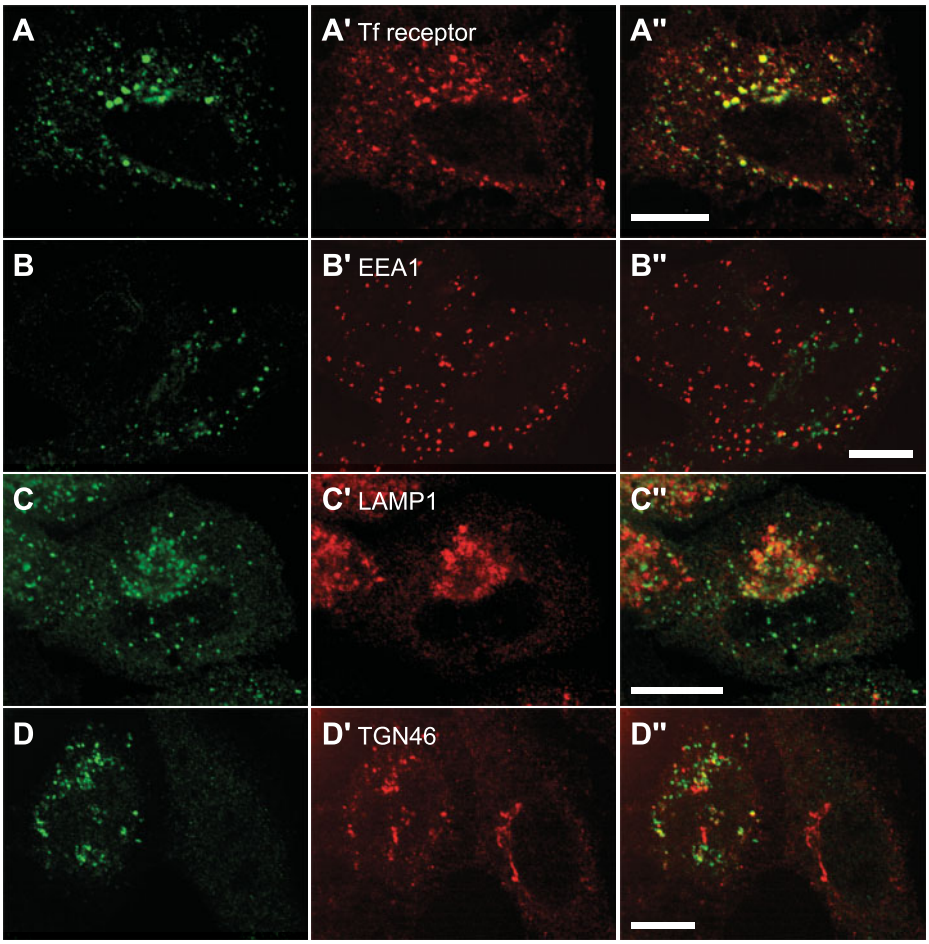

Fig. 4. **Immunofluorescence analysis of HeLa cells overexpressing MARCH-III.** HeLa cells transiently transfected with MARCH-III were doubly labeled with anti-MARCH-III antibodies and antibodies to Tf receptor (A–A″), EEA1 (B–B″), LAMP1 (C–C″), or TGN46 (D–D″). Signals for MARCH-III (green) and marker proteins (red) were observed by confocal microscopy. Bars, 10 μm.

change in the localization of the TGN protein TGN46, a human homolog of TGN38 (*22*, *23*), from the TGN to peripheral vesicles (Fig. 4, D–D″). TGN46 is known to cycle between the plasma membrane and TGN via endosomes (*23–25*). These results suggest that overexpression of MARCH-III perturbs the transport pathway taken by TGN46.

Association with MARCH-II and Syntaxin 6—The above mentioned intracellular distribution of MARCH-III is very similar to that of MARCH-II previously determined (*14*). We therefore examined their possible colocalization by expressing wild-type MARCH-II and GFP-tagged MARCH-III (GFP-MAR3) in HeLa cells followed by staining with anti-MARCH-II antibodies. The intracellular location of GFP-MAR3 corresponded well with that of MARCH-II, as seen in a merged image of their staining patterns (Fig. 5A). We next examined their possible association by immunoprecipitation analysis. HeLa cells were transfected with HA-tagged MARCH-II (HA-MAR2) and Myc-tagged MARCH-III (Myc-MAR3), and then extracted with 1% Triton X-100. The extracts were incubated with anti-HA-agarose beads. After extensive washing, proteins bound to the beads were subjected to Western blot analysis with anti-HA and anti-Myc antibodies. The results of immunoblotting with anti-Myc antibodies and anti-HA antibodies showed that Myc-MAR3 was co-immunoprecipitated with HA-MAR2 (Fig. 5B), suggesting a relatively tight association of MARCH-III and MARCH-II.

Our finding of an interaction between MARCH-II and MARCH-III raises the possibility that MARCH-III might form a complex with syntaxin 6. When HeLa cells coexpressing GFP-MAR3 and FLAG-tagged syntaxin 6 (FLAG-syn6) were stained with anti-FLAG antibodies, colocalization of the two proteins was readily detected (Fig. 5C). Next, we performed immunoprecipitation analysis with Triton extracts of HeLa cells expressing FLAG-syn6 and Myc-MAR3. The anti-FLAG affinity gel pulled down Myc-MAR3 as well as FLAG-syn6 (Fig. 5D). These results indicate that certain amounts of MARCH-III are associated with MARCH-II and syntaxin 6.

Importance of the PDZ-Binding Motif and RING Finger for MARCH-III Localization—We previously demonstrated that the PDZ-binding motif of MARCH-II is essential for its targeting to a destination, at least for translocation from the ER (*14*). MARCH-III also contains a characteristic 3-amino-acid sequence at the C terminus that matches the consensus sequence of a class I PDZ-binding motif [(S/T)*X*Φ, where Φ represents a hydrophobic residue, Fig. 1A (*26*)]. To investigate the importance of this motif for MARCH-III localization, we generated a GFP-tagged MARCH-III construct lacking four amino acid residues at the C-terminus. When the deletion mutants (GFP-MAR3-ΔPDZ) were expressed in HeLa cells, they remained in the ER, as judged from their colocalization with the ER marker calreticulin (Fig. 6A). To evaluate the importance of the PDZ binding affinity

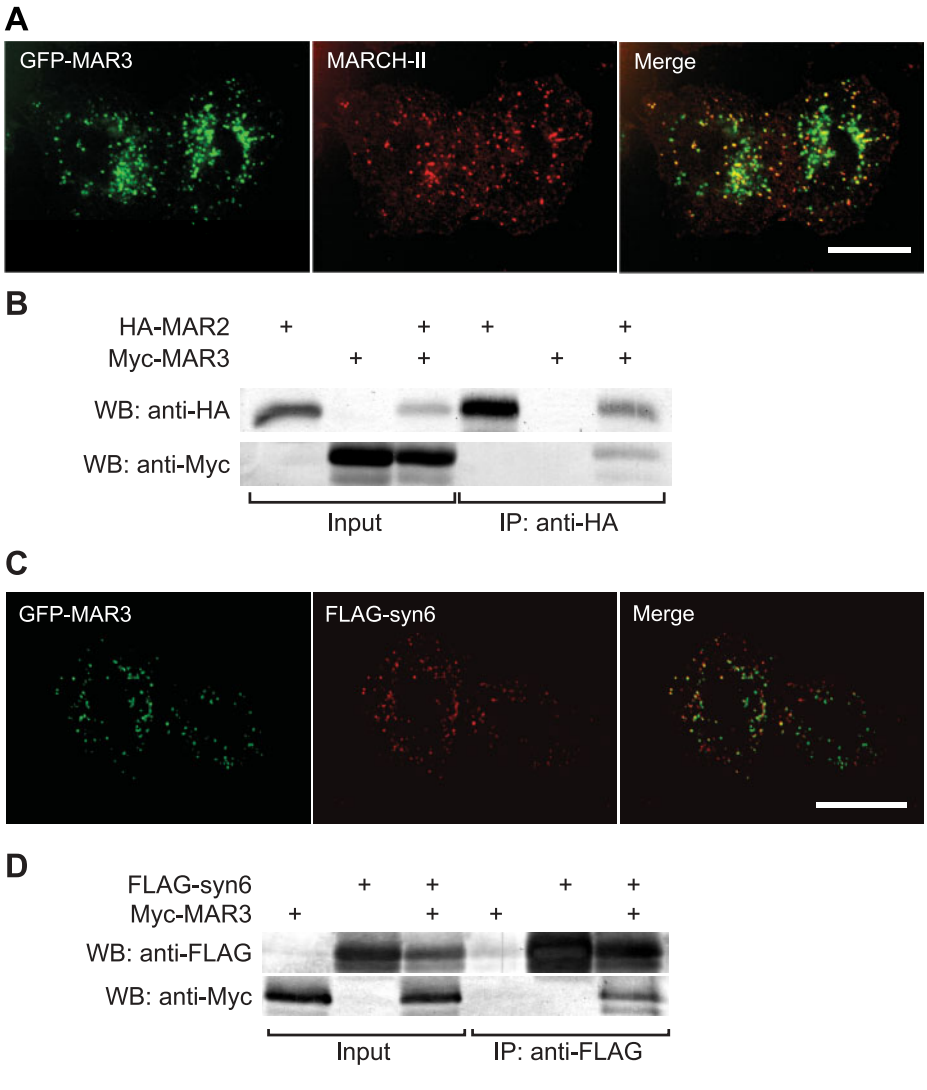

Fig. 5. **Association of MARCH-III with MARCH-II and syntaxin 6.** (A) HeLa cells cotransfected with GFP-MAR3 and MARCH-II were stained with anti–MARCH-II antibodies. Signals for GFP-MAR3 (green) and MARCH-II (red) were observed by confocal microscopy. Bar, 10 μm. (B) Lysates of HeLa cells transfected with Myc-MAR3 and/or HA-MAR2 were subjected to immunoprecipitation with anti–HA-agarose beads. The lysates (2.5% of input) and eluates were analyzed by Western blotting with anti-HA (top panel) or anti-Myc (bottom panel) antibodies. (C) HeLa cells cotransfected with GFP-MAR3 and FLAG-syn6 were stained with anti-FLAG antibodies (red). Bar, 10 μm. (D) Lysates of HeLa cells transfected with Myc-MAR3 and/or FLAG-syn6 were subjected to immunoprecipitation with anti-FLAG affinity gel. The lysates (2.5% of input) and eluates were analyzed by Western blotting with anti-FLAG (top panel) or anti-Myc (bottom panel) antibodies.

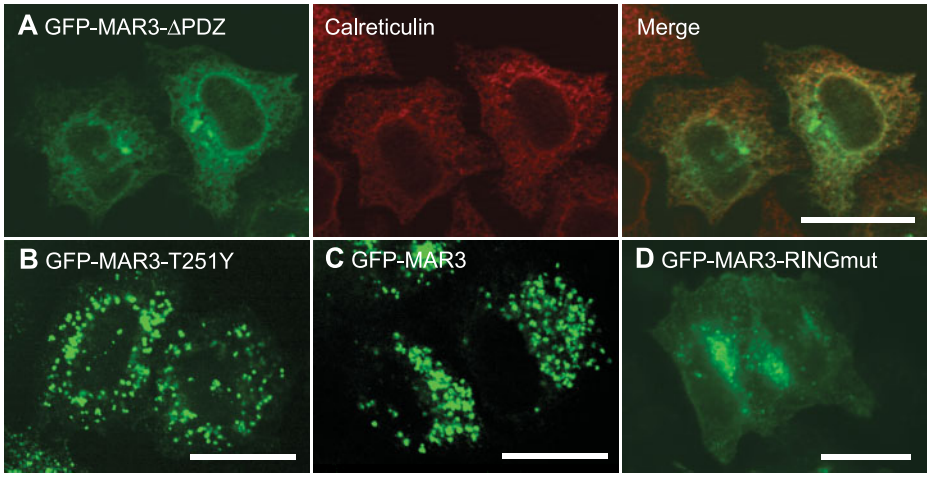

Fig. 6. **Subcellular localization of MARCH-III depends on the PDZ-binding motif and RING finger.** (A) HeLa cells expressing GFP-MAR3-ΔPDZ (green) were stained with anti-calreticulin antibodies (red) and then examined by fluorescence microscopy. (B–D) HeLa cells transfected with GFP-MAR3-T251Y (B), GFP-MAR3 (C), or GFP-MAR3-RING$_{mut}$ (D) were examined by fluorescence microscopy. Bars, 20 μm.

and selectivity, the motif was changed to a class II PDZ-binding one [(F/Y)*X*Φ] by replacement of Thr-251 with Tyr (GFP-MAR3-T251Y). This modification had little or no effect on the subcellular localization (Fig. 6B), as seen for the wild-type protein (Fig. 6C). This nonphenotypic change of Thr-to-Tyr suggests that the PDZ protein interacting with MARCH-III is not a typical class I. These results indicate that the intact PDZ-binding sequence is necessary for proper exit from the ER.

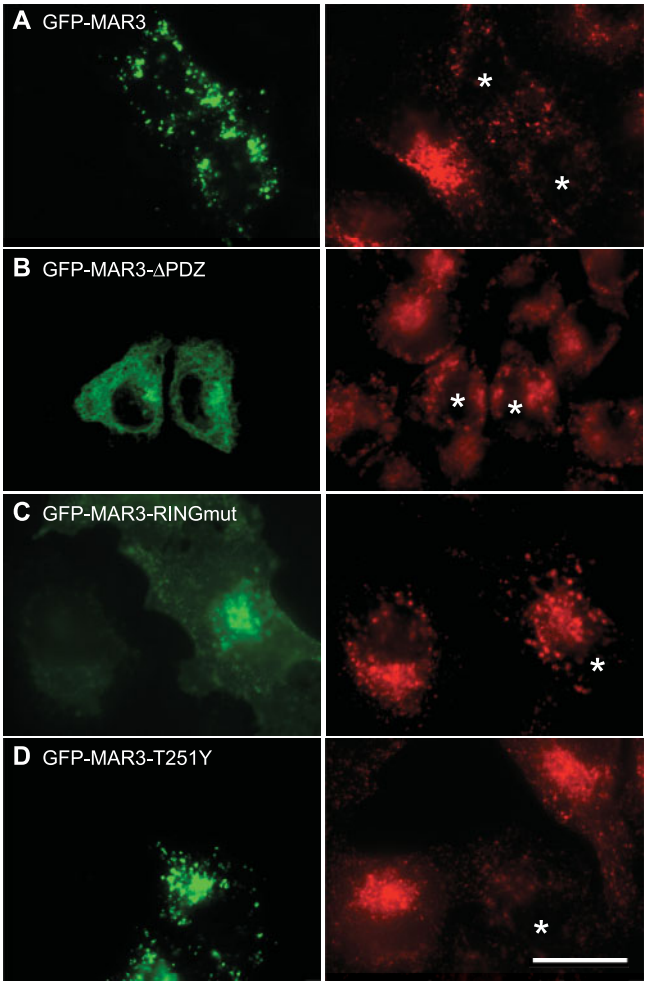

Fig. 7. **Inhibition of Tf uptake on MARCH-III overexpression.** HeLa cells were transfected with GFP-MAR3 (A), GFP-MAR3-ΔPDZ (B), GFP-MAR3-RINGmut (C), or GFP-MAR3-T251Y (D). At 24 h after transfection, cells were incubated with TMR-Tf (red) at 37°C for 30 min, fixed, and then subjected to fluorescence microscopy. Asterisks indicate transfected cells. Bar, 20 μm.

We next examined whether or not the RING finger plays a role in the subcellular localization of MARCH-III. Serine substitutions were made at two critical RING cysteine residues (C71 and C74) of GFP-MAR3. The mutant (GFP-MAR3-RINGmut) was transfected into HeLa cells and its localization was examined by immunofluorescence microscopy. GFP-MAR3-RINGmut was diffusely distributed in the intracellular membranes and weakly in the plasma membrane (Fig. 6D). This suggests the RING finger is necessary for targeting to peripheral vesicles.

Inhibition of Tf Uptake by MARCH-III Depends on Its Subcellular Localization—Given the fact that overexpression of MARCH-III results in relocalization of TGN46, we examined whether or not Tf uptake is affected by MARCH-III overexpression. HeLa cells transfected with GFP-MAR3 were incubated in serum-free medium in the presence of fluorescent-labeled Tf (TMR-Tf) at 37°C for 30 min. A marked reduction in Tf uptake was observed in cells expressing GFP-MAR3 (Fig. 7A), suggesting that overexpression of MARCH-III interfered with the endosomal recycling pathway. We next examined the effects of deletion of the PDZ-binding motif and of the mutations introduced in the RING finger of MARCH-III. The mutants that remained in the ER (ΔPDZ) or showed a diffuse distribution (RINGmut) had no effect (Fig. 7, B and C), whereas the mutant (T251Y) that has a mutation in the PDZ-binding motif but shows normal targeting to the final destination exhibited an unimpaired inhibitory effect on the Tf uptake (Fig. 7D). These results suggest that effective MARCH-III activity requires its proper subcellular targeting.

DISCUSSION

In this study we performed the identification and initial characterization of MARCH-III, a member of the RING-finger transmembrane protein family. In the mammalian MARCH family, MARCH-III is the closest homologue of MARCH-II. We have previously shown that MARCH-II is localized to a subpopulation of endosomes and binds to syntaxin 6, and that overexpression of MARCH-II perturbs the endosomal transport pathway (*14*). It appears that MARCH-III has similar properties to those of MARCH-II, based on the following observations. First, an immunofluorescence study of HeLa cells demonstrated that endogenous MARCH-III proteins were localized to a subpopulation of early endosomal vesicles positive for Tf receptor (Fig. 3D). Overexpressed MARCH-III proteins exhibited a similar distribution, and were partially colocalized with Tf receptor, EEA1, and LAMP1 (Fig. 4). Second, MARCH-III could be immunoprecipitated with syntaxin 6, and both proteins were colocalized in peripheral vesicles (Fig. 5, C and D). Third, overexpression of MARCH-III resulted in significant inhibition of Tf uptake (Fig. 7) as well as in redistribution of TGN46 (Fig. 4). Both Tf and TGN46/38 have been shown to pass through recycling endosomes (*24*, *25*, *27*, *28*). Possibly, overexpression of MARCH-III inhibits the recycling of Tf receptor and TGN46. These results suggest that MARCH-III might function in the regulation of vesicular transport along the endosomal recycling pathway.

We pointed out the possibility that MARCH-II and MARCH-III are present as a heteromeric oligomer based on the observations that (i) the exhibit essentially identical patterns of subcellular localizations, (ii) an immunoprecipitation study of Triton X-100 extracts demonstrated their tight association (Fig. 5), and (iii) a single population of cultured cells expresses both MARCH-II and MARCH-III mRNAs (Fig. 1C). Both showed a wide tissue distribution, but their relative message levels did not parallel each other, those for MARCH-III being low in muscle, heart and liver (Fig. 1B). This may reflect differences in the stability and turnover rate of MARCH-III mRNA in different tissues, or alternatively the ratio of MARCH-II and MARCH-III in the oligomer is different in different types of cells and, in extreme cases, oligomers of MARCH-II alone or MARCH-III alone may exist. Their stoichiometry and the functional significance of oligomerization remain to be determined.

MARCH-III is composed of multiple domains, one of which, the C-terminal PDZ-binding motif, was characterized here in considerable detail. Mutational analysis

indicated that the motif is recognized by PDZ protein(s) that facilitates the export of MARCH-III from the ER to its destination, which is required for MARCH-III activity. The interaction may initiate the loading of MARCH-III onto transport vesicles; the mutant MARCH-III not bound to PDZ domain, therefore, remains in the ER. PDZ domains consisting of 80–100 amino acid residues have now been found in a growing number of multidomain-scaffolding proteins involved in the formation of a complex network of protein–protein interactions necessary for the transport, localization, and assembly of membrane proteins (*29*). It has been reported that the PDZ-binding motifs found in several integral membrane proteins, such as proTGF-α (*30*), NMDA receptor (*31*, *32*), AMPA receptor (*33*), and dopamine transporter (*34*), are essential for their ER exit and proper subcellular targeting. Our results suggest the presence of ER-resident PDZ protein(s) controlling the MARCH-III transport.

An *in vitro* ubiquitination assay demonstrated that the RING-CH finger, another characteristic domain, possesses E3 ubiquitin ligase activity with specific E2 enzymes (*i.e.*, ubcH5C, ubcH6, and ubcH9). It is possible that MARCH-III acts as an E3 ubiquitin ligase *in vivo*, although its substrate remains to be identified. Several MARCH proteins have been shown to exhibit distinct E2 specificity in their E3 activities (*1*). However, the specificity of MARCH-III significantly overlapped that of MARCH-II (Fig. 2). This may reflect the high sequence similarity between the RING fingers of the two proteins. Mutations of the conserved cysteines of the RING finger resulted in not only loss of E3 activity but also mislocalization of GFP-MAR3-RINGmut. We observed an essentially identical effect of the RING-finger variant on the MARCH-II localization (data not shown). Our findings constitute evidence for a role of the RING fingers of MARCH-III and MARCH-II in their endosomal localization. Similarly, the RING fingers of PEX2 and a viral protein, ICP0/Vmw110, have been shown to be involved in their subcellular localization (*i.e.*, peroxisomes and ND10/POD/PML nuclear bodies, respectively) (*35*, *36*), although the mechanisms that link the RING fingers to subcellular targeting are poorly understood. Ubiquitination has now been found to serve as a sorting signal for endosomal compartments (*37*). If disruption of the E3 activity of MARCH-III effects the alteration in localization, one possible explanation is that the endosomal localization of MARCH-III might be mediated by ubiquitination of MARCH-III itself or of an unidentified substrate(s) that regulates the MARCH-III localization.

In conclusion, MARCH-III is expected, from its location and associated molecules, to be a component of the machinery regulating the trafficking and fusion of the transport vesicles in endosomes. Future studies on molecular characterization of other MARCH family members and a search for the PDZ proteins interacting with them should provide insight into the mechanism underlying the regulation of protein trafficking.

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